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ORIGINAL PAPER

Genetic analysis and molecular mapping of a seedling crown rust resistance gene in oat

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Abstract

Key message **Genetic analysis and genome mapping of a major seedling oat crown rust resistance gene, designated *PcKM,* are described. The chromosomal location of the *PcKM* gene was identified and linked markers were validated.**

Abstract Crown rust (*Puccinia coronata* Corda f. sp. *avenae* Eriks) is the most important foliar disease of oats and can cause considerable yield loss in the absence of appropriate management practices. Utilization of novel resistant genes is the most effective, economic and environmentally sound approach to control the disease. Crown rust resistance present in the cultivar 'Morton' was evaluated in a population developed from the cross OT3019 × 'Morton' to elucidate the genetic basis of resistance. Crown rust reaction evaluated in field nurseries and greenhouse tests demonstrated that resistance provided by 'Morton' was controlled by a single gene, temporarily designated as *PcKM*. The gene was initially linked to a random amplified polymorphic DNA band and subsequently converted into a sequence characterized amplified region (SCAR) marker. Genotyping with the *PcKM* SCAR on the 'Kanota' × 'Ogle' population, used to create the first oat chromosome-anchored linkage map, placed the *PcKM* gene on chromosome 12D. Consensus map markers present in the same region as the *PcKM* SCAR were tested on the OT3019 × 'Morton' population and two additional phenotyped populations segregating for *PcKM* to identify other markers useful for marker-assisted selection. Three markers were perfectly linked to the *PcKM* phenotype from which TaqMan and KBioscience competitive allele-specific PCR assays were developed and validated on a set of 25 oat lines. The assays correctly identified *PcKM* carriers. The markers developed in this study will facilitate fine mapping of the *PcKM* gene and simplify selection for this crown rust resistance.

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Introduction

Oat (*Avena sativa* L.) is one of the most important cereal crops in the world and has received significant attention in recent years due to its nutritional benefits. Oat contains the soluble fiber β-glucan, which has been linked to reduced risk of coronary heart disease (Maki et al. 2007), and a unique group of antioxidant compounds known as avenanthramides, which help in the prevention of atherosclerosis (Nie et al. 2006). Additionally, oat contains 12–20 % high-quality protein and low fat content (<8 %). For consumers to benefit from these nutritional characteristics, the grain quality and yield potential of the crop must be protected from disease to maximize returns to growers and maintain oat as a viable crop option.

Crown rust, caused by *Puccinia coronata* Corda f. sp. *avenae* Eriks, is the most important disease of oat and can cause severe damage in all regions where oat is cultivated (Simons 1985). In Canada, oat grown in the eastern Prairie region (Manitoba and eastern Saskatchewan) and southern Ontario is highly vulnerable to crown rust. The estimated yield losses averaged 5.1 % annually during the 5-year period from 2001 to 2005 (Chong unpublished data) with the highest loss reported at 11.2 % in 2001 (McCallum et al. 2007). Similarly, yield losses as high as 20 % have been reported in Minnesota and Louisiana (Carson 2011). While cultural control methods, such as crop rotation and early seeding, and fungicide application can reduce crown rust severity, host genetic resistance offers the most effective and economical means to deal with the disease.

The oat crown rust pathosystem displays a gene-for-gene interaction with approximately 100 host resistance genes reported (Gnanesh et al. 2014) and a similarly diverse pathogen virulence spectrum which has been partially characterized using an established nomenclature system (Chong et al. 2000). Initial resistance genes were identified within the cultivated oat gene pool, but since the 1960s most resistance genes have been derived from the hexaploid relative of cultivated oat, *A. sterilis* L. (e.g., *Pc38*, *Pc39*, *Pc48*, *Pc68*). Although these seedling resistance genes are highly effective, the presence of sexual reproduction within the pathogen has resulted in the creation of new virulent races which consistently overcome these genes within a few years of widespread use (McCallum et al. 2007). More recently, crown rust resistance genes have been derived from other *Avena* species, such as *Pc94* from the diploid species *A. strigosa* Shreb. (Aung et al. 1996) and *Pc91* from the tetraploid species *Avena magna* Murphy and Terrell (Rothman 1984). However, Carson (2009a) noted that no evidence exists to demonstrate that seedling resistance genes from diploid or tetraploid *Avena* species are more durable than those resistance genes from *A. sterilis*. Evidence to support this may be seen from Canadian isolates collected from both wild and cultivated oat that were virulent to either *Pc94* or *Pc91* (Chong et al. 2011). Similarly, one isolate collected from Spain was also found to be virulent against *Pc94* (Sánchez-Martín et al. 2012).

The ability of *P. coronata* to produce new races capable of overcoming previously effective resistance genes has led to a continuous search for new and effective sources of rust resistance to minimize yield losses (Cabral et al. 2014). Segregation of different resistance genes to defined geographic regions, gene pyramiding, creation of multilines (Carson 2009b) and incorporating adult plant resistance (Portyanko et al. 2005) are proposed strategies to increase the durability of resistance. To effectively and efficiently implement these approaches, it is necessary to genetically map the location of resistance genes within the oat genome. For example, it is often difficult to identify individual genes within an oat line containing several resistance genes, because crown rust isolates with the appropriate virulence combinations may not exist.

Molecular markers for more than 18 oat crown rust resistance genes have been reported (Gnanesh et al. 2014). Many of these markers, such as the random amplified polymorphic DNA (RAPD) markers linked to *Pc68* (Penner et al. 1993) or the random fragment length polymorphisms (RFLP) markers linked to *Pc71* (Bush and Wise 1998), have not been incorporated into high-throughput genotyping platforms and thus have not been widely used. In addition, the chromosome location of these crown rust resistance genes has not been determined, because only recently has a chromosome-anchored linkage map containing 21 linkage groups been available (Oliver et al. 2013). As a result, determining allelic relationships among the various resistance genes has been hindered. However, the recent availability of abundant single nucleotide polymorphism (SNP) markers and accompanying assays (e.g., Infinium HD and KASP) has allowed the position of crown rust resistance genes within the oat genome to be defined, for example *Pc91* (Gnanesh et al. 2013), *Pc94* (Gnanesh personal communication) and an adult plant resistance gene derived from MN841801 (Lin et al. 2014).

