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M. Acevedo
USDA-ARS, maricelis.acevedo@ars.usda.gov

E. W. Jackson
USDA-ARS

A. Sturbaum
USDA-ARS

H. W. Ohm
Purdue University

J. M. Bonman
USDA-ARS

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Genetics and resistance/Génétique et résistance

An improved method to quantify *Puccinia coronata* f. sp. *avenae* DNA in the host *Avena sativa*

M. ACEVEDO[1], E. W. JACKSON[1], A. STURBAUM[1], H. W. OHM[2] AND J. M. BONMAN[1]

[1]*USDA-ARS Small Grains and Potato Germplasm Research Unit, 1691 S. 2700 W., Aberdeen, ID 83210, USA*
[2]*Department of Agronomy, Purdue University, West Lafayette, IN 47907, USA*

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Abstract: Identification and genetic mapping of loci conferring resistance to polycyclic pathogens such as the rust fungi depends on accurate measurement of disease resistance. We converted an absolute quantification assay of *Puccinia coronata* DNA to a relative assay by adding a TaqMan® primers/probe set specific to the oat β-actin gene to simplify and improve quantification of fungal infection. The new multiplex assay estimates the amount of fungal DNA in a sample relative to the amount of host DNA and requires fewer and less labour-intensive steps than previous assays. The relative fungal DNA assay (RFDNA) reliably detected and quantified both host and pathogen DNA over five orders of magnitude and was at least as sensitive as either digital image analysis (DLA) or absolute estimation of fungal DNA (AFDNA) in repeated greenhouse studies using 12 oat cultivars with different resistance responses to *P. coronata* isolate LGCG. Measuring crown rust resistance to LGCG using DLA, AFDNA and RFDNA in a P8669/P94163 recombinant inbred line population produced segregation ratios that did not differ from the 1:1 Mendelian ratio expected for a single gene. Compared to the AFDNA assessment method, the RFDNA assay is equally sensitive, yet faster and much easier to use than the AFDNA method for precise quantification of the crown rust pathogen in oat leaves. The method will be especially useful for streamlining measurement of partial resistance, since uncovering small differences in resistance requires phenotypic evaluation of large populations.

Keywords: crown rust, disease resistance, fungal detection, genetic mapping

Résumé: L'identification et la cartographie génétique des locus qui confèrent la résistance aux agents pathogènes polycycliques, tels que les champignons causant la rouille, découlent de l'évaluation précise de la résistance aux maladies. Nous avons converti une analyse par quantification absolue de l'ADN de *Puccinia coronata*en analyse relative en y ajoutant une amorce et une sonde TaqMan® spécifique du gène β-actine de l'avoine pour simplifier et améliorer la quantification de l'infection fongique. La nouvelle analyse multiplexe permet d'évaluer la quantité d'ADN fongique dans un échantillon équivalant à la quantité d'ADN de l'hôte et requiert moins d'étapes et de main-d'œuvre que les analyses précédentes. L'analyse relative de l'ADN fongique (RFDNA) a permis de détecter et de quantifier précisément l'ADN de l'hôte et de l'agent pathogène sur plus de cinq ordres de grandeur, et était aussi sensible que l'analyse d'image numérique (AIN) ou que l'évaluation absolue de l'ADN fongique (AFDNA) découlant d'études successives effectuées en serre sur 12 cultivars d'avoine qui réagissaient différemment à l'isolat de *P. coronata* LGCG sur le plan de la résistance. L'évaluation de la résistance à la rouille couronnée, causée par l'isolat LGCG, à l'aide de l'AIN, de l'AFDNA et de l'RFDNA dans une population de lignées recombinantes fixées P8669/P94163 a produit des ratios de ségrégation qui ne différaient pas de la proportion mendélienne 1:1 prévue pour un gène unique. Comparativement, l'analyse RFDNA est aussi sensible quoique plus rapide et beaucoup plus facile à utiliser que l'analyse AFDNA pour ce qui est de quantifier avec précision l'agent pathogène de la rouille couronnée dans les feuilles d'avoine. La méthode s'avérera particulièrement utile pour rationaliser l'évaluation de la résistance partielle étant donné que la découverte de différences minimes quant à la résistance requiert l'évaluation phénotypique de fortes populations.

Mots clés: cartographie génétique, détection fongique, résistance aux maladies, rouille couronnée

Correspondence to: M. Acevedo. E-mail: maricelis.acevedo@ars.usda.gov

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Introduction

Crown rust, caused by *Puccinia coronata* f. sp. *avenae* P. Syd. & Syd, is the most important disease of cultivated oat (*Avena sativa* L.) causing significant yield loss and reduced seed quality (Simons *et al*., 1979; Simons, 1985). Crop resistance is an effective and economical method of managing crown rust. Crown rust resistance genes with major effects (*Pc* genes) have been identified and deployed in cultivated oat (Simons, 1985). In addition, partial resistance to the disease has been studied as a means to develop cultivars with more durable resistance (Luke *et al*., 1972; Diaz-Lago *et al*. 2002, 2003; Portyanko *et al*., 2005; Barbosa *et al*. 2006).

