Phenotyping ripe rot resistance in the *Actinidia chinensis* (kiwifruit) mapping population

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Abstract Resistance to ripe rot (*Cryptosporiopsis actinidiae*) in kiwifruit is likely to be controlled by quantitative trait loci (QTL). Accurate phenotyping of ripe rot resistance is crucial to locating QTL on the kiwifruit genomic linkage map, and may facilitate identification of candidate genes for breeding. A detached fruit assay was developed to compare resistance to ripe rot in the mapping population. Fruit, at or near eating ripeness, were inoculated by inserting mycelial plugs (3 mm diameter) under shallow skin flaps, and then lesion development was monitored at 20°C. Results suggested that resistance was quantitative. Assay variability was reduced by pipetting spore inoculum into uniform wounds (5 mm) in the fruit surface. Preliminary analysis using single marker t-tests showed that marker Ke316, which may be involved in acyl lipid metabolism/transfer, was associated with ripe rot susceptibility. The association weakened as more genotypes were tested, and more analyses are necessary to determine whether Ke316 detects an actual QTL and/or if further QTLs are detectable.

Keywords ripe rots, *Cryptosporiopsis actinidiae*, kiwifruit, *Actinidia chinensis*, quantitative trait loci, resistance, mapping population.

INTRODUCTION

Cryptosporiopsis actinidiae Johnst. et al. is one of the key causal agents of ripe rot, the major postharvest disease of *Actinidia chinensis* Planch. var. *chinensis* 'Hort16A', which is the world's main gold-fleshed commercial cultivar (Manning et al. 2003). Infection occurs predominantly during flowering, after which the pathogen resides latent within the surface layers of the fruit pre-harvest, but does not become symptomatic until 12 or more weeks of postharvest storage, when soft pits develop on the fruit surface (Fullerton et al. 2007). Postharvest storage in air, rather

than controlled atmosphere, has been shown to significantly reduce incidence of the disease (Manning & Lallu 1995), but otherwise there is no current disease control. Consequently, breeding for disease resistance will be important in the development of new cultivars to maintain New Zealand's competitive edge in the global market.

Resistance can be classified as either qualitative or quantitative. Qualitative resistance is expressed in large and discontinuous increments (i.e. an "all or nothing" response), and is usually associated

with a small number of major genes, which have large observable effects. Conversely, quantitative resistance is expressed as a continuous distribution between maximum and minimum levels, and is normally under the control of a larger number of minor genes, each with small additive effects. Positions on the genome that can be attributed to detectable differences in phenotype due to gene effects are known as quantitative trait loci (QTL). Preliminary assays of *Cryptosporiopsis* resistance in various *A. chinensis* populations (K.V. Wurms, unpublished data) suggest the latter type of resistance.

Using gene markers developed from microsatellites in Plant & Food Research's (PFR) *Actinidia* expressed sequence tag database, a genetic map has been formed of the full sibling progeny resulting from a single cross of two diploid *A. chinensis* parents (CK51_05 x CK15_02) (Fraser et al. 2009). This family, referred to below as the mapping family, is planted in the PFR Te Puke research orchard. To identify and locate possible QTL associated with *Cryptosporiopsis* resistance, there is a need to search for co-segregation of phenotypic traits in the mapping population and markers on the genetic map. Hence accurate phenotyping of the mapping family is a crucial prerequisite to locating putative QTL. Phenotype bioassay data are required over several seasons, and other requirements of the assay are (1) it must be carried out in the lab to avoid introducing inoculum into the field mapping population and (2) fruit collected must be as uniform as possible with respect to physiological maturity.

The immediate objectives of this project were to develop a bioassay to quantify *Cryptosporiopsis* resistance, and to use the assay to phenotype resistance in the mapping population, with longer aims of identifying QTL associated with resistance, and studying these loci to identify candidate genes.

MATERIALS AND METHODS

Selection of the bioassay technique

To develop a bioassay to quantify resistance, three different techniques were first investigated in 2006/2007, as outlined below.

(1) Disease incidence was measured after normal postharvest storage. Fruit were artificially inoculated pre-harvest, harvested, stored in a controlled atmosphere (2% O₂ 5% CO₂) for 20 weeks at 1.5°C and then assessed for ripe rot incidence in terms of the percentage of fruit per tray expressing fully-developed infections (fungal pits) and lenticel spots (small halos on the fruit that contain the pathogen, but have not developed into full infections).

