Core ideas

- Oat crown rust resistance gene, *Pc96,* was mapped to Chromosome 7D between 60.4 and 91.6 cM in a RIL population using Illumina SNP markers
- The *Pc96* QTL location was validated in two additional biparental populations
- Haplotype composed of nine markers for *Pc96* looked to be very predictive in a group of broad germplasm

Mapping and identification of molecular markers for the *Pc96* gene conferring resistance to crown rust in oat

Sidrat Abdullah [1] Tyler Gordon [2] Belayneh Admassu-Yimer[3] Edyta Paczos-Grzęda[4] Stephen A. Harrison[5]James G. Menzies[6] Kathy Esvelt Klos[2]

[1]Oak Ridge Institute for Science and Education (ORISE) Research Participant, Small Grains and Potato Germplasm Research Unit, Agricultural Research Service, United States Department of Agriculture, Aberdeen, ID 83210, USA

[2] Small Grains and Potato Germplasm Research Unit, Agricultural Research Service, United States Department of Agriculture, Aberdeen, ID 83210, USA

[3]University of Idaho, Department of Entomology, Plant Sciences, 1693 S. 2700 W., Aberdeen, ID 83210, USA

[4]Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences, Adademicka 13 St, 20-950 Lublin, Poland

[5] Morden Research and Development Centre, Agriculture and Agri-Food Canada, 101 Route 100, R6M 1Y5, Morden, MB, Canada

[6]School of Plant, Environmental and Soil Sciences, Louisiana State University Agricultural Centre, Baton Rouge, LA 70803

Abbreviations

CDL, Cereal Disease Laboratory; gDNA, Genomic deoxyribonucleic acid; IT, Infection type; *Pca*, *Puccinia coronata* f. sp. *avenae*; APR, Adult Plant Resistance; MAS, Marker Assisted Selection

Abstract

Oat crown rust caused by *Puccinia coronata* Cda f. sp. *avenae* Erikss (*Pca*), is a major constraint to oat (*Avena sativa* L.) production in many parts of the globe, with host resistance applied as a common strategy to curb crown rust epidemics. Combining multiple sources of crown rust resistance (for example, *Pc* genes conferring seedling resistance) in a single genetic background can be expedited by marker-assisted selection using molecular markers that are tightly linked with resistance loci. *Pc96* is a race-specific crown rust resistance gene originating

from cultivated oat that has been deployed in North American oat breeding programs. SNP markers linked with *Pc96* were identified using multiple interval mapping with 899 polymorphic SNPs in a recombinant inbred line population developed from a cross between the oat crown rust differential known to carry *Pc96* and the differential line carrying *Pc54*. A single resistance locus was identified on chromosome 7D between 60.4 and 91.6 cM with a peak LOD at 87.3 cM. The resistance locus and linked SNPs were validated in two additional biparental populations, Ajay x Pc96 and Pc96 x Kasztan. Genotype state at a combination of two linked SNPs predicted the absence of *Pc96* in a broad group of germplasm with 5% miss-classification. SNPs that are closely linked to the gene may be beneficial as PCR-based molecular markers in marker-assisted selection.

1 INTRODUCTION

Oat (*Avena sativa* L.) is a widely grown cereal, used for grain, forage, and straw production (Halima et al., 2015). Oat is of special interest from a human health perspective because of the presence of -glucans, which have been shown to lower blood cholesterol levels and the risk of cardiovascular diseases (Karin and Eliasson, 2009). The primary disease limiting oat production globally is crown rust caused by *Puccinia coronata* Cda f. sp. *avenae* Erikss (*Pca*). This economically important disease occurs in most areas where oat cultivars and wild oats are grown (Nazareno et al., 2018, Simons, 1985). Oat yield losses due to crown rust generally vary from 1% to 5% per year, but more severe losses are routinely reported (Nazareno et al., 2018, Long & Hughes, 2001). Crown rust management strategies include early planting, scouting and removal of the alternate host, Buckthorn (*Rhamnus* spp.), fungicide application and genetic resistance (McCallum et al., 2007; McCartney et al., 2011; Nazareno et al., 2018; Simons, 1970).