Development of marker-trait associations for use in marker-assisted breeding requires genetic mapping of the targeted trait. Mapping results depend on the accuracy of genotypic and phenotypic assessments. In recent years, substantial progress has been made in genotyping, including advanced marker technology, improved assays for better resolution between marker alleles at each locus, and automated data collection, reducing marker-allele calling errors (Dearlove, 2002; Collard & Mackill, 2008; Moose & Mumm, 2008). In contrast, relatively little has been done to improve phenotyping of disease resistance in plants. Historically, infection type ratings and diseased leaf area (DLA) have been used to estimate crown rust disease (Simons, 1985). Diaz-Lago *et al*. (2003) used digital image analysis to improve DLA estimates on oat leaves compared to visual ratings. The use of this phenotyping method has been substantiated in multiple mapping studies (Hoffman *et al*., 2006; Jackson *et al*., 2006, 2007, 2008). To further improve crown rust phenotyping, a new assay based on absolute quantitative real-time polymerase chain reaction (q-PCR) measurement of pathogen DNA content in the host plant was developed and used to measure crown rust resistance (Jackson *et al*., 2006). The assay enhanced mapping precision of a major gene, enabled identification of a quantitative trait locus (QTL) not discernable using visual ratings or digital image analysis, and achieved shorter QTL intervals versus visual or digital assessment (Jackson *et al*., 2007). Drawbacks to the absolute q-PCR assay are the tedious measurements required to obtain dry weights for infected tissue samples and the time required to assure DNA uniformity prior to fungal DNA estimation. Converting the absolute assay to a relative assay by multiplexing the fungal primers/probe set with a primers/probe set specific to an internal control, gene of known copy number overcomes these drawbacks. Measurement of pathogen DNA content relative to host DNA content using q-PCR is a proven concept in several pathosystems, including downy mildew [*Plasmopara viticola*Berk. et Curtis ex. de Bary] in grapes and late blight [*Phytophthora infestans* (Mont.) de Bary], pink rot [*Phytophthora erythroseptica* Pethybr.], leak [*Pythium ultimum* Trow], dry rot [*Fusarium sambucinum* Fuckel], soft rot [*Erwinia carotovora* subsp. *carotovora* Jones and subsp. *atroseptica* Hellmers and Dowson] and verticillium wilt [*Verticillium dahliae* Kleb.] in potato (Valsesia *et al*., 2005; Atallah & Stevenson, 2006; Atallah *et al*., 2007).

We report the conversion of the absolute q-PCR assay to a relative assay by simultaneous amplification and quantification of fungal and host DNA in one multiplex reaction. Results from the relative assay were compared with results from the absolute q-PCR and digital image assessment of DLA in 12 oat cultivars with various levels of crown rust resistance, as well as a recombinant inbred oat population segregating for crown rust resistance.

Materials and methods

Fungal isolates and inoculum production

The *P. coronata* races LGCG (isolate 98MNB245) (Chong, 2000; Chong *et al*., 2000) obtained from the USDA-ARS Cereal Disease Laboratory (CDL), St. Paul, MN was used to develop the relative q-PCR assay and to evaluate crown rust resistance in the population. The isolate produces a range of resistance reactions on the 12 oat cultivars used to develop the RFDNA assay, is highly virulent on the oat line P8669, causing large uredinia with and without chlorosis (infection type 3 and 4), and is avirulent on the oat line P94163 (infection type 0). Urediniospores were purified from a single pustule and subsequently increased on seedlings of the susceptible cultivar 'Provena' (Erickson *et al*., 2003) as described previously (Jackson *et al*., 2008). Spores were collected from infected 'Provena' plants using a vacuum collector and re-suspended in light mineral oil (Soltrol 170) to prepare fresh inoculum. Final inoculum concentrations for greenhouse and field inoculations were adjusted with a hemacytometer to 10^5 urediniospores ml^{-1}.

Primer design and amplification

Fungal DNA (FDNA) was amplified using the *P. coronata*-specific primers developed previously (Jackson *et al*., 2006). A TaqMan® primer-probe set was designed based on an oat β-actin mRNA fragment (NCBI accession AF234528) for amplification of oat DNA. The specificity of the non-labelled β-actin primers (forward, 5' -GAG CTA CGA GCT TCC TGA TGC; reverse, 5' -TCC ACG

