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Genetic Relationship of Stripe Rust Resistance Genes *Yr34* and *Yr48* in Wheat and Identification of Linked KASP Markers

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Abstract

The Australian continent was free from wheat stripe rust caused by *Puccinia striiformis* f. sp. *tritici* until exotic incursions occurred in 1979 and 2002. The 2002 incursion enabled the identification of a new stripe rust resistance gene (*Yr34*) in the advanced breeding line WAWHT2046. In this study, we developed and validated markers closely linked with *Yr34*, which is located in the distal region in the long arm of chromosome 5A. Four kompetitive allele-specific polymerase chain reaction (KASP) and three sequence-tagged site (STS) markers derived from the International Wheat Genome Sequencing Consortium RefSeq v1.0 scaffold-77836 cosegregated with *Yr34*. Markers *sun711*, *sun712*, *sun725*, *sunKASP_109*, and *sunKASP_112* were shown to be suitable for marker-assisted selection in a validation panel of 71 Australian spring wheat genotypes, with the exception of cultivar Orion that carried the *Yr34*-linked alleles for *sunKASP_109* and *sunKASP_112*. Markers previously reported to be linked with adult plant stripe rust resistance gene *Yr48* also cosegregated with *Yr34*. Wheat genotypes carrying *Yr34* and *Yr48* produced identical haplotypes for the *Yr34*-linked markers identified in this study and those previously reported to be linked with *Yr48*. Phenotypic testing of genotypes carrying *Yr34* and *Yr48* showed that both genes conferred similar seedling responses to pre-2002 and post-2002 *P. striiformis* f. sp. *tritici* pathotypes. Further testing of 600 F_2 plants from a cross between WAWHT2046 and RIL143 (*Yr48*) with *P. striiformis* f. sp. *tritici* pathotype 134 E16A+Yr17+Yr27+ failed to reveal any susceptible segregants. Our results strongly suggest that *Yr34* and *Yr48* are the same gene, and that *Yr48* should be considered a synonym of *Yr34*.

Stripe rust is a widely distributed disease of wheat, occurring in almost all continents, except Antarctica (Chen 2005). It is relatively new in Australia, with the first incursion of *Puccinia striiformis* f. sp. *tritici* pathotype 104 E137A− occurring in 1979 in eastern Australia (O'Brien et al. 1980). Since then, 21 variants of the original pathotype have been reported, through acquisition of virulence for stripe rust resistance genes *YrA*, *Yr6*, *Yr7*, and *Yr17* either individually or sequentially in different combinations. In 2002, another incursion occurred in Western Australia (WA). It was pathotyped as 134 E16A+ and named as the "WA pathotype". This pathotype differed from the eastern Australian pathotypes and appeared to be similar to those reported by Chen (2005) in the United States and Hovmøller and Bayles (2005) in Europe. The key feature of this pathotype compared with the earlier group was its combined virulence for genes *Yr8* and *Yr9* and avirulence for *Yr3*, *Yr4*, and *Yr34* (Bariana et al. 2006; Wellings et al. 2003). Since the original incursion, several new variants from the WA pathotype involving virulence for *Yr10*, *Yr17*, *Yr24/Yr26*, *YrJ*, *YrT,* and *Yr27* have been detected (Singh et al. 2010; Wellings 2007).

The incursion of the WA pathotype in Australia resulted in high yield losses and increased expenditure on chemical control (AU$40 to 90 million) during the crop seasons of 2003 to 2005 (Wellings 2007). Several Australian cultivars that carried resistance to pre-2002 pathotypes were susceptible to the WA pathotype, while cultivar Rubric and the advanced breeding line WAWHT2046 from the Department of Primary Industries and Regional Development, WA expressed higher levels of resistance (Bariana et al. 2006). Rubric was shown to carry *Yr4* (Bansal et al. 2010), whereas the resistance gene carried by WAWHT2046 was mapped 12.2 centimorgans (cM) distal to the awn inhibitor locus *B1* in the long arm of chromosome 5A and was formally named *Yr34* by Bariana et al. (2006) using a WAWHT2046/Carnamah doubled-haploid (DH) population. *Yr34* remains effective against all variants of the WA pathotype detected thus far in Australia.