Understanding the genetic basis of resistance to *P. coronata* and developing molecular markers linked to crown rust resistance are important milestones in effectively deploying crown rust resistance genes in breeding programs. The objectives of this research were to: (1) study the inheritance of a major seedling resistance gene initially identified in the cultivar 'Morton', (2) genetically map the resistance gene to a specific oat chromosome, (3) develop allele-specific PCR assays to the resistance gene for the purpose of marker-assisted selection (MAS) and (4) evaluate the utility of the assays in a panel of oat germplasm.

Materials and methods

Mapping populations

Segregation of the seedling crown rust resistance gene was studied in three F_5-derived RIL populations, derived from the crosses OT3019 × 'Morton', 'CDC Weaver' × 'Kame' and OT9001 × OT3060, with population sizes of 100, 72 and 72 lines, respectively. This gene will be subsequently referred to using the temporary designation *PcKM*. The populations were developed at the Crop Development Centre (CDC), University of Saskatchewan (Saskatoon, SK).

Table 1 Oat lines used to validate the utility of *PcKM*-linked markers

Line	Pedigree	*PcKM* status[a]
SA130054	99Ab11787/SA081139	Carrier
SA081139	OT3019/'Morton'	Carrier
MN07104	'Morton'/IL95-1241	Carrier
'Morton'	ND880922/B605X	Carrier
'Kame'	B605X//'Dane'/'Newdak'	Carrier
OT3060	OT3019/'Morton'	Carrier
OT3019	W97601/SA96482	Not carrier
'CDC Weaver'	OT369/W95116	Not carrier
OT9001	SW96290/SW00263	Not carrier
'AC Medallion'	Pc68/7*'Dumont'	Not carrier
'Makuru'	'Forward'/'Milford'	Not carrier
MN841801	'Florad'/Coker 58-7/3/CI7558//'Black Mesdag'/Aberdeen 101	Not carrier
'AC Assiniboia'	Pc68/7*'Robert'	Not carrier
'HiFi'	'Amagalon'/ND820712//ND852107/3/ND900118	Not carrier
'Stainless'	ND931475/'AC Assiniboia'//'HiFi'	Not carrier
'Newburg'	'HiFi'/ND990232	Not carrier
'AC Morgan'	OT526//'Fidler'/'Cascade'	Not carrier
'Leggett'	OT294/Pc94	Not carrier
OT3033	OT293/'CDC Dancer'	Not carrier
S42	Sun II*7/RL1697	Not carrier
'Summit'	'AC Ronald'/OT299	Not carrier
OT3024	'AC Assiniboia'/S42//'CDC Dancer'	Not carrier
'Ronald'	W89329 (dwarf)/'AC Medallion'	Not carrier
'CDC Sol-Fi'	N979-5-1/OT366	Not carrier
'CDC Dancer'	OT344/W90279	Not carrier

[a] Status based on reaction to crown rust isolate CR258 in the greenhouse. Carrier indicates the allele associated with *PcKM* resistance; Not carrier indicates the allele not associated with *PcKM* resistance

'Morton', 'Kame' and OT3060 are carriers of *PcKM*, while OT3019, 'CDC Weaver' and OT9001 lack the *PcKM* gene. 'Morton' is a variety from North Dakota State University (Fargo, ND) with the pedigree ND880922 × B605X (McMullen et al. 2005), while 'Kame' is a variety bred at the University of Wisconsin (Madison, WI, USA) and is derived from the cross B605X × ('Dane' × 'Newdak'). OT3060 is an advanced breeding line from the CDC with the pedigree OT3019 × 'Morton'. OT3019 is also an advanced breeding line from the CDC with the pedigree W97601 × SA96482, while 'CDC Weaver' is a registered variety from the CDC with the pedigree OT369 × W95116. OT9001 is an advanced breeding line from Lantmännen (Svalov, Sweden) with the pedigree SW96290 × SW00263.

To validate the utility of newly developed allele-specific *PcKM* marker assays, a set of 25 oat lines (6 resistant lines known to carry *PcKM* and 19 susceptible cultivars which are non-carriers for *PcKM*) were used. Details pertaining to these oat lines are presented in Table 1. The marker assays were also evaluated on 19 oat crown rust differential lines grown in the Guelph crown rust field nursery (Table 2) and an *A. sterilis* accession (F169; CAV5050), from which *Pc45* was originally identified, to determine the identity of the *PcKM* gene.

Phenotyping

Field screening

Crown rust reaction and severity were evaluated in the OT3019 × 'Morton' population in crown rust nurseries located at Guelph, Ontario, Canada (2008, 2009, 2010) and Saskatoon, Saskatchewan, Canada (2009, 2010). The 'CDC Weaver' × 'Kame' population was evaluated in the same nurseries except the Guelph and Saskatoon nurseries in 2010. The nurseries grown in 2008 and 2009 consisted of a single replication at each location, while the 2010 Guelph and Saskatoon nurseries consisted of two replications grown in an alpha lattice design. Each line evaluated was grown as a hill plot consisting of 30 seeds.

The Guelph nursery was inoculated with aeciospores produced on common buckthorn (*Rhamnus cathartica* L.). The spreader rows were seeded to a mixture of susceptible varieties. Nineteen oat differential lines carrying different resistance genes were grown as controls in the nurseries (Table 2), as was the highly susceptible variety 'AC Morgan' which does not carry major resistance genes. In the Saskatoon nursery, spreader rows were seeded to 'AC Morgan'. The Saskatoon nurseries were inoculated with a

Table 2 Crown rust reactions and *PcKM*SNP1 TaqMan assay genotypes of 19 oat differential lines grown in the Guelph crown rust field nursery over 5 years