Single (*Pc*) gene resistance can be very effective against specific *Pca* races and is easy to incorporate into new cultivars. For this reason, incorporation of single gene resistance into cultivated oat germplasm has been a common strategy for managing oat crown rust (Litzenberger, 1949; Simons et al., 1978, Gnanesh et al., 2015). Oat crown rust qualitative resistance genes with known genomic locations include *Pc38* (Wight et al., 2004), *Pc39* (Sowa et al., 2020; Zhao et al., 2020), *Pc45* (Kebede et al., 2019), *Pc48* (Wight et al., 2004), *Pc50-5* (Toporowska et al., 2021), *Pc53* (Yimer et al., 2018), *Pc58*a (Hoffman et al., 2006; Jackson et al., 2007), *Pc68* (Kulcheski et al., 2010), *Pc71* (Bush and Wise, 1998), *Pc91* (Gnanesh et al., 2013), *Pc94* (Chong et al., 2004) and *Pc98* (Zhao et al., 2020). Unfortunately, single gene resistance is typically only effective for a few years due to rapid changes in *Pca* virulence (Carson, 2008; Carson, 2011; Chong and Kolmer, 1993; Leonard, 2002; Miller et al., 2020). Oat crown rust qualitative resistance genes are controlled by single major gene and pyramiding multiple major genes in a single cultivar have been proposed as a method to provide long lasting, effective resistance (McCarthy et al., 2011). Developing molecular markers that are closely linked with major genes can allow

for reliable introgression of multiple major genes in a marker assisted selection (MAS) breeding program.

In an ongoing effort to identify the genomic locations of all known *Pc* genes, the purpose of this study was to genetically map the location of the *Pc96* gene. *Pc96* was obtained from the oat accession MG 85039, which was designated by the National Research Council Germplasm Institute, Bari, Italy. The *Pc96* single gene differential line has the pedigree: Makuru x MG 85039 (Chong and Brown, 1996). Although *Pca* isolates with virulence to *Pc96* have been documented (Miller et al., 2020; Sowa and Paczos, 2021), this gene has provided a moderate level of resistance against a diverse group of *Pca* isolates in the Matt Moore Buckthorn Nursery in St. Paul, MN since 2016 (T. Gordon, Personal communication) in Canada since 2010 (Menzies et al., 2019). The Buckthorn nursery consists of oat lines planted among the alternate host of crown rust, *Rhamnus cathartica* L. and has been in continuous use for oat crown rust research since the 1950s (Carson, 2009); therefore, performance of *Pc* genes within this nursery is impacted by a sexually recombining, highly diverse crown rust population.

The objectives of this study were to: i) identify the chromosome region linked with *Pc96* in a biparental population, and ii) develop closely linked molecular markers, and iii) validate the genetic position of *Pc96* in two other biparental populations.

2 MATERIALS AND METHODS

2.1 Plant materials

A recombinant inbred line (RIL) population (*Pc54* x *Pc96*) of 122 F_5-derived RILs was developed from a cross between the oat crown rust differential known to carry *Pc96* (Makuru × MG 85039) originally identified by Chong and Brown (1996) and the oat differential known to carry *Pc54* (Pendek*2 ×x CAV1832) described by Martens et al., (1980). Two biparental populations were developed for QTL validation: one was comprised of 139 F_3-derived lines from a cross between the susceptible oat cultivar 'Ajay' (74AB1952 × 74AB2608) and the *Pc96* oat crown rust differential (Ajay × Pc96). The second population consisted, of 169 F_2 lines and 168 $F_{2:3}$ lines from a cross between the *Pc96* differential line and the susceptible cultivar 'Kasztan' (Dawid × CHD 1685/84). Allele frequencies of linked markers in the Collaborative Oat Research Enterprise (CORE) association mapping panel (Esvelt Klos et al., 2016) were used to evaluate marker performance in diverse oat germplasm. For comparison, 114 susceptible lines of the CORE and 30 *Pc* differential lines were defined as non-carriers of *Pc96* (Supplemental Table S1) with a susceptible reaction with all 10 isolates used in the Esvelt Klos et al., (2016) study and with severity ratings >30% at all field location years were assumed to not carry *Pc96* (Esvelt Klos et al., 2017; Source: http://triticeaetoolbox.org/oat/).