The advent of next-generation DNA sequencing and high-throughput marker genotyping has played a significant role in speeding up the process of developing trait-linked markers and identifying causal genes for trait variation. Several technologies now exist for high-density genotyping, including Diversity Array Technology (DArT; www.diversityarrays.com), Illumina iSelect single-nucleotide polymorphism (SNP) array (Wang et al. 2014), and genotyping by sequencing (He et al. 2014). Such technologies, in combination with the International Wheat Genome Sequencing Consortium (IWGSC) assembly for common wheat variety Chinese Spring and the more recent release of the annotation for the wheat genome (https://wheat-urgi.versailles.inra.fr/About-us/News/IWGSC-RefSeq-v1.0-annotation-is-now-available), now make it possible to physically localize trait-linked polymorphism in the wheat genome to develop new markers to saturate a genomic region of interest, and to prioritize candidate genes for functional analysis. For example, markers linked with *Sr56* and *Lr52/Yr47* were developed using these genomic resources (Bansal et al. 2014; Qureshi et al. 2017).

Despite the effectiveness of *Yr34* against all post-2002 pathotypes, this gene has not been widely used in Australian breeding programs, presumably due to expression of high seedling infection type (IT) 23C-33C and the unavailability of closely linked molecular markers. Adult plant stripe rust resistance gene *Yr48* was located in chromosome 5AL by Lowe et al. (2011), and these workers placed it distal to *Yr34* based on a comparison of their map with that presented by Bariana et al. (2006).

This study was planned to produce a detailed map of the *Yr34* region on the long arm of chromosome 5A using various genomic resources to identify closely linked markers for effective pyramiding of this gene with other marker-tagged rust resistance genes, and to study the relationship between *Yr34* and *Yr48*.

Material and Methods

Plant material. The WAWHT2046/Carnamah DH population of 106 lines from Bariana et al. (2006) was used in this study. Another WAWHT2046/Avocet S-derived recombinant inbred line (RIL) population (68 lines) was used to validate the association of *Yr34*-linked

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markers. A set of 71 Australian cultivars was used to validate the utility of closely linked markers for marker-assisted selection (MAS) of *Yr34* and *Yr48* in breeding programs.

An F_2 population derived from a cross between WAWHT2046 (*Yr34*) and RIL143 (*Yr48*; derived from the cross UC1110/PI610750) (Lowe et al. 2011) was developed to determine allelism between *Yr34* and *Yr48*.

Greenhouse tests. The DH and RIL populations were screened under greenhouse conditions using *P. striiformis* f. sp. *tritici* pathotype 134 E16A+Yr17+Yr27+ (University of Sydney accession 617). Eight seeds of each line and four lines per pot were sown in 9-cm pots filled with a potting mix comprising pine bark and river sand (2:1). Parents WAWHT2046, Carnamah, and Avocet S were included as controls in each experiment. After sowing, pots were placed in a rust-free microclimate room set at 20°C. Seedlings were inoculated at the two-leaf stage with urediniospores of *P. striiformis* f. sp. *tritici* pathotype 134 E16A+Yr17+Yr27+. The urediniospores were suspended in light mineral oil Isopar-L (https://www.ascc.net.au/) and atomized onto seedlings using an aerosol pressure pack. Inoculated seedlings were incubated in a dark dew chamber set at 9°C for 24 h and then transferred to a microclimate room set at 17°C. Stripe rust responses were assessed 14 to 16 days after inoculation using the 0 to 4 infection type (IT) scale outlined by McIntosh et al. (1995), where IT 0 to 3 were considered resistant and 3^+ and 4 were considered susceptible.