Differential line	Crown rust reaction[a]					*PcKM* status[b]
	2008	2009	2010	2011	2013	
Pc38	8	7	7	9	8	Carrier (het)
Pc39	8	5	6	9	6	Not carrier
Pc40	8	3	6	6	4	Carrier
Pc45	4	2	2	4	1	Carrier
Pc46	7	3	7	8	6	Not carrier
Pc48	7	NA	7	9	8	Not carrier
Pc50	3	1	3	8	NA	Not carrier
Pc51	6	1	6	8	7	Not carrier
Pc52	7	4	7	7	8	Not carrier
Pc54	6	1	3	6	7	Not carrier
Pc56	9	4	6	9	9	Carrier (het)
Pc58	3	1	1	3	0	Not carrier
Pc59	4	0	4	7	6	Carrier
Pc62	6	3	3	2	6	Not carrier
Pc64	5	1	2	2	3	Not carrier
Pc68	8	6	7	8	NA	Not carrier
Pc91	1	1	0	1	1	Not carrier
Pc94	1	0	0	7	7	Not carrier
Pc96	6	1	0	3	8	Not carrier

[a] Lines were rated on a 0–9 scale (0–4: resistant; 5–9: susceptible). NA indicates the line died and was not rated

[b] Status based on marker assay genotype. Carrier: indicates the allele associated with *PcKM* resistance, Not carrier: indicates the allele not associated with *PcKM* resistance, het: indicates heterozygous

mixture of urediniospores of the local *P. coronata* isolates. Urediniospores were suspended in Bayol® (Esso Canada, Toronto, ON, Canada) and inoculated with a Micron Micro fit Herbi Flex herbicide applicator (Micron Sprayers Ltd, Bromyard, UK). Oat differential lines carrying *Pc91* and *Pc94* were also grown as controls in the nursery. At the Guelph nursery, crown rust reaction and severity were evaluated on a 0–9 scale: 0, 1 and 2 were resistant with a few uredinia on the lower leaves; 3 and 4 had additional uredinia, but not over halfway up the plant; 5 and 6 had some uredinia on the flag leaf and were susceptible; 7, 8 and 9 were highly susceptible, with varying levels of uredinia on the flag leaf. For the purpose of classifying lines, those rated 0–4 were considered resistant, while those rated 5–9 were considered susceptible. In Saskatoon, crown rust severity was evaluated with the modified Cobb scale (Peterson et al. 1948) and infection type. The modified Cobb scale described the percentage of infected leaf area using the following categories: tr (trace), 1, 5, 10 and then increments of 5 up to 100. Infection type which described lesion development on the leaf was expressed as follows: 0, immune; R, resistant with necrotic flecks; MR, moderately resistant with small uredinia surrounded by necrosis/chlorosis; MS, moderately susceptible with medium to large uredinia surrounded by chlorosis; and S, susceptible with large uredinia lacking chlorosis or necrosis. A combination of infection types (e.g., RMR) was used to rate lines that fell between the categories. For the purpose of classifying lines, those rated MR or lower were considered resistant, while those rated MRMS or higher were considered susceptible. In both the Guelph and Saskatoon nurseries, lines were rated for crown rust reaction once the panicles had emerged from the boot (Zadok's growth stages 51–59).

Greenhouse screening

Seedling tests were carried out on all three RIL populations (OT3019 × 'Morton', 'CDC Weaver' × 'Kame' and OT9001 × OT3060) at the Cereal Research Centre (Winnipeg, MB, Canada) in 2013. *Puccinia coronata* f. sp. *avenae* isolate CR258 (race NTGG) was used to test segregation of *PcKM* in the greenhouse. Seedlings at the one-leaf stage were inoculated by spraying urediniospores (4 mg/450 µl) suspended in Bayol® on the leaves. Approximately, ten seedlings from each RIL were inoculated. The inoculated plants were incubated in a Percival model I-60D dew chamber (Percival Scientific, Perry, IA, USA) overnight at 15 °C and then grown in a greenhouse maintained at a temperature between 18 and 22 °C with 16 h of daylight supplemented with high-pressure sodium lighting (approximately, 300 µmol m^{-2} s^{-1}). Crown rust infection types (ITs) were scored 12 days after inoculation on a 0–4 scale (Murphy 1935): 0 (immune), ; (fleck), 1 and 2, resistant; 3 and 4, susceptible. After rust scoring, the infected leaves were trimmed and the plants were raised to the fourth-leaf stage for DNA collection.

Data analysis

The bimodal distribution of crown rust reaction in the populations allowed the lines to be classified as resistant or susceptible. χ^2 analyses were performed for each field and greenhouse environment to determine the goodness-of-fit of the observed crown rust reactions against the expected segregation ratio for a single gene. For the field nurseries, crown rust segregation data were tested against a 1:1 (resistant:susceptible) ratio. Greenhouse crown rust data were evaluated against a 47:47:6 (resistant:susceptible:segregating) ratio in the OT3019 × 'Morton' population and a 34:34:4 (resistant:susceptible:segregating) ratio in the 'CDC Weaver' × 'Kame' and OT9001 × OT3060 populations. These ratios represent the expected segregation numbers from an F_5-derived population. Segregating lines could be evaluated in the greenhouse because single plants were being scored, while field hill plots could not be assessed accurately for the presence of segregation within the line. The genotypic status of each RIL with respect to *PcKM* was deduced from the seedling crown rust reaction data obtained in the greenhouse and used for genetic mapping.

PcKM marker development

Seed of lines from the OT3019 × 'Morton' population was germinated for 5 days on moistened cotton balls and DNA was isolated from coleoptile tissue using a CTAB extraction method (Procunier et al. 1991). Bulked segregant analysis was conducted using DNA from ten resistant lines and ten susceptible lines to form the bulks. Forty 10-mer RAPD primers (Operon Technologies Inc., Alameda, CA, USA; University of British Columbia, Vancouver, BC, Canada) were tested across the bulks. Primers identified as being polymorphic between the bulks were tested across the entire set of lines in the population.

RAPD PCR was carried out in 25-µl reactions containing 20 mM TrisHCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 0.6 µM of primer, 1 unit of *Taq* polymerase (Life Technologies, Grand Island, NY, USA) and 100 ng of DNA. Cycling consisted of an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 60 s, 37 °C for 60 s and 72 °C for 90 s, followed by a final hold at 72 °C for 3 min. PCR products were loaded on 1 % agarose gels containing ethidium bromide at 0.1 µg/ml and electrophoresed in 0.5× Tris-borate-EDTA (TBE) for 2 h at 115 V. Gels were photographed under UV light.