TCG CAC TTC ATG A) was tested using template DNA from the 12 oat cultivars (Table 1) along with DNA from *P. coronata* races LSLG (isolate 93MNB236) and LGCG (isolate 98MNB245) using conventional PCR. PCR amplifications were conducted in an ABI Gene Amp 9700 PCR system (Applied Biosystems, Foster City, CA) with the following protocol; an initial denaturation at 98 °C for 2 min, 29 denature/anneal/extension cycles of 98 °C for 10 s: 60 °C for 15 s: 72 °C for 15 s, followed by a 72 °C extension for 5 min. All reactions were done in a total volume of 25 µL containing 1× PCR reaction buffer, 200 nM dNTPs, 400 nM primers, 1 Unit Red Taq (Sigma-Aldrich, St. Louis, MO) and 60 ng DNA template. PCR products were visualized on 2% agarose (Sigma-Aldrich, St. Louis, MO) gels stained with ethidium bromide using a Fluorochem 8800 Image System (Alpha Innotech Corp., San Leandro, CA). Specificity of the labelled TaqMan®(Applied Biosystems, Foster City, CA) primers-probe sets for FDNA and oat β-actin quantification was tested on the same 12 oat cultivars and two *P. coronata* isolates using real-time q-PCR using an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). The amplification protocol used was an initial incubation at 50 °C for 2 min followed by initial denaturation at 95 °C for 15 min, 40 denature/anneal-extension cycles at 95 °C for 15 s and 60 °C for 60 s.

Table 1. Oat- β-actin and fungal DNA (FDNA) cycle threshold (Ct) values from q-PCR amplification of DNA of 12 oat cultivars and two *P. coronata* isolates used to evaluate specificity of oat β-actin and FDNA primer pairs and probes.

Oat line	β-actin Cq values[a]	SD	FDNA Cq values[a]	SD
CDC Boyer	30.9	0.62	NA	. . .
IA98822-2	31.2	0.14	NA	. . .
Makuru	31.4	0.21	NA	. . .
Mn841801-1	28.5	0.26	NA	. . .
Noble-2	30.7	0.48	NA	. . .
Ogle1040	31.3	0.06	NA	. . .
Otana	29.4	0.42	NA	. . .
P8669	32.2	0.35	NA	. . .
P94163	28.2	0.35	NA	. . .
Provena	30.0	0.15	NA	. . .
TAM 301	28.0	0.47	NA	. . .
TAM 405	30.3	0.47	NA	. . .
P. coronata LSLG	NA	. . .	28.3	0.17
P. coronata LGCG	NA	. . .	32.0	0.64

Notes: [a]Quantification cycle (Cq) values of the real-time PCR assay. NA = no amplification.

Primers and probes efficiency

The efficiency for each DNA amplification (efficiency = $10^{(1\text{-slope})} - 1$) was tested on a serial dilution (five-fold) of *P. coronata* DNA and *A. sativa* DNA (150 ng μL^{-1}, 30 ng μL^{-1}, 6 ng μL^{-1}, 1.2 ng μL^{-1} and 0.24 ng μL^{-1}). Each serial dilution was tested in the presence of both TaqMan® sets (multiplexed) since the assay must quantify both FDNA and host DNA (HDNA) in a single reaction. The influence of high quantities of FDNA on the quantification of HDNA was tested by adding a total of 450 ng of FDNA to the *A. sativa* DNA serial dilution reactions. The same protocol was followed using 450 ng of *A. sativa* DNA and the FDNA serial dilution reactions. All q-PCR amplifications were conducted in duplicates on an ABI Prism 7000 Sequence Detection System. The TaqMan® FDNA probe was labelled with the fluorescent reporter dye 6FAM (6 –Carboxyfluorescein; emission at 517 nm) and the oat β-actin probe was labelled using VIC reporter fluorescent dye (emission at 552 nm) so that the FDNA and HDNA amplifications could be detected in the same reaction well. Reactions were performed in 25 µL volumes containing 1× Universal master mix (Applied Biosystems), 300 nM forward and reverse FDNA primers, 400 nM forward and reverse oat β-actin primers, 200 nM each FDNA and β-actin MGB TaqMan® probes and 90 ng total DNA.

Evaluation of QPCR assays on oat cultivars with different levels of crown rust resistance

To compare the efficacy of the AFDNA and RFDNA estimation assays on detecting differences in response to crown rust, seeds of 12 oat cultivars (Table 1) having different levels of crown rust resistance were used. Four seeds of each genotype were sown in three 6 cm pots containing 1:2:1 (v:v:v) mix of sand, peat moss and vermiculite. Plants were grown in a greenhouse with a 14–15 h photoperiod and day/night temperature of 21±1 °C/13 ±1 °C. At the second leaf stage, leaves were inoculated with an uredinospore suspension (10^5uredinospores mL^{-1}) using a spray inoculator. After inoculation, plants were allowed to dry and then incubated overnight (16 h) in a dew chamber at 15.5±1 °C and 100% relative humidity. Ten days after inoculation, the second leaf of each plant was excised and digital images were taken using a SP-510UZ Olympus digital camera (3072 × 2304 pixel resolution) mounted on a portable stand at a fixed distance of 30 cm. Immediately after photographing, a 4 cm long section at the mid-point of each leaf was excised, and placed in individual 1.0 mL microcentrifuge tubes for DNA extraction. The tissues were lyophilized and weighed before DNA

extraction. DNA was extracted using a CTAB mini prep method (Stewart & Via, 1993) and quantity and quality were checked using a spectrophotometer (*A*260nm and *A*280nm). All DNA q-PCR amplifications were conducted in duplicated multiplexed reactions as described above and repeated twice.