(2) Lesion spread of inoculated wound sites (wound flaps) was assessed. Fruit were harvested and surface sterilised by spraying and wiping with ethanol. The pathogen was introduced by placing a 3 mm diameter inoculum plug (sourced from 14-day-old *Cryptosporiopsis* cultures grown on potato dextrose agar) face-down under a skin flap cut into the sterilised fruit surface using a sterilised flat-edged scalpel. Wounds were sealed with Sellotape® and the fruit were left in closed trays for 1 week at 20°C before any lesions originating from the flaps were measured using digital callipers. Growth rates (mm/day) were calculated.

(3) Skin colonisation of nitrate non-utilising (NIT) mutants was measured. These mutants were unaltered in pathogenicity compared with the wild type (R. Beever, Landcare Research, unpublished data), but were used to facilitate tracking of the original inoculum. After pre-harvest inoculation with nitrate non-utilising mutants, approximately 10 surface-sterilised skin pieces/fruit from five fruit/vine were sampled 2 weeks after inoculation and again at harvest, repeatedly subbed to obtain pure cultures, then plated onto Vogels medium, with nitrate as nitrogen source, to select for the mutants. Percentage skin colonisation was recorded after incubation at 20°C.

Standardising maturity

Fruit maturity is known to influence kiwifruit resistance to postharvest pathogens, such as *Botrytis cinerea* (Wurms et al. 1998; Wurms 2004), and therefore it was important to harvest fruit of comparable physiological maturity to minimise effects of this variable on resistance phenotype.

In commercial operations, climacteric fruit are harvested when fully grown but unripe, since they are better able to withstand the rigours of postharvest handling/long distance shipping than when fully mature. However, the mapping dataset has been collected from vine-ripened fruit that is ready for consumption. Kiwifruit is deemed to be eating-ripe when the flesh yields slightly when squeezed between the thumb and index finger. Unfortunately this is not a quantitative measure, so one of the aims of the bioassay development was to quantify eating ripeness by correlating the finger squeeze test with quantitative maturity measurements.

In season 1, historical harvest dates were used as a guide for harvest, but fruit were only harvested according to the finger squeeze test. To examine the relationship between the finger squeeze test and quantifiable maturity parameters, flesh firmness (kgF); brix or fruit soluble sugars (% SS); and flesh colour as quantified by hue angle (h°), were measured at harvest on five fruit/genotype deemed to be eating-ripe on the basis of the finger squeeze test. Analysis of historical harvest data provided by the PFR kiwifruit breeders and mapping team made it possible to establish data ranges for each of the maturity variables. The total percentage of eating-ripe fruit harvested falling within the designated maturity ranges was then calculated.

To determine whether maturity parameters ought to be considered as covariants during QTL analysis, the relationships between the maturity parameters and *Cryptosporiopsis* lesion growth were first examined using matrix scatter plots and then covariant analysis was carried out for any factors showing a distinct linear relationship in the scatter plots.

Bioassay season 1

The inoculated wound site bioassay (method (2) of the initial bioassays) was used to phenotype *Cryptosporiopsis* resistance in all fruiting female vines (130 in total) of the PFR mapping family in season 1 (2007/2008). Fruit harvests were staggered over 3 months according to criteria described below, so as to obtain fruit of equivalent maturity.

An initial exploratory genome-wide scan of the PFR mapping family for putative QTL was carried out in SAS by running a series of t-tests to compare *Cryptosporiopsis* growth rates with single markers, and results were output as a list of markers with corresponding P-values. Separate analyses were done with both the male and female marker maps. To control the error rate associated with multiple comparisons, the Bonferroni correction method (Miller 1981) was used.

For markers identified as significant by the single marker QTL analysis, polymerase chain reaction (PCR) was used to amplify the alleles of the marker(s) in DNA samples from all the mapping population genotypes. PCR products were subjected to capillary gel electrophoresis in an ABI Prism® 3100 Genetic Analyser (Filter Set D, ROX™ size standard), and analysed with GeneMapper™ Software Version 3.0 (Applied Biosystems). The analyses resulted in a list of allelic segregation patterns by genotype for the marker(s). When combined with the phenotype information, this gave an indication of whether the marker contributes to resistance, either directly or by linkage with functional allele(s).

Bioassay season 2

In season 2 (2008/2009), *Cryptosporiopsis* resistance was determined in the 16 most resistant and 16 most susceptible genotypes identified from season 1.

The inoculated wound site assay (which was method (2) in initial assay development) was the most promising of the original assays, but this technique was refined by looking at three different methods of wound site inoculation to further reduce assay variability.