The polymorphic fragment linked to the *PcKM* gene was excised from the agarose gel, transferred to a 1.5-ml microfuge tube and allowed to soak in distilled water for 15 min. An aliquot of the soaking solution was then used to re-amplify the marker using the same PCR cocktail and conditions as described above. The presence of a single band of the same molecular weight as the original RAPD band was confirmed by running an aliquot of the PCR reaction on an agarose gel. The remaining PCR reaction was used to clone the band using the TA cloning kit (Invitrogen). Vector inserts were sequenced by Sanger Sequencing at the National Research Council (Saskatoon, SK, Canada).

Primers were designed based on the resulting sequence information to amplify the polymorphic fragment. Sequence characterized amplified region (SCAR) PCR was carried out in 25-µl reactions and contained 20 mM TrisHCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 2 µM of each primer, 1 U of *Taq* polymerase and 100 ng of DNA. PCR conditions consisted of an initial hold at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 65 °C for 45 s and 72 °C for 60 s, followed by a final hold at 72 °C for 5 min. PCR products were visualized on agarose gels as described above.

PcKM TaqMan assay

The SCAR marker (named *PcKM*SCAR1) amplified a single fragment of different size in both resistant and susceptible lines and was cloned and sequenced as described above. Sequence comparison identified a T/C substitution which became the basis of a bi-allelic TaqMan® Assay (Life Technologies) which was named *PcKM*SNP1 (Table 3). TaqMan reactions were carried out as 10-µl reactions containing 1× Fermentas Maxima Master Mix, 0.5× TaqMan Minor Groove Binder Assay (Life Technologies) and 1 µl of crude extract prepared from a 2-mm^2 leaf sample (as the source of DNA) based on the method of Klimyuk et al. (1993). Real-time PCR was conducted on the ABI StepOne Plus (Life Technologies, Grand Island, NY, USA) in ABI MicroAmp Fast Optical 96-well reaction Plates with ABI Optical Adhesive Covers. Reaction conditions consisted of a pre-PCR read at 60 °C for 30 s and a hot-start activation at 95 °C for 10 min, followed by 40 cycles of 92 °C for 15 s and 60 °C for 30 s, and finally a post-PCR read at 60 °C for 30 s.

Chromosome assignment and genetic mapping of *PcKM*

The *PcKM*SNP1 TaqMan assay was screened across the 52 RILs from the 'Kanota' × 'Ogle' population used in the creation of the first oat 21 linkage group chromosome-anchored map (Oliver et al. 2013). Marker scores from these lines were entered into MultiPoint (http://www.multiqtl.com) and evaluated in conjunction with 320 polymorphic SNP markers which had already been mapped in this population using the 6K oat Illumina Infinium SNP array (Oliver et al. 2013). The physical chromosome location of the *PcKM* gene was determined based on the previously assigned chromosome location of SNPs present on

Table 3 Primer and probe information for two markers tightly linked to the *PcKM* crown rust resistance gene and validated in a panel of 25 oat lines

Marker name	Marker type	Primer/probe sequences (5′–3′)
*PcKM*SNP1	TaqMan	Forward: TGGGCCTACCTTCTTACTAAGATTG Reverse: TTCGAGTCATGTCATCTACAAATGCA Resistant allele probe: CTAGATATGGAGGAATTG Susceptible allele probe: CTAGATATGGAGGGATTG
I05-0874-KOM16c1	KASP	Common primer 1: CTGCAACAACGCAATACCTTATTGACATT Common primer 2: TTGACATTACATATGCACACAACCAACAAA Resistant allele primer: TAG1-GGAACTGATCACTGTAGCGGGAT[a] Susceptible allele primer: TAG2-GAACTGATCACTGTAGCGGGAC[a]

[a] Note that the 5′ tag sequences are not provided by KBioscience, but are necessary for the assays to function

the array. Using the latest consensus map data provided by Dr. Nick Tinker (AAFC-Ottawa) and Dr. Jessica Schlueter (University of North Carolina at Charlotte), 17 6K oat Infinium SNPs (Tinker et al. 2014) and 3 genotyping-by-sequencing (GBS) SNPs (Huang et al. 2014) surrounding the *PcKM* gene, along with 6 SNPs present within the original RAPD fragment, were converted to KASP assays as described below and screened on all three RIL populations to saturate the region and identify additional markers linked to *PcKM*. Recombination fractions among all *PcKM*-linked markers were calculated using the software program MapDisto v. 1.7 (Lorieux 2012) and converted to centimorgans (cM) using the Kosambi mapping function (Kosambi 1943). Logarithm of odds (LOD) score of 3.0 was used to determine the significance of genetic linkages. MapChart v. 2.1 (Voorrips 2002) was used to construct and align genetic maps for a visual inspection of map order.

Kompetitive Allele Specific PCR (KASP) assay

DNA was prepared from leaf tissue using the DNeasy Plant DNA extraction kit (Qiagen, Toronto, ON, Canada). All 26 SNPs spanning the *PcKM* locus were converted to KASP SNP genotyping technology assays (LGC Genomics LLC, Beverly, MA, USA). Two allele-specific forward primers and two common reverse primers were designed for each SNP. Thirteen assays were polymorphic in at least one of the three RIL populations and are listed in Table 3 and Supplementary Table S1. KASP assays were performed as described by Gnanesh et al. (2013). Fluorescence detection of the reactions was done using an Omega Fluorostar scanner (BMG LABTECH GmbH, Offenburg, Germany) and the data were analyzed using the KlusterCaller 2.23 software (LGC Genomics LLC, Beverly, MA, USA).

Validation of *PcKM* assays and identity of *PcKM* gene

The *PcKM*SNP1 TaqMan assay and four KASP assays (I05-0090-KOM13c1, I05-0874-KOM16c1, GMI_ES02_c16987_268 and GMI_DS_oPt-17084_293) that were linked to the *PcKM* gene in the three RIL populations were evaluated on the panel of 25 oat lines (Table 1) to validate the ability of these assays to predict the *PcKM* phenotype. In addition, the *PcKM*SNP1 TaqMan assay was tested on the 19 oat crown rust differential lines (Table 2) and the *A. sterilis* accession (F169; CAV5050) in an attempt to determine the identity of the *PcKM* gene.