Evaluation of q-PCR assays on recombinant inbred lines using polycyclic single-race field experiment

Parents and 88 randomly selected recombinant inbred lines from a P8669/P94163 cross were sown in irrigated field plots at the University of Idaho Research and Extension Center in Aberdeen, ID in May 2007. A randomized complete block design (RCBD) with two replicates was used to evaluate the lines for crown rust resistance. Within each block, approximately 25 seeds of each parent and RIL were sown in a single hill. The hills were planted 35.5 cm apart on 36 cm centres. A single hill of the susceptible cultivar 'Provena' was sown adjacent to each test plot throughout the block as a 'spreader'. A wheat border was used to reduce edge effect.

To create the environmental conditions necessary for disease development under dry climate conditions, the blocks were surrounded by a 6 μm thick polyethylene sheet attached to a wooden frame as described by Jackson *et al*. (2006). Beginning at the five leaves growth stage, spreaders were inoculated at dusk twice per week for three consecutive weeks using a hand-held garden sprayer. The blocks were irrigated 30 minutes prior to inoculation then covered overnight (14 h) with the polyethylene sheeting. After the last inoculation, the blocks were covered four additional times at three day intervals to promote disease development.

Forty days after the first inoculation, four representative flag leaves per plot were removed and individually photographed as described for the greenhouse experiment. Immediately after photographing, a 2 cm^2 section at the mid-point of each leaf was excised; the sections were pooled together and carefully placed in one 1.0-mL microcentrifuge tube per genotype to reduce spore loss before DNA extraction.

Data analysis

Normality of trait distribution was estimated using the Shapiro–Wilk test. For statistical analysis, absolute AFDNA and RFDNA data were transformed using the square root, since the transformation improved the normality of the frequency distribution curve. Mean values for each RIL/replicate were calculated separately and used to determine phenotypic correlations. Pearson correlations were calculated to determine the relationship between disease measurements from each assay. Disease measurement means across the 12 oat cultivars were compared using the Tukey's-HSD test. All statistical analyses were performed using JMP 6.0 statistical software (SAS Institute, Cary, NC).

DLA was assessed from the digital images using the Assess image analysis software (Lamari, 2002). The threshold settings used for DLA estimation were 103 and 60 as previously described by Jackson *et al*. (2007). These settings take into account pustules and a portion of the adjacent chlorotic area.

AFDNA content was estimated based on the FDNA Cq values for each sample, taking into account sample concentration (dilution factor) and sample dry weight, while RFDNA content was calculated by the equation $2^{(Cq\beta - Cqp)}$where $Cq\beta$ is the quantification cycle for the oat β-actin amplification and Cqp is the quantification cycle for the pathogen DNA amplification in each reaction. Since eight separate q-PCR runs were used to estimate FDNA in this study, two replicates of the serial dilution standards (five-fold) were included in each run and replicated samples of each genotype were randomized between runs. The efficiency curves (slope, intercept, $R^2$and efficiency) for the standards in each run were compared across all plates to allow valid comparisons. RILs were classified as resistant or susceptible based on comparison with the parental lines. The resulting segregation ratios were compared to common Mendelian ratios and a Chi-square (χ^2) value was calculated for each replicate. The data were then converted into a phenotypic marker for each replicate. The six phenotypic markers were tested for linkage to 437 polymorphic DArT markers between the P8669/P94163 parental lines using MapManager QTXb20 (Manly *et al*., 2001) The markers were grouped using the 'ri self' linkage evaluation with a search linkage criterion of $P = e^{-6}$. The best overall marker order containing the phenotypic markers was determined using the ripple function with a linkage criterion of $P = e^{-6}$. The final map was calculated using the Kosambi distances and drawn using MapChart 2.2 (Voorrips, 2002).

Results

Primer and probe design and efficiency

The β-actin primers amplified a 149 bp fragment in all 12 oat cultivars, but not in fungal isolates (Fig. 1). Cq-values from oat β-actin TaqMan® primers and probe set ranged from 28.0 to 32.2 when using the template from the oat cultivars, whereas fluorescence did not reach the cycle

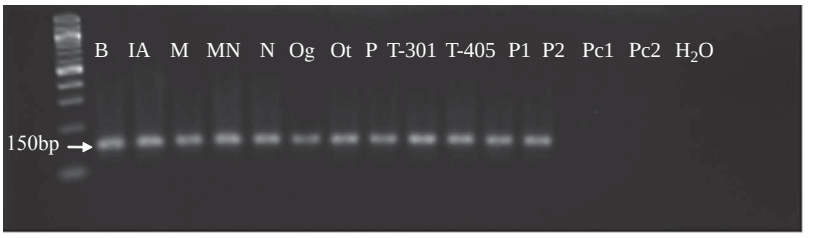

Fig. 1. Assessment of specificity to β-actin of a TaqMan primer set on 12 oat lines and two *Puccinia coronata* isolates using conventional polymerase chain reaction (PCR) analysis and PCR product visualization. B = 'CDC Boyer', IA = IA98822-2, M = 'Makuru', MN = MN841801, N = 'Noble-2', Og = 'Ogle1040', Ot = 'Otana', P = 'Provena', T-301 = TAM 301, T-405 = TAM 405, P1 = 8669, P2 = P94163, Pc1 = *P. coronata* race LSLG, Pc2 = *P. coronata* race LGCG.