- (1) Inoculation of a wound flap, (the inoculation method used in initial assay development and in season 1).
- (2) Stab inoculation, where a 5 mm deep $\times$ 1 mm wide well was created by inserting the broad end of an inner pipette tip from a P25 positive-displacement pipette into the sterilised fruit surface, then 10 μ l of a suspension containing 2×10^7 *Cryptosporiopsis* spores/ml was pipetted into each well.

- (3) Inoculation by injection, where 10 µl of 2×10^7 spores/ml of *Cryptosporiopsis* inoculum was dispensed via a repetitive dispensing pipette attached by tubing to a needle that was inserted approximately 3 mm under the surface-sterilised skin.

After inoculation, the trays were left at 20°C and lesion development was measured after 6 to 8 days. Growth rates (mm/day) were calculated.

RESULTS

Selection of the bioassay technique

Given the low fruit numbers of many genotypes in the mapping population, and the stringent requirement that inoculum is not introduced into the field, wound site inoculation (method 2) represented the best compromise between variability, cost sample size and speed (Table 1).

Standardising maturity

Assessment of current and historical harvest data from the mapping population indicated that harvest of eating-ripe fruit using the finger squeeze test equated to a firmness of ≤ 4 kgF

and a brix of $\geq 14\%$. A hue angle flesh colour of $\leq 104^\circ$ of 'Hort16A' is also considered as a guide to physiological maturity (Minchin et al. 2003), with 98–100° corresponding to eating ripeness (B. Jordan, Plant & Food Research, personal communication).

Table 2 shows the relationship between the finger squeeze test and maturity parameters. The maturity parameter most closely associated with eating ripeness, as determined by the finger squeeze test, was firmness (84%) followed by brix (82%) then colour (78%). The relationship was true regardless of whether parameters are considered singly or in combination (Table 2). Increasing the number of parameters measured decreased the relationship with the finger squeeze test (Table 2).

The matrix scatter plots (data not shown) suggested that relationships between *Cryptosporiopsis* lesion growth rate and maturity variables were weak. The most promising relationship, between decreasing *Cryptosporiopsis* growth rate with increasing firmness, still showed a large scatter of growth rates at any given range

Table 1 Advantages and disadvantages of three *Cryptosporiopsis* resistance bioassay methods. The units measured and standard errors of the population means (SEM) are shown.

Unit measured	Population mean $\pm$ SEM	Advantages	Disadvantages
(1) Normal postharvest storage			
% fruit infected	55.9 $\pm$ 4.4%	Best mimics natural epidemiology. Lowest variation relative to the mean.	Requires field inoculation. Requires ≥ 75 fruit/genotype. Results take ≥ 16 weeks in a controlled atmosphere (2% O ₂ 5% CO ₂).
(2) Wound site inoculation			
lesion diameter (mm)	9.6 $\pm$ 1.3 mm	Inoculation takes place in the lab. Results obtained within 1–2 weeks. Requires ≥ 15 fruit/genotype.	Wound size is not controlled. Amount of mycelium varies from plug to plug.
(3) Skin colonisation			
% pathogen isolated	10.2 $\pm$ 4.6%	Can distinguish nitrate non-utilising mutant from wild type <i>Cryptosporiopsis</i> . Requires ≥ 5 fruit/genotype.	Most variable results. Costly in terms of labour and consumables. Results take ≥ 3 weeks.

Table 2 Proportion of *Actinidia chinensis* fruit harvested according to a qualitative finger squeeze assay that also fall within specified ranges of the quantitative maturity parameters: firmness, brix and colour.

Maturity parameters measured and harvestable ranges thereof	Percentage of finger-squeezed fruit within specified maturity parameter ranges
Firmness (≤ 4 kgF)	84%
Brix ($\geq 14\%$)	82%
Colour ($98 \leq h^{\circ} \leq 104$)	78%
Firmness and brix	76%
Brix and colour	71%
Firmness and colour	70%
Firmness and brix and colour	65%

of firmness. A mixed model fitted to mean lesion growth rate, with and without the kgF covariate, showed that the vine-vine variance component changed only by $\sim 8\%$ (from 0.2298 to 0.2482), indicating there is not much gain in precision in QTL detection by adjusting for the covariates.