Results

Segregation of *PcKM* resistance

Suitable crown rust infection was observed in all field and greenhouse nurseries where the three RIL populations were evaluated, as indicated by the susceptible ratings obtained on the susceptible control variety ‘AC Morgan’ (Supplementary Table S2). In the Guelph nurseries, the *PcKM* carrying parent of each RIL population (‘Morton’, ‘Kame’, OT3060) displayed strong resistance, while the susceptible parents (OT3019, ‘CDC Weaver’, OT9001) showed high infection levels (Table 4, Supplementary Table S2). Over the 3 years of the study, and in two subsequent years in which lines carrying *PcKM* were evaluated and rated resistant (data not shown), only the *Pc45*, *Pc58* and *Pc91* oat differential lines were also consistently resistant in the Guelph nursery (Table 2). In the Saskatoon 2009 field nursery, ‘Morton’ and ‘Kame’ also displayed strong resistance, but OT3019 and ‘CDC Weaver’ showed only moderate susceptibility (Table 4, Supplementary Table S2). However, in the Saskatoon 2010 field nursery, ‘Morton’ displayed strong resistance, while OT3019 showed a high level of susceptibility (Table 4, Supplementary Table S2). In both 2009 and 2010, the *Pc91* and *Pc94* controls in the Saskatoon nursery were resistant (data not shown). The range of crown rust infection across all nurseries was very wide, encompassing both high levels of resistance and susceptibility for each of the three RIL populations (Table 4, Supplementary Table

Table 4 Crown rust reaction summary data for three recombinant inbred line (RIL) populations (OT3019 × 'Morton', 'CDC Weaver' × 'Kame' and OT9001 × OT3060) segregating for the *PcKM* resistance gene

Population (S × R)	Nursery	Nursery type	Crown rust reaction[a]			*PcKM* segregation				
			Susceptible parent	Resistant parent	RIL population	Resistant	Susceptible	Segregating	χ^{2b}	*P* value
OT3019 × 'Morton'	Guelph 2008	Field	7	1	0–9	50	50	–	0.00	1.00
	Guelph 2009	Field	6	1	1–8	57[c]	41	–	2.61	0.11
	Saskatoon 2009	Field	10MRMS	0	0–85S	50	50	–	0.00	1.00
	Guelph 2010	Field	6	1	0–8	48	52	–	0.16	0.69
	Saskatoon 2010	Field	55S	10R	5R–100S	47[d]	52	–	0.36	0.55
	Winnipeg 2013	Greenhouse	4	0	0–4	49	50	1	4.44	0.11
'CDC Weaver' × 'Kame'	Guelph 2008	Field	8	2	0–8	32	40	–	0.89	0.35
	Guelph 2009	Field	5	2	1–8	32[d]	39	–	0.51	0.48
	Saskatoon 2009	Field	trS	0	0–85S	28	44	–	3.56	0.06
	Winnipeg 2013	Greenhouse	4	0	0–4	31	35	6	1.29	0.61
OT9001 × OT3060	Winnipeg 2013	Greenhouse	4	0	0–4	34	31	7	2.51	0.28

[a] RIL populations were evaluated with mixed inoculum in five field nurseries and with crown rust isolate CR258 in the greenhouse. Guelph 2008, 2009 and 2010 field nurseries rated on a 0–9 scale (0–4: resistant, 5–9: susceptible), Saskatoon 2009 and 2010 field nurseries rated using a modified Cobb scale (Peterson et al. 1948) and infection type (0: immune; R: resistant, MR: moderately resistant, MS: moderately susceptible, S: susceptible), Winnipeg 2013 greenhouse nursery rated on a 0–4 scale (0–2: resistant, 3–4: susceptible; Murphy 1935)

[b] Field nursery data were tested against a 1:1 (resistant:susceptible) segregation ratio. Greenhouse nursery data were tested against a 47:47:6 (resistant:susceptible:segregating) ratio for the OT3019 × 'Morton' population and a 34:34:4 (resistant:susceptible:segregating) ratio for the 'CDC Weaver' × 'Kame' and OT9001 × OT3060. These ratios represent expected segregation numbers from an F_5-derived population

[c] Two lines were not phenotyped (died in field)

[d] One line was not phenotyped (died in field)

S2). However, the susceptible control variety 'AC Morgan' displayed a lower infection level in the Guelph 2009 field nursery than in the other nurseries (Supplementary Table S2) which may account for the higher number of lines classified as resistant in the OT3019 × 'Morton' population (Table 4). Each population displayed a bimodal distribution for resistance and susceptibility across all nurseries, with ratings generally very consistent for a given line (Table 4, Supplementary Table S2). Where discrepancies occurred, this was most often a result of: (1) a segregating phenotype being assigned to a line based on greenhouse data, while a resistant or susceptible phenotype was assigned to the line in a given field nursery, or (2) a line receiving a rating that was near the boundary which differentiated resistance from susceptibility (Supplementary Table S2). χ^2 analysis of the segregation data for each RIL population in all environments indicated that the populations did not significantly deviate from a 1:1 (resistant:susceptible) ratio expected for a single gene (Table 4).

PcKM marker development and TaqMan assay

After screening a total of 40 RAPD primers, Operon primer OP-I05 (5′-TGTTCCACGG-3′) produced a distinct band of approximately 1,300 bp in the susceptible bulk that was absent in the resistant bulk. When this marker was evaluated across the bulk constituent lines, it was observed to be perfectly correlated with crown rust reaction. Upon sequencing the polymorphic fragment and designing primers to specifically amplify the fragment (forward: 5′-ATATGGCCGGT AGACGTGTT-3′; reverse: 5′-AGCCAGGAACTGAAAAAT GG-3′), a co-dominant SCAR marker (*PcKM*SCAR1) pattern was revealed with a band of approximately 1,200 bp segregating with susceptible lines and a band of approximately 1,050 bp segregating with resistant lines (Fig. 1). The basis of this difference in size was a 159-bp insertion present in susceptible lines (Fig. 1).