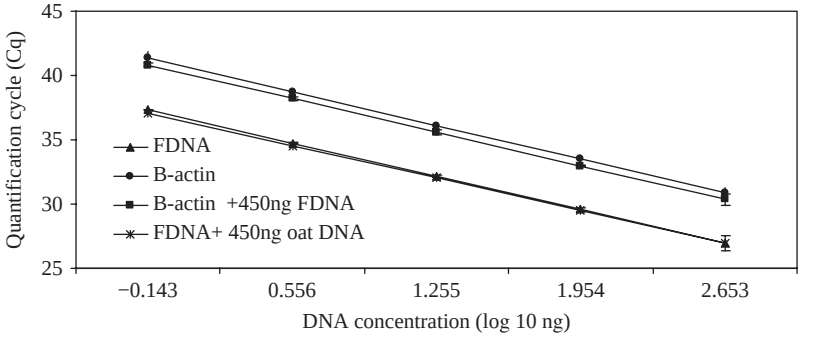

Fig. 2. Efficiency curve for fungal DNA and oat DNA (oat β-actin) across a five-fold serial dilution in the presence and absence of 450 ng of oat DNA and fungal DNA, respectively. Oat β-actin, y = –3.579x + 40.266 R^2 = 0.996; oat β-actin + 450 ng FDNA, y = –3.767x + 40.853 R^2= 0.991; *P. coronata* DNA (FDNA), y = –3.693x + 36.785 R^2 = 0.996; *P. coronata* (FDNA) + 450 ng oat DNA, y = –3.622x + 36.559 R^2 = 0.999.

threshold when using template from the *P. coronata* isolates (Table 1). The mean amplification efficiency for both individual and multiplexed FDNA and HDNA q-PCR reactions was 95%. Cq-values for FDNA and HDNA did not differ (P > 0.05) when 450 ng of oat or fungal DNA were added to the respective reactions (Fig. 2).

Evaluation of q-PCR assays on oat cultivars with different levels of crown rust resistance

Differences on mean disease reaction to *P. coronata* race LGCG were evident across the 12 oat cultivars for all three disease measurements (Table 2). In the first experiment, mean comparisons of DLA and RFDNA were able to separate the cultivars into five distinct groups while the AFDNA assessment detected only four groups. Changes in ranking order between the cultivars across disease measurements within or between experiments were evident mostly between cultivars showing intermediate levels of DLA, AFDNA and RFDA. The overall major trends were consistent as all three assays consistently detected higher levels of resistance in the oat cultivars TAM-O-405, P94163, TAM-O-301 and IA98822-2, whereas 'Provena' showed higher levels of susceptibility to LGCG. In the first experiment, 'Ogle-1040' had the highest mean values for DLA and AFDNA but ranked in a lower category for RFDNA. Differences between cultivars were less significant in the second experiment. DLA and AFDNA separated the cultivars into three different groups, while RFDNA separated the cultivars into four distinct groups.

Evaluation of q-PCR assays on recombinant inbred lines in a polycyclic single-race field experiment

Resistance (no disease signs or symptoms) and susceptibility were consistent in all plants of P94163 and P8669, respectively. Comparisons between the eight different q-PCR runs used to estimate RFDNA in the population were possible without additional calibration since the standard deviation around the mean efficiency line equations for AFDNA (slope = –3.52, SD = 0.12; intercept = 37.61, SD = 0.31; R^2 = 0.99, SD = 0.005) and HDNA (slope = –3.27, SD = 0.06; intercept = 36.73, SD = 0.15, R^2 = 0.99, SD = 0.005) standards were low (Fig. 3). Highly significant differences (P < 0.0001) were observed between the two parent lines for all three disease measurements in both replicates (Table 3). DLA,

Table 2. Mean diseased leaf area DLA, absolute fungal DNA (AFDNA) and relative FDNA (RFDNA) of oat cultivars used to evaluate effectiveness of the three methods in detecting differences in response to *Puccinia coronata* race LGCG in two greenhouse experiments[w].