Bioassay season 1

Results from the season 1 bioassay indicated a continuous distribution of *Cryptosporiopsis* resistance phenotypes ranging from resistant (0.08 mm/day lesion growth) to susceptible (2.14 mm/day lesion growth) (Figure 1). When a smoothed density plot was overlaid on the histogram of mean *Cryptosporiopsis* growth rates,

a skewed normal distribution resulted rather than bimodal one (Figure 2), which may indicate a mixed distribution of phenotypes that could be associated with differences in marker genotypes.

Preliminary single marker QTL analysis of 43 female genotypes indicated that high *Cryptosporiopsis* growth rates (i.e. *Cryptosporiopsis* susceptibility in females) were significantly associated with only one marker (Ke316) in the male map, with an unadjusted P-value of $9.55E-06$, with reference to a Bonferroni threshold at $\alpha = 0.05$ of $1.36E-04$. However, when a larger sample size of 81 female genotypes was considered, the observed P-value was 0.0056, which was greater than the threshold Bonferroni

Figure 1 Range of *Cryptosporiopsis actinidiae* resistance phenotypes (mean lesion growth rate in mm/day) in the *Actinidia chinensis* diploid mapping population.

Figure 2 Data distribution as shown by a smoothed Kernel Density plot overlaid on the histogram of mean *Cryptosporiopsis actinidiae* resistance phenotypes (mean lesion growth rate in mm/day).

of 1.36×10^{-4} . Hence the association with Ke316 is non-significant in the larger sample size. The allelic substitution effect ('n' by 'p' allele) seen in Figure 3 indicated that the 'p' allele carried by the male parent was responsible for slightly increased susceptibility to *Cryptosporiopsis*.

The PFR expressed sequence tag (EST) database showed that marker Ke316 was derived from EST 245787 from an *Actinidia chinensis* petal library. It is part of the acyl lipid metabolism family with a putative molecular function in lipid binding and transport.

Genotype analysis indicated that there was not a specific combination of Ke316 marker alleles associated with the *Cryptosporiopsis* resistance phenotype, i.e. there was not a 100% correlation between susceptibility and the presence of the 'p' allele.

Bioassay season 2

Figure 4 showed that the stab and injection inoculation methods produced much smaller lesions than inoculation of fruit skin flaps in the inoculated wound site assay. The stab and inject techniques ranked genotypes similarly in terms

of levels of *Cryptosporiopsis* resistance, but were different to the rankings obtained from the flap inoculation technique (Figure 4).

A comparison between the data for seasons 1 and 2 showed that inoculation of wound flaps did not produce consistent results, since the correlation coefficient for the association was 0.0153, i.e. strongly non-linear (Figure 5).

DISCUSSION

A bioassay has been developed to quantify *Cryptosporiopsis* resistance, including quantification of the finger-squeeze assay that is currently used to determine eating-ripeness. Using the information from this bioassay, the data suggest that *Cryptosporiopsis* resistance in the *A. chinensis* mapping family appears to be quantitative.

Of the three bioassay methods initially tested, method (1), in which fruit were inoculated in the field pre-harvest then subjected to normal postharvest storage, best mimicked natural disease epidemiology and had the lowest variability, but was impractical because of the

Figure 3 Box plot of inheritance patterns of the *Actinidia chinensis* diploid mapping population for marker Ke316, as associated with the *Cryptosporiopsis actinidiae* resistance phenotype, where nn = female parent inheritance and np = male parent inheritance.

Figure 4 Mean *Cryptosporiopsis actinidiae* lesion growth rates (mm/day) on 32 genotypes of *Actinidia chinensis* fruit, resulting from three different techniques of inoculation in the inoculated wound site assay: (i) mycelial plug placement under shallow skin flap wounds (“flap”); (ii) spore suspension inoculation into a stab wound (“stab”); and spore suspension injected under the skin (“inject”). Growth rates were measured after 6–8 days incubation at 20°C in 2009. Error bars indicate $\pm$ standard error of the mean.

introduction of spores into the field and the need for high sample sizes. Skin colonisation (method (3)) was also impractical because of the extremely high variation accounting for nearly half of the population mean, and the expensive and time consuming process of repeated culture isolation. Wound inoculation (method (2)) had variability and sample size requirements intermediate between the other 2 assays, the fastest results generation, and represented the most practical choice. It was successfully used in season 1 to assay all the females in the mapping population and to demonstrate that resistance to *Cryptosporiopsis* was quantitative rather than qualitative. However, in an attempt to further refine this assay and reduce variability, three different methods of inoculation were tested in season 2 – inoculation of a wound flap (the existing inoculation method), stab inoculation and inoculation by injection. Lesions were much larger and more variable in the flap inoculation technique compared with the other two methods, because the initial wound area was larger, wound sizes and lesion shapes were irregular, and it was difficult to distinguish browning associated with the wound from lesions. Moreover the amount of

mycelium contained on each inoculum plug used in the flap technique could not be quantified, whilst the stab and inject techniques delivered controlled amounts of inoculum (10 μ l of 2×10^7 spores/ml) to uniform wound sites, 1 mm in diameter. Consequently the stab and inject methods performed similarly to each other in