Sequencing of the original *PcKM*-linked RAPD fragment revealed a number of SNPs. One such polymorphism, a T/C substitution located at position 360 of the consensus sequence, was used to create a TaqMan assay, *PcKM*SNP1 (Fig. 1; Table 3). *PcKM*SNP1 was interrogated across the OT3019 × 'Morton' population and showed perfect agreement with the disease phenotype of the lines.

Chromosome assignment and genetic mapping of *PcKM*

Segregation data from the *PcKM*SNP1 marker in the 'Kanota' × 'Ogle' population indicated that the *PcKM* resistance gene co-segregated with marker

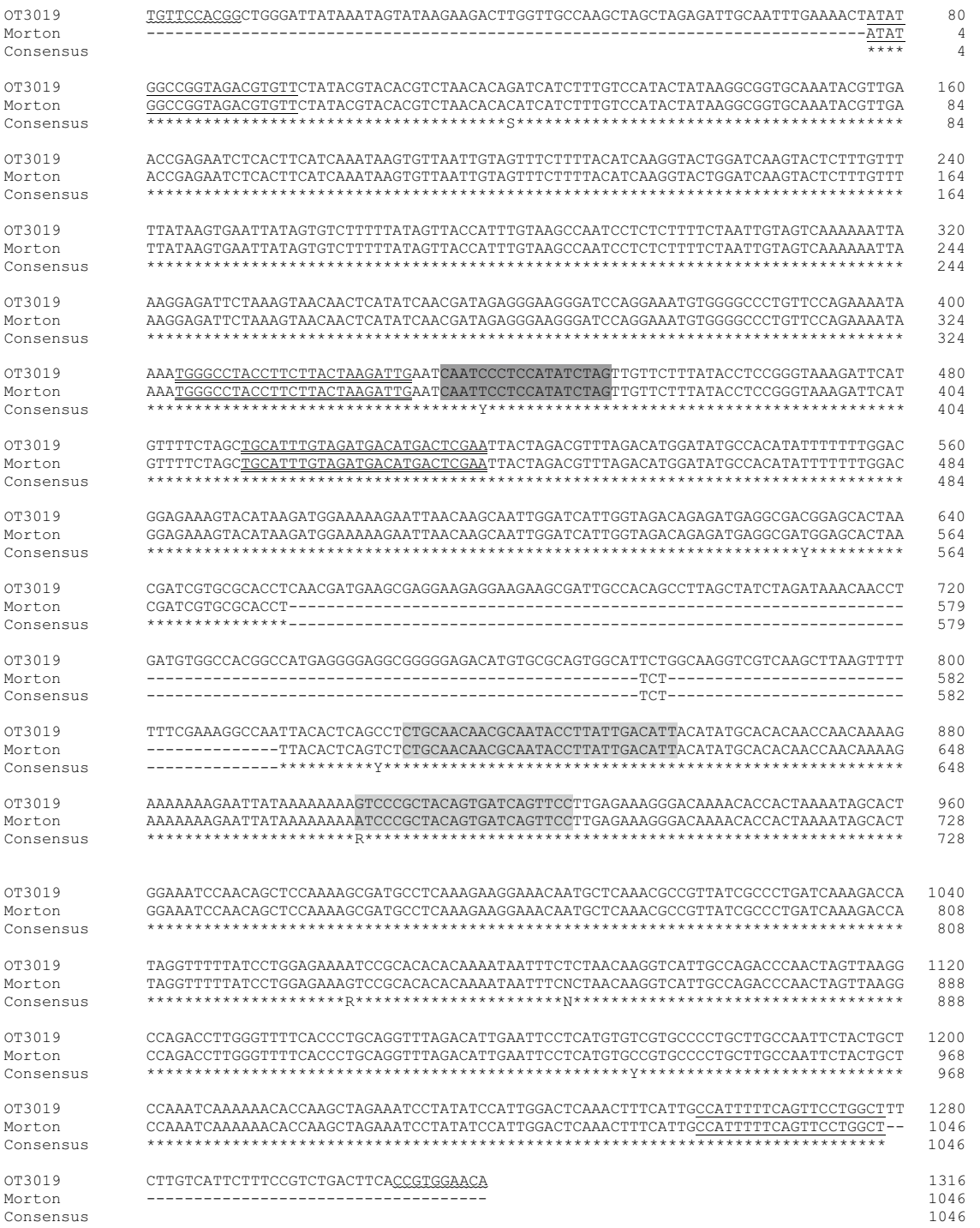

Fig. 1 Sequence alignment of the *PcKM*-linked DNA fragment amplified in OT3019 (*PcKM* non-carrier) and 'Morton' (*PcKM* carrier). The original OP-I05 RAPD primers used to amplify the fragment in OT3019 are *underlined* with a *single wavy line* and the *PcKM*SCAR1 SCAR primers are *underlined* with *single straight lines*. The *PcKM*SNP1 TaqMan primers are *underlined* with *double straight lines* and the allele-specific probes are highlighted in *dark gray shading* with the polymorphic SNP indicated in the consensus sequence. The I05-0874-KOM16c1 KASP assay primers are indicated in *light gray shading* with the polymorphic SNP indicated in the consensus sequence