	Experiment 1						Experiment 2					
Cultivar	DLA (%)[x]		AFDNA (ng/mg)[y]		RFDNA[z]		DLA (%)[x]		AFDNA (ng/mg)[y]		RFDNA[z]	
'Ogle-1040'	33.3	a	19.8	a	22.4	bcd	17.04	a	11.22	ab	29.95	a
'Provena'	30.6	a	7.8	abcd	37.6	a	9.24	abc	18.99	a	32.71	ab
P8669	23.3	ab	18.2	ab	23.7	ab	20.45	a	3.50	bc	8.26	cd
'Otana'	23.0	ab	14.4	abc	24.0	ab	9.50	abc	11.15	abc	21.10	abc
'Noble'-2	21.3	abc	17.2	abc	16.9	bcde	9.66	ab	11.80	abc	17.76	abcd
MN841801-1	12.7	bcd	9.2	bcd	6.3	de	19.23	a	1.80	bc	9.96	bcd
'CDC Boyer'	10.8	cd	14.6	abc	10.0	de	12.36	ab	10.79	ab	23.93	abc
'Makuru'	9.9	cde	7.0	cd	10.9	cde	2.33	bc	8.50	abc	10.46	bcd
TAM-O-301	3.2	de	2.0	d	0.7	e	0.54	bc	0.34	c	1.65	cd
IA98822-2	1.8	de	0.7	d	0.8	e	3.57	abc	4.80	abc	3.13	cd
TAM-O-405	0.1	e	0.7	d	0.2	e	0.03	bc	2.78	bc	16.79	abcd
P94163	0.0	e	2.4	cd	1.4	e	0.07	c	1.06	c	0.55	d

Notes: [w]Means within the same column followed by the same letter are not significantly different ($P < 0.05$) based on Tukey's means comparison test.
[x]Mean per cent of diseased leaf area was based on digital image analysis of four cm sections of 4 leaves each from three replicates using Assess image analysis software.
[y]Mean square root of AFDNA was based on ng FDNA per mg of oat tissue. DNA was extracted from 4 cm sections of four leaves from each of three replicates. Each reaction was performed in duplicate.
[z]Mean square root of RFDNA was calculated using the equation $2^{(Cq\beta - Cqp)}$ where $Cq\beta$ is the quantification cycle for the oat β-actin amplification and Cqp is the quantification cycle for the pathogen DNA amplification in each reaction. DNA was extracted from 4 cm sections of four leaves from each of three replicates. Each reaction was performed in duplicate.

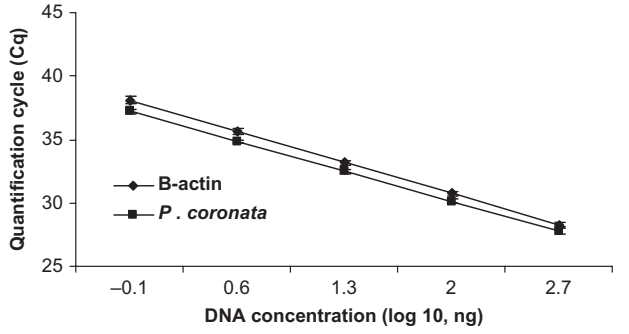

Fig. 3. Efficiency curve for fungal DNA and oat DNA (oat β-actin) from a five-fold serial dilution in duplex. Lines represent the mean value for the eight separate reactions performed for the amplification of all infected tissue samples collected from *P. coronata* single isolate field experiment. Oat β-actin, $y = -3.5x + 37.61$, $R^2 = 0.993$; *P. coronata*, $y = -3.4x + 36.73$, $R^2 = 0.991$.

AFDNA and RFDNA distributions of RIL were skewed toward the resistant parent with most of the RILs positioned between the parental lines (Table 3). RFDNA results on samples collected from the population were highly correlated with the AFDNA estimates and DLA assessments (Table 4). Higher correlations were observed between replicates of DLA estimates than for replicates of AFDNA and RFDNA.

Segregation ratios using AFDNA and RFDNA disease assessments did not differ significantly from the expected 1:1 resistant/susceptible (44R:44S) Mendelian model for a single gene controlling crown rust resistance against LSLG (Table 5). When each replicate was analyzed separately the DLA ratio did not fit the expected ratio of one gene controlling crown rust resistance to LSLG ($P = 0.03$, 0.001). However, when DLA estimates for both replicates were combined and RIL were re-classified based on the parents' DLA mean, a ratio not different from the expected 1:1 (36:51 resistant; susceptible) was observed (Table 5).

The six phenotypic markers were grouped together with 30 DArT makers using multipoint linkage analysis. The best maker order of the group placed all six phenotypes into an 18.9cM cluster 20.3cM from the nearest DArT marker oPt13431 (Fig. 4). Overall, the marker of the DLA phenotypes varied by 4.1cM while the marker of the AFDNA and RFDNA phenotypes varied by 4.2cM and 6.8cM, respectively (Fig. 4). The variation in map position between AFDNA and RFDNA did not vary within replicates (1.3cM for rep1 and 1.3cM for rep 2), however, the variations for both AFDNA and RFDNA

Table 3. Mean values for diseased leaf area (DLA) from digital images, relative fungal DNA (RFDNA), and absolute fungal DNA (AFDNA) of the two parents P8669 and P94163 and 88 recombinant inbred lines (RIL) infected tissue samples from two replicates tested with *P.coronata* race LGCG in the field.