2.2 Phenotyping

Field Screening:

Crown rust disease screening with 122 RILs of the Pc96 × Pc54 mapping population and the *Pc54* and *Pc96* parents was carried out in the Louisiana State University crown rust nursery in Baton Rouge, LA. Of the 122 field tested lines, we received data from 107 RILs as 15 lines were not germinated. Each RIL was planted in a nonreplicated meter long row with 38 cm between rows, along with three replicated rows of parents. Crown rust was allowed to naturally infect the crown rust nursery and the population was evaluated on April 20, 2018, for crown rust severity when lines were at the flag leaf stage (Zadoks et al., 1974). Reaction of RILs to crown rust was recorded as resistant or susceptible based on the infection response (IR) produced on each line.

Controlled environment screening:

Seedlings of all three populations were evaluated in a growth chamber for their reaction to crown rust as previously described by Yimer et al. (2018). Seedling tests were conducted at USDA-ARS, Aberdeen, ID; except the Pc96×Kasztan population which was tested at the Morden Research and Development Centre, Agriculture and Agri-Food Canada between December 2018 and July 2019. *Pc* genes that were effective against each *Pca* isolate used in these experiments are shown in Supplemental Table S2 (Chong et al., 2000; Menzies et al., 2019). Parents were included as internal checks spaced every 50 entries. Approximately two weeks after planting, when seedlings were at the two-leaf stage, they were inoculated with uredinia suspended in Soltrol 170 isoparaffin oil (Chevron Phillips, The Woodlands, TX) and adjusted to a concentration of 2.0 x 10^5 mL^{-1}. Seedlings were grown in a growth chamber set at 20°C with a 14 h photoperiod and assessed approximately 14 days post inoculation (dpi) for infection type based on scoring guide developed by Stakman et al. (1962) where infection type (IT) can range between 0 = no uredia, immune and 4 = large uredia; very susceptible.

Controlled environment phenotypes of Pc54 × Pc96 RILs were taken to differentiate resistance due to *Pc96* from any potential effect of the *Pc54* gene. About 4 seedlings from each of the 122 RILs were evaluated against *Pca* race NQBK where the *Pc54* differential had an IT of '1+' and the *Pc96* differential had an IT of '0'. Because of a shortage of seed, hypothesized *Pc96* carrier status was checked by evaluating 4 additional seedlings sampled from the F_5 generation. Non-confirmed scores were set as missing. At least 20 seedlings from each $F_{2:3}$ family were evaluated for the Ajay × Pc96 and Pc96 × Kasztan populations. The Ajay × Pc96 (n=139) population was tested against *Pca* race MBTG. It produced an IT of '3;' or '4;' with large uredia on Ajay, and an IT of '0;' on the *Pc96* differential. The F_2 (n=169) and $F_{2:3}$ (n=168) families of the (Pc96 × Kasztan) population was screened with *Pca* race BRBG-94 which produced an IT of '4' or '4+' with large uredia on susceptible parent Kasztan, and an IT of '0' on the resistant *Pc96* parent.

2.3 Genotyping and SNP marker development

The Pc54 × Pc96 and Ajay × Pc96 populations were genotyped from gDNA

using the 6K Illumina Infinium iSelect oat SNP chip at the Cereal Crops Research Unit of ARS-USDA in Fargo, ND. SNPs were called automatically using the Genome Studio 2.0 DBSCAN procedure and were manually assessed for call accuracy based on instructions from the manufacturer (Illumina, San Diego, CA, 2016). Genotyping in the CORE and 31 *Pc* differential lines were as previously described by Esvelt Klos, et al. (2016). Genotype calls for markers of interest on CORE lines were obtained from the T3 database (http://triticeaetoolbox.org/oat/) except where the quality control process described by Esvelt Klos et al. (2016) resulted in elimination of a marker of specific interest to this study. The Pc96 × Kasztan population was genotyped using SNP assays designed from Illumina SNP sequences with the following protocol:

Competing SNP allele assays were designed and run according to instructions provided by the manufacturer of PACE genotyping master mix, 3CR Bioscience (Harlow, UK). Briefly, each SNP assay was designed with two allele-specific forward primers and one common reverse primer for each putative SNP. SNP assay reactions were prepared in a final volume of 10 l which was comprised of 3 l of genomic DNA (50 ng l^{-1}), 5 l of 2 ×PACE reaction mix (StdRox), and 0.14 l of primer mix (including 12 l of each forward primer and 30 l common primer) adjusted with water. The PCR protocol was run on a CFX96 (BioRad, Hercules, CA) with an initial denaturation for 15 min at 94° for, 10 touchdown cycles of 94° for 20 s, 65 C for 60 s (dropping down by0.6 C per cycle), and 30 cycles of 94 C for 20s, followed by extension at 55 C for 60 s and a plate read at 25°C after 60 s.

