PCR-Based High-resolution Melt Markers for Pc genes/QTL. Part 1: SNPs linked to QCr.cdl11-13A, QPc.crc-14D, Pc58a, and Pc91.

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This short paper is a first installment on a series of informal releases of molecular marker information for crown rust resistance genes. The USDA lab in Aberdeen, Idaho is tasked with developing molecular markers for adult plant crown rust resistance (APR) QTL and seedling stage resistance (Pc) genes. Ultimately, our aim is to develop a full panel of molecular marker assays with the capability of diagnosing which of the known Pc genes and APR QTL are possessed by any given line. No marker currently available has passed through the level of validation needed by a diagnostic marker for application to germplasm from all sources. However, the process of validating markers to this level of stringency produces many markers not suitable for diagnosis, but with potential for use in populations known to be segregating for specific genes or QTL. In the spirit of OatGlobal.org, we intend to release information on assays developed for these markers as they become available, along with details of their performance in bi-parental populations and samples of unrelated oat lines.

Our lab uses the PCR-based High-resolution Melt (HRM) assay technology for marker validation, because of the low cost of assay development and the low reagent cost compared to KASP and TaqMan. Both of those types of markers will be developed later for potentially diagnostic SNPs, but assays for the SNPs in this release are based on HRM technology. HRM is an assay where alleles are differentiated based on the sequence-specific temperature at which double-stranded amplicon DNA disassociates into single-stranded DNA (Tm). We use a BioRad CFX thermocycler with real-time PCR detection system, including the BioRad Precision Melt Analysis software. However, these protocols could be adapted to other real-time PCR systems. The basic assay protocol is as follows:

1. Prepare a master mix as indicated in Table 1, with volumes adjusted for the number of samples to be run.
2. Vortex the mixture briefly and spin to clear mix from the sides of the tube.
3. To 17.5 uL of master mix add 2.5 uL of DNA in each well of a PCR plate.
4. Cover with optical clear adhesive seal (Microseal, BioRad Laboratories), and centrifuge briefly to ensure that all reagents are at the bottom of the well.
5. Program the PCR conditions (Table 1) into the real-time PCR thermocycler.
6. Create a plate layout file specific for your samples, ensuring that the correct fluorophore is defined.
7. Run the thermal cycling protocol and subsequent plate read step for your sample plate.
8. View the data, and make the allele calls.

Table1. Reagents and PCR condition for High-resolution Melt Assay

Master Mix (per sample)			PCR Conditions		
Reagent	**Volume (uL)**		**Temp**	**Time**	**Cycle**
H_2O	8.25		98°C	2 min	1
F-Primer (10 uM)	1.5		98°C	2 sec	39x
R-Primer (10 uM)	1.5		60°C	5 sec	
Precision Melt Supermix (BioRad)	6.25		65°C	5 sec	
DNA (20ng)	2.5		95°C	0.1°C incr	

The BioRad Precision Melt Supermix includes the buffer, cofactors, and DNA polymerase needed for PCR amplification, along with a fluorophore used to collect the melt curve data during qPCR. The Tm and the shape of the melt curve in 0.1 °C increments are collected for each sample, both of which can be used in allele-calling. Homozygotes generally differ most in Tm, while the heterozygotes can be distinguished by differences in the shape of the melt curve. BioRad's Precision Melt Analysis Software has tools for normalizing the data to account for variation in background fluorescence and for clustering melt curves into genotype classes.

Primers for SNPs reported linked to APR QTL QCr.cdl11-13A (Babiker *et al.* 2015) and QPc.crc-14D (Lin *et al.* 2014) and Pc58a (Esvelt Klos *et al.* 2014) are in Table 2. In addition, we used the placement of RFLP and DArT markers on the consensus map of Chaffin *et al.* (2016) to identify the SNP linked to Pc91 (McCartney *et al.* 2010). The sequence around all these SNPs is available on T3/Oat at http://triticeaetoolbox.org/oat/ should you need to design primers for a different assay type.

Table 2. HRM marker primers for SNPs linked to crown rust resistance QTL and Pc genes.