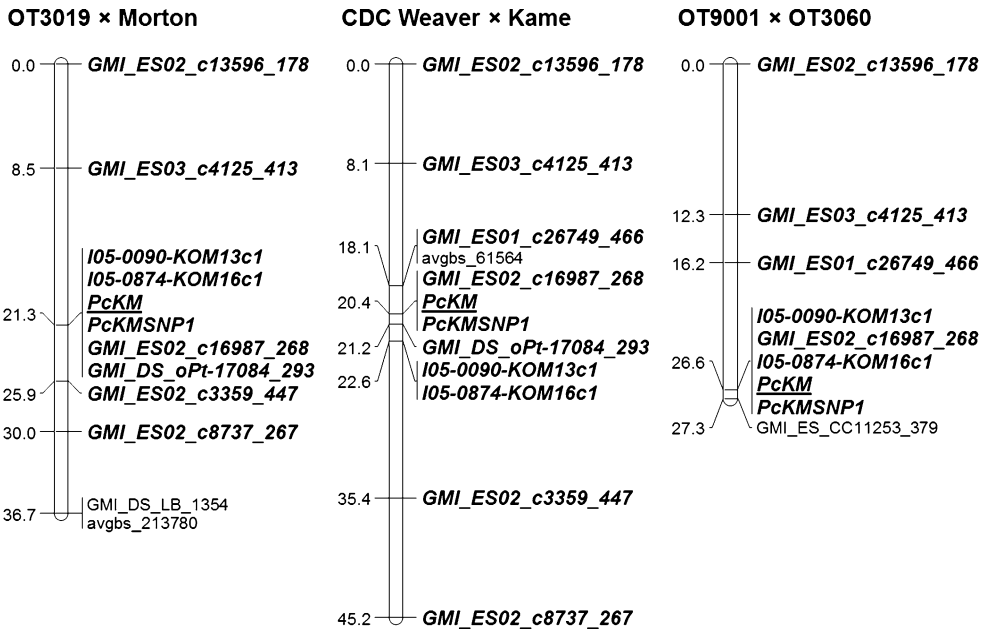

Fig. 2 Genetic linkage maps created in three recombinant inbred line (RIL) populations showing the location of the *PcKM* crown rust resistance gene on oat chromosome 12D. Markers beginning with GMI are from the 6K oat Illumina Infinium SNP array, those beginning with avgbs were identified using genotype by sequencing, those starting with I05 are from the original RAPD marker and the TaqMan marker is denoted *PcKM*SNP1. Markers common to all three populations are indicated in *bold*, *italicized font* and the *PcKM* gene is *underlined*

GMI_ES_CC11253_379 (data not shown) located at position 66.5 cM on oat chromosome 12D (Oliver et al. 2013). Among the various SNPs which resided in the vicinity of the *PcKM*SNP1 marker, two RAPD-derived, nine 6K oat and two GBS SNP-derived KASP assays (Table 3, Supplementary Table S1) were mapped on the OT3019 × 'Morton', 'CDC Weaver' × 'Kame' and OT9001 × OT3060 populations, along with the original *PcKM*SNP1 marker (Table 3), to produce linkage groups containing 11, 11 and 8 markers, respectively (Fig. 2). There was very good correspondence in the order of those markers present on the three linkage groups and the oat consensus map (Fig. 2), confirming that *PcKM* is located on oat chromosome 12D.

Validation of *PcKM*-linked markers

Five markers linked to the *PcKM* phenotype in the three RIL populations (Fig. 2) were evaluated on the panel of 25 oat lines (Table 1) to determine their ability to accurately predict the *PcKM* phenotype. In addition to the *PcKM*SNP1 TaqMan assay mentioned previously, two RAPD-derived KASP assays, I05-0090-KOM13c1 (Supplementary Table S1) and I05-0874-KOM16c1 (Fig. 1; Table 3), based on a G/C and G/A. substitution, respectively, and two 6K oat SNP-derived KASP assays, GMI_ES02_c16987_268 and GMI_DS_oPt-17084_293, based on an A/G and T/C substitution, respectively (Supplementary Table S1), were also tested. The *PcKM*SNP1 TaqMan assay and KASP SNP I05-0874-KOM16c1 accurately predicted the oat lines carrying the *PcKM* resistance gene (Supplementary Fig. S1, Supplementary Table S3), whereas the I05-0090-KOM13c1, GMI_ES02_c16987_268 and GMI_DS_oPt-17084_293 KASP assays failed to accurately predict the presence of the *PcKM* gene within the oat panel (Supplementary Table S3). The I05-0090-KOM13c1 and GMI_ES02_c16987_268 KASP assays both misidentified one line ('AC Assiniboia') as a carrier of *PcKM*, while the GMI_DS_oPt-17084_293 KASP assay misidentified ten lines as *PcKM* carriers.

Screening of the *PcKM*SNP1 TaqMan assay across the 19 differential lines grown in the Guelph crown rust nursery indicated that 5 lines carried the allele (either in the homozygous or heterozygous state) associated with resistance (Table 2). The *Pc45* differential was among these lines. Additionally, the *Pc45* progenitor *A. sterilis* accession (F169; CAV5050) also carried the *PcKM*SNP1 allele linked to resistance (data not shown).

Discussion

The current study identified several markers tightly linked to a major seedling crown rust resistance gene, temporarily designated *PcKM*, and placed the gene on oat chromosome 12D. This information complements recent studies which have mapped the chromosomal location of other crown rust resistance genes, such as *Pc58* (Oliver et al. 2013), *Pc91* (Gnanesh et al. 2013), *Pc94* (Gnanesh personal communication) and an adult plant resistance gene derived from MN841801 (Lin et al. 2014). Collectively, these studies begin the process of understanding the distribution and potential allelic relationships among currently described and future crown rust resistance genes.

The crown rust resistance provided by *PcKM* over the course of this study (and in several subsequent years of

study) was consistently effective in the naturally infected field nurseries in which the populations were grown. Among the 19 crown rust differential lines present in the nurseries, only those lines containing *Pc45*, *Pc58* and *Pc91* were equally effective. These observations indicated that *PcKM* has utility within oat breeding programs (at least within the Canadian oat growing regions) and that efforts should be made to extend the useful life of the gene. Pyramiding *PcKM* with other currently effective major genes, such as *Pc91* and *Pc94*, or with partial resistance genes, such as that found in MN8419801 (Gnanesh et al. 2014), could prolong the durability of *PcKM* resistance. Another possible option, which has been implemented for the orange wheat blossom midge resistance gene *Sm1* (Smith et al. 2004; Vera et al. 2013), would be the use of varietal blends in which a refuge variety within the blend is susceptible to the pathogen. These strategies are needed, since the relatively rapid development of virulence to new and widely distributed crown rust genes has been well documented (McCallum et al. 2007).