2.4 Genetic map construction and statistical analysis

JMP Genomics v. 10.0 (Cary, NC) was used for all statistical analyses. Markers were removed from the F_5-derived population when they had a minor allele frequency (MAF) < 0.20, or missing data > 0.20, or were not present on the Chaffin et al. (2016) consensus map. A final total of 822 polymorphic SNP markers were retained for linkage analysis and the Chaffin et al. (2016) consensus map was used to assign markers to linkage groups. Chromosome designations have been translated from the linkage group names of Chaffin et al. (2016) to the corresponding chromosome names ("*Avena sativa* – OT3098 v2, PepsiCo, https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico"). During analysis, the IT reactions and field scores were converted to binary scores where the susceptible *Pc54* was coded as '0' and the resistant reaction in *Pc96* was coded as '2'. Multiple interval mapping (MIM), described by Kao et al. (1999), was used to identify quantitative trait loci (QTL) associated with field and seedling crown rust resistance. A forward search for QTL main effects was run to locate peak QTL positions and detect possible epistatic-effect QTL interactions.

SNP assays were designed and used to validate markers associated with resistance in the Pc96 × Kasztan population (Supplemental Table S3). Markers that distinguished the parents, *Pc96* and Kasztan, into two different clusters

were evaluated.

A Pearson's Chi-squared (^{2}) goodness of fit test was used to estimate the number of genes segregating within each population.

3 RESULTS

3.1 Crown rust reaction and inheritance of resistance

The *Pc96* differential was resistant, *Pc54* was consistently susceptible, and the population segregated roughly 1:1 with 52 susceptible and 55 resistant RILs in the LSU field nursery (Table 1). The *Pc96* differential line exhibited a resistant reaction in all the seedling tests with an IT of '0' or '0;'' ' to races NQBK, MBTG and BRBG-94. The susceptible parents exhibited varying IT to the tested *Pca* races. The *Pc54* differential had an IT of '1+' to race NQBK, Ajay had an IT of '3+;' to race MBTG and Kasztan had an IT reaction of '3' or '4' when challenged with *Pca* race BRBG-94.

Resistance in the Pc54 × Pc96 population fit a single dominant gene segregation ratio in both the field and seedling tests (Table 1). When the Ajay × Pc96 population was tested under a single gene model, it did not fit the ratio and likely segregated for an additional *Pc* gene detectable using the MBTG race. However, the model free linkage analysis used in this study is expected to be robust to multiple genes segregating in a population. Segregation in the Pc96 × Kasztan F_2 population fit a single dominant gene model, but the F_2:$_3$ population did not (Table 1). Given the fit with the expected one gene ratio using F_2 plant phenotypes we hypothesize that this is a false negative result that could be due to greater than expected heterozygosity because of such factors as sampling error and/or plants escaping infection.

Population	
Pc54 × Pc96	
Ajay × Pc96	
Pc96 × Kasztan	

$_a$Lines were rated as resistant if they had seedling IT below "3" and field response of "0" all lines with ratings

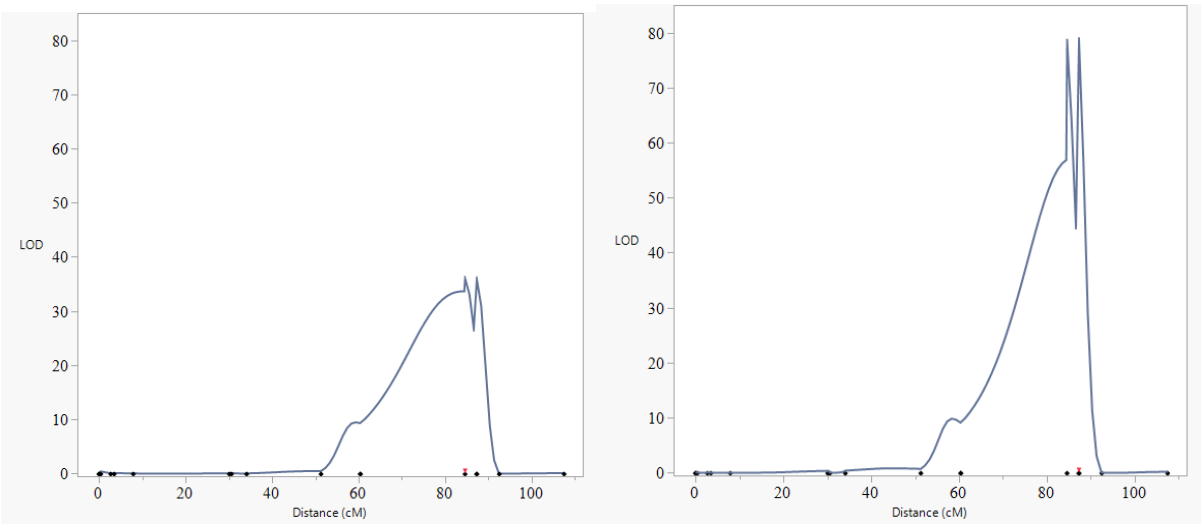
Table 1. Crown rust reaction segregation ratios in three biparental populations