QTL/Pc gene	SNP	HRM primers
QCr.cdl11-13A	GMI_ES_CC13970_83	F-5'-TGAGCATTCTGGAGGATGTG R-5'-GGCTGCAGTCCTGGTATCTC
	GMI_ES01_lrc22746_326	F-5'-GAATGCGATGTAACTACAAA R-5'-CGCAAAGTACACGCAGAAGA
QPc.crc-14D	GMI_DS_LB_6480	F-5'- TCATGCAAGTAGTGTAGGGTTAGC R-5'-TGCCTTTGCGAAGAAAACTT
Pc58a	GMI_DS-LB_6614	F-5'- AATTCTGTATTATGTAGGCTAAACAGG R-5'-CATATGCAATCGGAAATCTCA
	GMI_ES17_c19068_531	F-5'-TGTATCTCGAGGAATGAATCTGTG R-5'-CAGCCCGATCAAAAGTCTTC
Pc91	GMI_ES05_c11155_383	F-5'-TGTGCTGACTCTTTCCCCTG R-5'-TGACCGACGAAAACAAACAACA

The QCr.cdl11-13A, QPc.crc-14D, Pc58a, and Pc91 SNP assays correctly place the donor parents (94197A1-9-2-2-2-5, MN841801, TAM O-301, and HiFi, respectively) and susceptible parents (Provena and CDC Boyer, Makuru, Ogle, and CDC Sol-Fi, respectively) from the mapping populations into different genotype classes. The performance on other germplasm is presented in Table 3.

Table 3. Genotype calls (as a cluster number) for 7 SNPs linked to APR QTL and Pc genes.

Line	Notes	QCr.cdl11-13A		QPc.crc-4D	Pc58a		Pc91
		GMI_ES_CC13970_83	GMI_ES01_lrc22746_326	GMI_DS_LB_6480	GMI_DS-LB_6614	GMI_ES17_c19068_531	GMI_ES05_c11155_383
94197A1-9-2-2-2-5	QCr.cdl11-13A donor	1	1	3	3	3	NA
Provena	Susceptible mapping parent	3	3	1	3	3	3
MN841801	QPc.crc-14D donor	3	3	1	1	1	3
TAM O-301	Pc58a donor	3	1	3	1	1	3
Ogle	Susceptible mapping parent	3	3	3	3	3	3
HiFi	Pc91 mapping parent	3	3	3	3	3	1
CDC-Dancer	Susceptible	3	3	3	3	3	NA
CDC Sol-Fi	Susceptible mapping parent	3	3	3	1	3	**3**
AC Morgan	Susceptible	3	3	3	3	3	3
Ajay	Susceptible	3	3	1	3	3	3
Amagalon	Pc91 donor	3	3	3	3	3	1
CDC Boyer	APR resistance	3	3	3	3	3	3
Coker 234	Seedling resistance from Pc61	3	3	3	3	3	3
Exeter	Susceptible	3	3	3	3	3	3
Fidler	Susceptible	3	3	3	3	3	NA
Florida 501	Some seedling resistance	3	3	1	3	3	3
Gem	Susceptible	3	3	3	1	3	NA
Goslin	Seedling resistance from Pc48	3	3	3	3	3	3
Horizon	Susceptible	3	3	3	1	1	NA
Hudson	Susceptible	3	NC	3	3	3	NA
Kangaroo	Susceptible	3	3	1	1	3	3
Leggett	Seedling resistance from Pc94	3	3	3	3	3	3
Makuru	Susceptible	3	3	3	3	3	NA
MAM 17-5	APR resistance	3	3	3	3	3	3
Morton	Seedling resistance	3	3	3	3	3	NA
Noble 2	Susceptible mapping parent	3	3	3	3	3	NA

Otana	Susceptible	3	3	3	3	3	3
OT-62	Resistant Ogle/TAMO-301 RIL	3	3	3	1	3	NA
Pc45	Differential line	3	3	3	3	3	NC
Pc48	Differential line	3	3	3	3	3	3
Pc58	Differential line	3	1	3	1	1	NC
Pc59	Differential line	1	3	3	3	3	3
Pc68	Differential line	3	3	3	1	3	3
Pc91	Differential line	3	3	3	3	3	1
Sesqui	Seedling resistance from Pc51	3	3	3	3	3	3
Shadow	Susceptible	3	3	1	3	3	3
Tardis	Susceptible	3	3	3	3	3	NA
UFRGS 8	Susceptible	3	3	3	3	3	3

NC= no call; cluster 1 associated with resistant and cluster 3 with susceptible homozygote
NA= Not assayed.

The marker genotypes associated with resistance at the APR QTL and Pc58a are present in susceptible germplasm to some extent. This limits their use as diagnostic markers. However, because they're known to be in linkage with crown rust resistance genes/QTL, these markers could be used in marker-assisted selection in crosses where they are polymorphic between parents.

References

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