		Parents		RIL	
	Disease measurement	P8669	P99163	Mean ± SD	Range
Replicate1	Mean RFDNA	28.39	0.58	4.76 ± 8.42	0.00–38.72
	Mean FDNA by weight (ng/mg)	14849.45	16.27	1762.83 ± 2622.79	0.00–13870.00
	DLA (%)	69.42	0.43	13.0 ± 15.18	0.01–56.16
Replicate 2	Mean RFDNA	22.69	0.05	4.63 ± 8.71	0.00–50.11
	Mean FDNA by weight (ng/mg)	14261.01	68.50	1551.92 ± 2240.27	0.00–9870.50
	DLA (%)	84.76	1.59	13.94 ±15.73	0.04–60.15

Table 4. Pearson's correlation coefficients between diseased leaf area (DLA) from digital images, relative fungal DNA (RFDNA) and absolute fungal DNA (AFDNA) from two different replicates of data collected from single race field inoculation with *P. coronata* race LGCG.

Disease measurement	DLA1[a]	DLA2[a]	AFDNA1	AFDNA2	RFDNA1
DLA2	0.98[b]				
AFDNA1	0.84	0.85			
AFDNA2	0.80	0.82	0.83		
RFDNA1	0.88	0.89	0.93	0.75	
RFDNA2	0.80	0.84	0.81	0.94	0.75

Notes: [a]Number to the right of the disease measurement refers to the replicate. [b]All pairwise correlations were significant at the <0.0001 level of probability.

were different from DLA (> 8cM for rep 1 and >17.6cM for rep 2).

Discussion

Characterization and measurement of disease resistance can be complicated by effects of environment, plant growth stage, pathogen specificity, and the precision in which various levels of resistance can be quantified. Jackson *et al*. (2006) developed an 'absolute' q-PCR assay to estimate the amount of fungal DNA content in host tissue and successfully used the assay to more accurately define crown rust resistance in single-race field tests compared to conventional methods of assessment. Two drawbacks of the absolute q-PCR method are the time required to obtain dry weights for each tissue sample and the need for uniform DNA extractions resulting in similar concentrations for each sample so that additional DNA concentration manipulations are not required before the assay. Omitting these tedious steps could result in large error. To reduce these complexities, we converted the current assay to a relative assay which estimates the amount of fungal DNA relative to the amount of host DNA.

In this study, we converted the absolute assay to a relative assay by combining the previously developed fungal TaqMan® primers/probe set with a new TaqMan® primers/probe set designed to measure the concentration of the oat β-actin gene in the tissue samples. β-actin was used as a reference gene in our study and served as a DNA normalizer and internal DNA standard. Reference genes are commonly used as normalizers and internal controls in q-PCR assays (Livak & Schmittgen, 2001).

The standard curves of both AFDNA and HDNA developed for both reactions show that it is possible to reliably detect and quantify DNA over five orders of magnitude. Additionally, since aliquots from all concentrations of both original series were included in each test and the standard deviations from the mean efficiency equation line were low, comparison between samples from different tests were possible without the use of calibrator DNA in each plate.

To accurately quantify the pathogen DNA in host tissue in a single well, we used two different reporter dyes for the probes and both reactions had similar efficiencies. The reaction with pathogen DNA was unaffected by the presence of host DNA and the reaction with host DNA was unaffected by the presence of pathogen DNA (Figs. 2 and 3). Moreover, by having similar efficiencies for both reactions, we were able to report our results using an adapted version of the $2^{-\Delta\Delta Cq}$ method recommended for analysis of relative gene expression assays (Livak & Schmittgen, 2001). Reporting the results in the form of $2^{(Cq\beta-Cqp)}$allowed us to evaluate a linear form of the exponential amplification of both DNAs.

Highly significant correlations between DLA, AFDNA and RFDNA estimates suggested that all three methods are similar in their ability to detect differences in disease

Table 5. Crown rust reaction of the recombinant inbred lines of the P8669/P94163 cross based on diseased leaf area (DLA), absolute fungal DNA (AFDNA) and relative fungal DNA (RFDNA).

		Number of plants			
	Disease measurement	Resistant	Susceptible	χ^2(1:1)	P value[a]
Replicate 1	DLA	33	53	4.65	0.05–0.025
	AFDNA	44	44	0.0	1.00
	RFDNA	46	42	0.18	0.90–0.10
Replicate 2	DLA	29	59	10.2	0.005–0.001
	AFDNA	43	43	0.0	1.00
	RFDNA	45	41	0.19	0.90–0.10
Overall	DLA	36	51	2.58	0.90–0.10
	AFDNA	42	45	0.10	0.90–0.10
	RFDNA	42	45	0.10	0.90–0.10

Note: [a]Probability from χ^2test for goodness-of-fit of data to the expected ratio.