3.2 Linkage analysis

Linkage analysis in the Pc54 × Pc96 population identified single QTL on Chromosome 7D at 87.3 cM with a strong effect on both the seedling reaction to race NQBK and response to crown rust reaction in the field (Figure 1, Table 2, Supplemental Table S4). Peak LOD scores were 35.86 for the seedling reaction and 78.63 for the field response. Additive effect of the *Pc96* differential parent

alleles in the 1-LOD confidence region around the peak ranged from 0.57 to 0.98, and from 0.52 and 0.83, when resistance and susceptibility were coded as 2 and 0, for the field and seedling crown rust responses, respectively indicating the source of resistance to be *Pc96* (Supplemental Table S4). Consistent with the phenotype segregation results, two QTL were detected in the Ajay × Pc96 population (Supplemental Table S5). One QTL was detected on Chromosome 7D with a peak LOD at 87.3 cM which coincided with the QTL found in the Pc54 x Pc96 population. The peak LOD score in the Ajay x Pc96 population was 14.27 and the additive effect of the *Pc96* differential alleles ranged from 0.03 to 0.36 (Table 2, Supplemental Table S5). A second QTL was detected in the Ajay × Pc96 population on chromosome 6C with peak LOD at 15.7 cM (Supplemental Table S5). The allele conferring resistance was also contributed by the *Pc96* differential.

Fifteen SNPs in the *Pc96* gene region were selected for KASP marker assay development based on linkage and segregation in one or both Pc54 × Pc96 and Ajay × Pc96 populations (Table 2). In total we evaluated six markers on the Pc96 × Kasztan population. Four of the six SNPs did not segregate, and two SNPs were found to be polymorphic in the Pc96 × Kasztan population. The LOD score (LOD=12.42) of one marker, GMI_ES02_c1532_592, validated the presence of *Pc96* in this region.



1. Field b) Seedling

Figure 1. LOD profile generated for both field (a) and seedling (b) from a Pc54 × Pc96 F_5-derived oat population using crown rust phenotypes across chromosome 7D genetic distances where each SNP marker is represented by a single point

Table 2. SNP markers closely linked[a] with *Pc96* crown rust resistance in three biparental populations

Marker	Gro
GMI_ES03_c7453_413	7D
GMI_ES02_c1532_592	7D
GMI_ES15_c9981_469	7D
GMI_ES03_c95_413	7D
GMI_ES22_c2732_580	7D
GMI_ES01_c29247_156	7D
GMI_ES01_c2095_229	7D
GMI_ES01_c13079_243	7D
GMI_ES15_c15279_258	7D
GMI_ES03_c3011_446	7D
GMI_ES15_c6098_227	7D
GMI_DS_LB_10093	7D
GMI_ES22_c2813_554	7D
GMI_ES03_c9523_612	7D
GMI_DS_LB_4563	7D

[a]LOD for each linked marker based on crown rust field scores for the Pc54 × Pc96 population, and seedling infection types for Ajay × Pc96 and Pc96 × Kasztan, "-" markers rejected for poor quality or were monomorphic in the population; "na" are markers that were not polymorphic in the population or either marker failed in KASP assay; "*' " are markers not tested.