Figure 5 Scatterplot of two seasons of mean *Cryptosporiopsis actinidiae* lesion growth rates (mm/day) from flap wounds on 32 genotypes of *Actinidia chinensis* fruit, measured after 6–8 days incubation at 20°C. Details of a linear regression fitted to the data are shown.

terms of variability and resistance rankings. It was difficult to get a consistent penetration depth with the injection method, whereas the stab technique created wound sites 5 mm deep, as governed by the depth of a lip on the broad end of the displacement pipette tip. Thus the stab inoculation technique will be used in future.

Consistency of the bioassay can be further enhanced by assaying fruit of comparable maturity, as assessed by the finger squeeze assay and measurement of flesh firmness and brix. Firmness was the maturity parameter that aligned most closely with eating-ripeness as assessed by the finger squeeze test, whilst hue angle was the least predictive of eating-ripeness. Although hue angle is the parameter used to determine when to harvest *A. chinensis* 'Hort16A' (Zespri Group Ltd 2010), the wider population of *A. chinensis* is very variable in colour. This is especially true in the mapping population where fruit at eating-ripeness show a colour range from bright green, through green-yellow, to gold (L.G. Fraser, Plant & Food Research, personal communication). Using more than one maturity parameter to estimate eating ripeness decreased goodness of fit with the finger squeeze test, but is considered to be a more stringent test of maturity, so the finger squeeze test will be used in concert with the maturity limits of firmness ≤ 4 kgF and brix $\geq 14\%$.

Although a complete dataset of all mapping population females was only collected during season 1, and must be repeated because the bioassay was in a developmental stage, *Cryptosporiopsis* resistance in the *A. chinensis* mapping family appears to be quantitative rather than qualitative. There was a continuous spread of resistance phenotypes and data distribution was approximately normal rather than bimodal, the latter being suggestive of major gene resistance. Season 1 data also suggested a link between expression of marker Ke316 and increased *Cryptosporiopsis* lesion growth rates. Genotype analysis indicated that there was not one specific combination of Ke316 marker alleles associated with *Cryptosporiopsis* susceptibility, and this result is consistent with expression of a quantitative locus marker as opposed to a major resistance gene.

Preliminary analysis of season 1 phenotypic data with genotypes from 43 females suggested that marker Ke316 in the male map correlated with high *Cryptosporiopsis* growth rates. This marker represents an EST that is in an acyl lipid metabolism family, with a putative molecular function in lipid binding and transport. Literature supports the role of acyl lipids in host resistance (Dhondt et al. 2002; Rumbaugh 2007; Kachroo et al. 2008; Upchurch 2008), and La Camera et al. (2005) showed that a pathogen-inducible acyl lipid hydrolase facilitated fungal and bacterial colonisation of *Arabidopsis*. Ke316 is a fully informative marker where it is possible to determine which alleles come from each parent. The male alleles had a high t-test with the phenotype of the females, suggesting that the allele associated with susceptibility was inherited from the male parent of the population. However, the initial association between Ke316 and *Cryptosporiopsis* resistance was based on a limited number of genotypes (43) as well as the developmental phenotype assay. When a larger number of genotypes (81) were considered, the association between Ke316 and *Cryptosporiopsis* was no longer statistically significant. This illustrates that small sample sizes can sometimes lead to false positives. More individuals must be genotyped, and phenotyping repeated using the improved technique over preferably two to three seasons to determine whether Ke316 detects an actual QTL and/or if further QTLs are detectable. Even if Ke316 co-segregates with the QTL for resistance, but does not have direct involvement in resistance/susceptibility, it may still be useful as a marker in marker-assisted breeding.

Now that accurate phenotyping of ripe rot resistance is possible, the inoculated bioassay (using the stab inoculation technique) will be used to phenotype the mapping population for a further two to three seasons. This step is a crucial prerequisite to detecting QTL on the kiwifruit genomic linkage map, identifying candidate genes, and, through these, facilitating development of resistant cultivars.

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