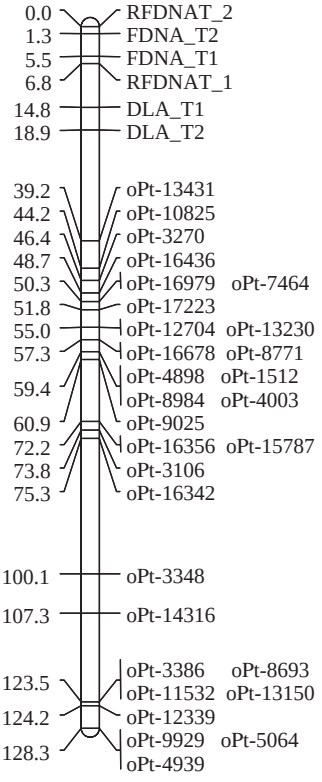

Fig. 4. Genetic linkage map of disease resistance loci in the P8669/P94163 population. oPt markers are based on DArT loci from the oat DArT array. Genetic distances on the left are in Kosambi. Phenotype markers are based on disease assessment using diseased leaf area (DLA), fungal DNA with absolute q-PCR (FDNA) and fungal DNA using relative q-PCR (RFDNA).

reactions. However, the RFDNA consistently detected smaller differences than the AFDNA assay resulting in higher numbers of groups or clusters of cultivars with significantly different levels of resistance in both experiments than the DLA in the second experiment. The RFDNA method likely discriminates levels of resistance more finely than the AFDNA estimate because errors associated with quantification and adjustment of DNA concentration and measuring sample weights are eliminated by using host DNA as a normalizer. Despite the high sensitivity of the RFDNA method in detecting difference between cultivars, DLA estimation showed higher correlations between replicates in field experiments. It is possible that due to lower resolution, DLA estimation does not account for smaller differences between sample replicates. DLA accounts only for symptoms and signs observed on the surface of the leaf, whereas AFDNA and RFDNA values represent the total biomass of the fungus present, including urediniospores in pustules and mycelial growth within the host tissue. Since crown rust resistance can vary with plant growth stage (Heagle & Moore, 1970; Lee & Shaner, 1982; Luke *et al*., 1984; Brake & Irwin, 1992), plant age and time from inoculation must be standardized when measuring the resistance of different cultivars or lines, as was done in the present study. One potential criticism of the RFDNA measure is that it does not account for any differences in DNA content between cultivars per unit host tissue. Although such effects should be small based on the correlation between RFDNA and AFDNA results, further work will be necessary to quantify these potential effects.

In the greenhouse, major trends were consistent between methods and between experiments comparing the 12 cultivars differing in their response to LGCG. For example, the oat cultivars ‘Provena’ and ‘Ogle 1040’ were consistently more susceptible than the resistant TAM-O-301, IA98822-2, TAM-O-405 and P94163

(Table 2). In a similar study, Jackson *et al*. (2006) reported higher susceptibility of 'Provena' compared with IA98822-2 and TAM-O-301 using a different *P. coronata* race.

Based on the segregation of the RIL for AFDNA and RFDNA for each replicate, a single gene is proposed as controlling the resistance to crown rust race LGCG in the P8669/P94163 population. The lack of fit to a 1:1 ratio by DLA in individual replicates may be due to the limitation of the DLA estimation method based on digital images. The software used for DLA estimation relies on differentiation of colour contrast from digital images, which in some cases may result in over-estimation of DLA if tissue quality or colour contrast is not adequate. However, when the data for both replicates was combined, a 1:1 ratio was observed. For breeding and marker assisted selection purposes using the overall data (mean values from both replicates combined) may be more informative since it takes into account intra-experiment variability. The one-gene model is supported by the placement of all six phenotypic markers together in one location.

A major limitation of genetic mapping in this study was the population size. As with many genetic studies, the number of markers used (n = 437) greatly exceeded the resolution of recombination for the population size (88 RILs). Based on the theoretical resolution of recombination in the current study, we could only resolve 2cM accurately on our genetic map. Taking this into account, the variation for all three measurements was similar. The FDNA phenotypes were placed in the same map location which was 4 to 8cM from the DLA phenotype. Previous QTL mapping studies using AFNDA (Jackson *et al*., 2008) and RFDNA (Acevedo *et al*., 2010) to map partial crown rust resistance showed that the FDNA measurements identified QTL with higher LOD values and reduced intervals compared to DLA. Thus, the placement of phenotypes based on FDNA estimates are probably more accurate than those placed based on DLA measurement. Further work should be done with larger population sizes and more replications to confirm this conclusion.

The high correlations among the three disease resistance assessments demonstrate that the RFDNA assay can be utilized as an accurate and easier technique compared with AFDNA for quantifying pathogen content in tissue as a measure of disease resistance. Since the RFDNA assay allowed quantification of pathogen DNA over a concentration range of five orders of magnitude, the method can be used to detect disease resistance at different infection stages as well as to evaluate genotypes with widely differing degrees of resistance. Future work will be done to evaluate the usefulness of the RFDNA method for mapping resistance loci, including quantitative trait loci associated with disease resistance.

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