[b]Linkage groups and genetic distances assigned according to Chaffin et al. (2016)

In order to estimate allele frequencies in the wider population of cultivated oat, genotype data for SNPs within the chromosomal region most likely to contain the *Pc96* gene was examined in the Collaborative Oat Research Enterprise (CORE) association mapping panel (Esvelt Klos et al., 2016) and in a separately genotyped panel of *Pc* differential lines. Data was examined only for markers that met the same QC standards for both panels. One-hundred-forty-four lines, including 30 *Pc* differential lines, presumed to not carry the *Pc96* gene based on pedigree data and on susceptible phenotypes in controlled environment tests were compared for genotype at 9 SNPs located on chromosome 7D between 72.7 to 87.3 cM for which the *Pc96* differential was observed to carry the rare allele (Supplemental Table S1). SNPs were found to misclassify between 16 to 21% of *Pc96* non-carriers. Of the 9 markers composing the resistant SNP haplotype we found three susceptible lines (Mortlock, PI263412-1 and Red Algerian) with all markers predicting the presence of *Pc96* where those markers with these three haplotypes across *Pc* differentials and susceptible lines misclassify 2%

3 DISCUSSION

In this study we used model free linkage analysis to map the genetic location

for *Pc96* in the Pc54 × Pc96 RILs and validated the map position in two additional populations. A 95% confidence interval can be inferred for the linked gene location within a region defined by markers with LOD scores of peak-1 or better. For the Pc54 × Pc96 and Ajay × Pc96 populations this is 60.4 to 91.6 cM and 85.2 to 91.6 cM, respectively, with the peak LOD at 87.3 cM in both. Thus, we consider the most probable location of the *Pc96* gene to be between 84.6 and 87.3 cM on the consensus map of Chaffin et al. (2016). Disappointingly, the two segregating markers evaluated in the Pc96 × Kasztan population were outside this region, although statistically significant evidence of linkage was still obtained at 72.7 cM. For the purposes of this discussion, we consider the location of the *Pc96* gene to be on chromosome 7D between 85.2 and 91.6 cM. Other investigations have identified *Pc* genes in this chromosomal region. Evidence of linkage in the *Pc96* gene region was reported for *Pc35* (Chong & Brown, 1996), *Pc38* (chromosome 9D; Wright et al., 2004), and *Pc54* (Yimer et al., 2022). Crown rust resistance *Pc38* clusters with *Pc62* and *Pc63* (Harder et al., 1980) and appears to overlap with the location of *Pc96*. On the same linkage group, but at least 30 cM proximal to *Pc96* are also *Pc58a* (Hoffman et al., 2006; Jackson et al., 2007), and *QPc.CORE.02* (Esvelt Klos et al., 2017).

PcA, later assigned the gene symbol *Pc96* (Chong and Brown, 1996), was found to be linked to *Pc35*, a gene derived from *A. sterilis* (McKenzie & Fleischmann, 1964). These two genes came from different sources, so they should be linked in repulsion phase. This suggests that combination within a single line would be rare, but a precise map location for *Pc35* remains to be identified. The crown rust resistance gene *Pc38* was originally identified in *A. sterilis* (Fleischmann and McKenzie, 1968; Simons et al., 1978), and mapped with restriction fragment length polymorphism (RFLP) markers by Wight et al. (2004). *Pc38* mapped to a region that corresponds to the Mrg02 consensus map linkage group between 73.3 and 118.5 cM (Chaffin et al., 2016). This position of *Pc38* overlaps with the most probable location of *Pc96*, and the recombination between *Pc38* and *Pc96* could be evaluated if this combination of genes is desired. Yimer et al. (2022) mapped *Pc54* to the same location as *Pc96*, in part using the Pc54 × Pc96 population analyzed in this study. A comparison of Pc54 × Pc96 phenotypes from growth chamber screening with NQBK (avirulent on *Pc96*) and LBMG (avirulent on *Pc54*; Yimer et al., 2022) indicate that these two genes are linked rather than allelic. Crossover events produced 2 RILs which were homozygous carriers of both genes and 4 RILs homozygous for both wild type alleles. Linkage analysis with carrier status of *Pc54* and *Pc96* both coded as markers indicated that *Pc54* was proximal to *Pc96* (not shown). These genes are 4.83 cM apart on chromosome 7D in this population (Yimer et al., 2022).