PcKM resistance was controlled by a single gene, similar to other seedling crown rust resistance genes such as *Pc38*, *Pc39* and *Pc48* (Wight et al. 2004), *Pc68* (Chen et al. 2006; Satheeskumar et al. 2011), *Pc71* (Bush and Wise 1998), *Pc91* and *Pc92* (McCartney et al. 2011; Rooney et al. 1994) and *Pc94* (Chong et al. 2004; Gnanesh personal communication). Knowing the genomic location of *PcKM* in relation to some of these crown rust genes should complement information about the virulence spectrum effectiveness of resistance genes and help breeders pyramid the best combination of resistance genes. The single gene mode of inheritance is highly amenable to creating gene pyramids and tracking the presence of the gene in varietal blends. Accomplishing these strategies is simplified further by the availability of the *PcKM*SNP1 and I05-0874-KOM16c1 markers. Both markers were very specific to the *PcKM* locus, as demonstrated when they were validated across the 25 oat line panels, which would minimize the occurrence of false positives.

The importance of validating markers potentially linked to *PcKM*, by screening a wider selection of oat lines, was evident by the inability of the I05-0090-KOM13c1, GMI_ES02_c16987_268 and GMI_DS_oPt-17084_293 KASP assays to accurately identify *PcKM* carriers. The poor predictive power of the GMI_DS_oPt-17084_293 KASP assay, which misidentified ten oat lines, is perhaps not surprising since it was also not polymorphic within one of the original three RIL populations (OT9001 × OT3060) used to evaluate *PcKM* resistance. These observations suggest that the GMI_DS_oPt-17084_293 marker is more distantly linked to the *PcKM* gene than indicated by the linkage groups created using the three *PcKM* segregating RIL populations. The misidentification of only one oat line, 'AC Assiniboia', by the I05-0090-KOM13c1 and GMI_ES02_c16987_268 KASP assays indicated that these markers are closer to the *PcKM* gene. It was surprising that the I05-0090-KOM13c1 marker is not completely predictive of the *PcKM* gene in the 25 oat lines, since the SNP on which it is based (position 43 of the consensus sequence in Fig. 1) is located only a few hundred nucleotides from the SNPs underlying the *PcKM*SNP1 and I05-0874-KOM16c1 markers.

For determining if *PcKM* was a novel resistance gene, or one of the approximately 100 named crown rust resistance genes, several lines of evidence suggest that it may be *Pc45*. 'Morton' and 'Kame' share a common parent, IAB605X, which McMullen et al. (2005) indicated was the source of effective crown rust resistance in 'Morton'. Both varieties also share a second parent, RL3038, which contains *Pc38* and *Pc39*. However, these genes are not *PcKM*, because during the breeding process McMullen et al. (2005) selected for crown rust resistance using races which were virulent on these two genes. IA B605X is a breeding line from Iowa State University that was selected from IA B605, a heterogeneous bulk developed from crosses involving multilines 'E70' (Frey et al. 1971a) and 'M70' (Frey et al. 1971b). An increase in virulence to both IA B605X and *Pc45* was noted within the *P. coronata* population surveyed from the US spring oat growing region from 2006 to 2009 (Carson 2011). Carson (2011) also observed a strong correlation among the isolates virulent on both *Pc45* and IA B605X. The virulence to *Pc45* over these years may be explained by the increased presence of 'Morton' and 'Kame' in commercial fields over the same period.

A second observation linking *PcKM* to *Pc45* is based on crown rust reactions of the differential lines grown in the naturally infected Guelph crown rust field nursery. In each of the 3 years of the study, and in two subsequent years in which lines carrying *PcKM* were resistant, the *Pc45* differential line was also consistently resistant. As noted above, only the *Pc58* and *Pc91* differentials were similarly consistent in their resistance across these years. However, both of these genes can be ruled out as likely candidates. Markers linked to *Pc58* map to oat chromosome 9D (Oliver et al. 2013), while *PcKM* was determined to be on oat chromosome 12D. The *Pc91* gene was originally identified in the tetraploid species *A. magna* Murphy and Terrell (Rothman 1984) and was mapped to chromosome 7C-17A (Gnanesh et al. 2013), while the *Pc45* gene was introduced into the common oat gene pool from an *A. sterilis* accession designated F169 (Fleischmann et al. 1971). If *PcKM* is *Pc45*, it is not clear which parents in the pedigrees of 'Morton' and 'Kame' would have donated the gene. It is interesting that the pedigrees of 'E70' and 'M70' (from which IA B605X was derived) contain four *A. sterilis* parents, although F169 is not one of them. Two of the parents are known

to carry *Pc51* and *Pc52* (Simons et al. 1978), but perhaps they unknowingly carried *Pc45* as well.

A final indication that *PcKM* may be *Pc45* comes from the *PcKM*SNP1 TaqMan marker data. The resistant allele associated with *PcKM* was amplified in the F169 (CAV5050) *A. sterilis* accession (from which *Pc45* was originally described) and in the *Pc45* oat differential line. Although the resistance allele was also amplified in four other differential lines, none of these other lines showed consistent resistance within the Guelph crown rust nursery over the 5 years that *PcKM*-containing lines were evaluated. The amplification of the allele associated with resistance in these other four lines likely reflects a common sequence associated with the *A. sterilis* background from which these resistance genes were derived.

The current study mapped a major seedling crown rust resistance gene, designated *PcKM*, to oat chromosome 12D. The gene provided consistent and effective resistance over 5 years of testing in field nurseries, which indicates that it will be a valuable addition for oat breeders wishing to incorporate crown rust resistance into varieties. Disease reactions of crown rust differential lines grown in field nurseries and molecular marker data suggest that *PcKM* is *Pc45*.

Author contributions BG was responsible for KASP marker development, greenhouse screening of populations, data analysis and manuscript writing. CM was responsible for Saskatoon field screening of populations, experimental design and manuscript review. PE participated in marker validation and manuscript review. JMF provided oat germplasm and reviewed the manuscript. JM provided crown rust isolates for greenhouse screening of populations and reviewed the manuscript. AB was responsible for experimental design, providing oat germplasm, co-ordinating the Guelph field screening of populations, TaqMan marker development, data analysis and manuscript writing.

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Conflict of interest The authors declare that they have no conflicts of interest.

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