Of the *Pc58* complex genes *Pc58a* and *Pc58c* are positioned at 10.8 cM (Jackson et al., 2010) on the consensus map which is more than 50 cM from the QTL region of *Pc96*. Whereas as *Pc58b* is at 110.4 cM (Hoffman et al., 2006; Jackson et al., 2007; Oliver et al., 2013). *Pc96* is implausible as a *Pc58* cluster gene. Using association mapping methods, Esvelt Klos et al. (2017) reported the

presence of the seedling resistance QTL *QPc.CORE.02* on linkage group Mrg02. This QTL was identified in the CORE collection of elite oat lines and influenced variation in crown rust disease response in field nurseries in Louisiana, North Dakota, and Manitoba in 2010 and 2011. *QPc.CORE.02* was best represented by the SNP GMI_GBS_94371 at 28.1cM, at least 30 cM proximal to *Pc96*, suggesting that *QPc.CORE.02* is unlikely to represent the effect of the *Pc96* gene. It seems that chromosome 7D contains multiple *Pc* genes (*Pc35, Pc38, Pc54, Pc58a*, *Pc62, Pc63* & *Pc96*). These characteristics make chromosome 7D a promising target for further investigation. For example, the map positions of *Pc62* and *Pc63* are still only roughly defined.

Interestingly, we observed an additional gene segregating in the Ajay × Pc96 population, also originating from the *Pc96* differential line. This was originally suggested by the lack of fit to a single segregating gene model (Table 1) and confirmed by evidence of linkage on chromosome 6C (Supplemental Table S5). Mapping crosses with the *Pc96* differential line were made under the assumption that this line carried a single gene conferring seedling resistance to crown rust. However, many of the oat *Pc* gene differential lines currently available were developed by phenotypic selection which would allow additional unobserved genes to remain in the line during development. This observation has already been reported within the differential line of *Pc50* where *Pc50-2* (Sebesta 1983), *Pc50-4* (Klenova et al., 2006) and *Pc50-5* (Toporowska et al., 2021) were selected from the original *Pc50* differential.

Markers identified in this study linked to the *Pc96* gene could be of interest to oat breeders for use in marker assisted selection (MAS) of segregating lines inheriting the *Pc96* gene from a known parental carrier. In a similar process, we chose to evaluate linkage in the Pc96 × Kasztan population by evaluating the RILs for genotype at 15 SNPs used to map *Pc96* in our other populations. This method captured information on segregation in Pc96 × Kasztan at only the proximal end of the most probable location of *Pc96*. MAS using markers that are linked to but fail to bracket the gene of interest risks retaining lines during selection with unobserved recombination between marker and gene. Additional polymorphic markers would be required for MAS in the Pc96 × Kasztan and this could also be a limiting factor in future populations. Information from this study should prove useful in identifying markers with potential for MAS based on their position across the most probable *Pc96* gene region.

Markers identified in this study linked to the *Pc96* gene could also be of interest to oat breeders and pathologists for use in attributing unknown crown rust resistance to the presence of *Pc96*. Markers used for this purpose must not only be linked to the gene of interest but should ideally be specific to the unique haplotype that surrounds the gene. SNPs that are present in other haplotypes within the population will mis-classify germplasm as carriers or non-carriers to some extent. We evaluated the ability of markers in the *Pc96* gene region to correctly classify susceptible germplasm and other *Pc* differential lines as non-carriers of the *Pc96* gene. The 9 SNPs we evaluated all mis-classified some non-carrier lines

as carriers, resulting in a 16 to 21% mis-classification rate (Supplemental Table S1). This is too high to qualify these SNPs as potential diagnostic markers for *Pc96*. However, the use of the genotype of SNP GMI_ES05_c14633_290 (72.7 cM) in combination with the genotype of any of the other six SNPs at 87.3 cM evaluated in this study would reduce the mis-classification rate to a more reasonable 5%. SNP markers closely linked with *Pc96* crown rust resistance in three biparental populations in this study were Blasted against the *Avena sativa* – OT3098 v2, PepsiCo (https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico). The markers are positioned on a fragment of the 7D chromosome between 460 and 490 Mbp. The availability of a consensus map as well as its reference genome sequence has expanded our understanding of the genetics of crown rust resistance in cultivated oat. Further investigation will be required to identify markers unique to the haplotype carrying the *Pc96* gene and capable of unambiguously diagnosing carrier status in unrelated germplasm.

Historical reports of the effectiveness of *Pc96* in producing an effective level of resistance to naturally occurring crown rust have been encouraging. Chong and Brown (1996) found that this gene was successful in controlling more than 97% of oat crown rust isolates collected from North American regions (US & Canada) during 1991 to 1994. Menzies et al. 2019 observed 95% of all isolates of *Pca* from Canada to have avirulence to *Pc96* during 2010 to 2015. In Eastern Europe this gene was also found effective against East European oat crown rust pathotypes. In 2006, *Pc96* was classified in Eastern Europe as a very efficient source of resistance, with a value of resistance efficiency score 0.857 (Klenová & Sebesta, 2006). Within the Matt Moore Buckthorn Nursery at St. Paul, MN, the *Pc96* differential line exhibited a consistent level of moderate resistance in 2016, 2019, and 2020 as indicated by severity ratings of 23%, 25%, and 20%, respectively (Tyler Gordon, unpublished). Given that the frequency of virulence remains fairly low in natural populations, it may be beneficial to use *Pc96* in oat breeding for crown rust resistance. It has been previously suggested that *Pc96* is a good candidate gene for combining with other effective *Pc* genes including *Pc42, Pc45*, *Pc48*, *Pc50*, *Pc62*, *Pc68*, and *Pc91* (Chong & Kolmer 1993; Chong & Seaman 1996; Chong et al., 2011). In addition to the moderate effectiveness of *Pc96* gene in the Matt Moore Buckthorn Nursery, we also observed moderate effectiveness of genes *Pc58*, *Pc64*, *Pc91* and *Pc94*. In a recent report of three years data (2016, 2019 and 2020), lines carrying those *Pc* genes had 19-32% rust severity in the field (Tyler Gordon, unpublished). This information suggests potential for utilization in combination with *Pc96*.

In conclusion, we mapped *Pc96*, which confers race-specific crown rust resistance in oat, to a region which corresponds to the oat consensus linkage group Mrg02 (chromosome 7D) between GMI_ES03_c7453_413 (60.4 cM) and GMI_DS_LB_4563 (91.6 cM). This increases the number of crown rust seedling resistance genes placed to the oat genome in the context of modern high-throughput molecular markers. In addition, we demonstrated the ability to identify linked markers in multiple bi-parental populations that could be used to track inheritance within a breeding program.

ACKNOWLEDGMENTS

The research at ARS-USDA in Aberdeen was supported in part by North American Millers' Association and by an appointment of the first author to the ARS Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture (USDA). All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA, ARS, DOE, or ORAU/ORISE. The authors are also very grateful to the Cereal Disease Laboratory of ARS-USDA, St. Paul, MN for providing *Pca* races. The authors acknowledge the support of Allyson Lunos in planting and maintaining plots at Baton Rouge, LSU research station and Ellen Kress, biological science technician at USDA-ARS, Aberdeen, ID to have her help to complete seedling reaction phenotyping. The first author would also like to thank Rawnaq Nazneen Chowdhury, USDA-ARS, Aberdeen, ID for reviewing and formatting the manuscript.

SUPPLEMENTAL MATERIAL

Supplemental S1. Genotype cluster calls at 6 SNPs on chromosome 7D at 87.3 cM for non-carriers of *Pc96* compared with the *Pc96* differential line

Supplemental S2. List of *Pc* differential virulence pattern when challenged with the *Puccinia coronata* races used in this study

Supplemental S3. Primer sequences of polymorphic Kompetitive allele-specific PCR (KASP) assays in the Pc96 × Kasztan population derived from 60K single nucleotide polymorphism (SNP) markers linked to *Pc96* quantitative trait loci (QTL)a

Supplemental S4. All polymorphic SNP markers from a Pc54 × Pc96 F_5-derived population rated for crown rust infection type and their field and seedling LOD and additive effect values

Supplemental S5. All polymorphic SNP markers from Ajay × Pc96 population and their LOD and additive effects for crown rust seedling phenotypes

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization: Sidrat Abdullah, Kathy Esvelt Klos.

Data curation: Sidrat Abdullah.

Genotyping: Sidrat Abdullah, Belayneh Admassu-Yimer

Field data: Belayneh Admassu-Yimer, Stephen A. Harrison

Formal analysis: Tyler Gordon.

Funding acquisition: Kathy Esvelt Klos.

Investigation: Sidrat Abdullah, Kathy Esvelt Klos. James G. Menzies.

Methodology: Sidrat Abdullah, Tyler Gordon, Belayneh Admassu-Yimer, Edyta Paczos-Grzęda,

James G. Menzies.

Project administration: Kathy Esvelt Klos.

Writing – original draft: Sidrat Abdullah, Kathy Esvelt Klos.

Writing – review & editing: All co-authors

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