The *Yr48*-carrying genotype RIL143 (Lowe et al. 2011) was also tested at the two-leaf stage with post-2002 and pre-2002 pathotypes 134 E16A+Yr17+Yr27+ and 110 E143A+, respectively, along with WAWHT2046, and susceptible controls Carnamah and Morocco to compare pathotypic specificities of these genes.

Field tests. The WAWHT2046/Carnamah DH population was grown at the Karalee and Lansdowne experimental field sites of the Plant Breeding Institute, Cobbitty, NSW, Australia and the WAWHT2046/Avocet S RIL population at the Horse Research Unit site as 1-m rows. The parents were sown along with each population. A 30-cm strip of the susceptible genotype Nyabing 3 was planted as a spreader after each block of 35 experimental rows to facilitate uniform disease development. The spreader rows were inoculated three times with *P. striiformis* f. sp. *tritici* pathotype 134 E16A+Yr17+Yr27+ at weekly intervals from the last week of July. Stripe rust responses were scored using a 1 to 9 scale (Bariana et al. 2007) after flag leaf emergence. Both populations were scored for segregation at the awn inhibitor locus *B1*.

DNA extraction and quantification. Genomic DNA was extracted from 2-week-old seedlings of both populations and their parents using the modified cetyltrimethylammonium bromide procedure described in Bansal et al. (2014). DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and diluted to a concentration of 30 ng/µl.

Simple sequence repeat and sequence-tagged site genotyping. Previously reported simple sequence repeat (SSR) and sequence-tagged site (STS) markers (*gwm410*, *cfa2149*, *gpw2181*, *wPt-7061*, *gwm291*, *SNF-A2*, *gwm595*, and *gwm6*) were tested on both populations (Lowe et al. 2011; Röder et al. 1998; Somers et al. 2004; Sourdille et al. 2001) (http://maswheat.ucdavis.edu/protocols/index.htm) following the procedure described by Bansal et al. (2014).

SNP genotyping. Selective genotyping using the iSelect 90K Infinium SNP assay (Wang et al. 2014) on eight homozygous resistant (*Yr34Yr34*) and eight homozygous susceptible (*yr34yr34*) lines from the WAWHT2046/Carnamah DH population was undertaken to identify *Yr34*-linked SNP. Sequences flanking the linked SNP (Wang et al. 2014) were used to design kompetitive allele-specific polymerase chain reaction (KASP) assays with two allele-specific forward primers and one common reverse primer (https://www.lgcgroup.com/kasp/#.WUyFu-uGOUk).

KASP genotyping. KASP assays were performed in 8-µl reaction volumes containing 165 nM each specific forward primer, 412 nM common reverse primer, 90 ng of genomic DNA, and 1× KASP buffer mix containing $MgCl_2$, Taq polymerase, dNTP, universal FAM and HEX fluorescence resonance energy-transmitted cassettes, and ROX reference dye (KBioscience). Polymerase chain reaction (PCR) thermocycling was performed using a Bio-Rad CFX96 Touchdown real-time PCR system with an initial denaturation step of 94°C for 15 min, followed by 9 touchdown cycles at 94°C for 20 s and 61°C for 1 min (dropping 0.6°C per cycle), and 38 cycles at 94°C for 20 s and 55°C for 1 min. SNP genotype calling was performed using Bio-Rad CFX Manager software (Bio-Rad).

STS marker development and genotyping. DArT marker *wPt-7061*, previously reported to be linked with *Yr48*, was used in BlastN queries against the genome sequence assembly of cultivar Chinese Spring (https://urgi.versailles.inra.fr/blast/?dbgroup=wheat_all&program=blastn). Scaffold-77836, carrying the linked markers, was used to design primers with Primer3 v4.0 software (Koressaar and Remm 2007; Untergasser et al. 2012) for amplification of STS markers from the parental lines WAWHT2046 and Carnamah. The STS amplification products were checked for size variation on agarose gels and products not revealing polymorphism were Sanger sequenced at the Australian Genome Research Facility (www.agrf.org.au) and compared for nucleotide variation using Sequencher software (www.genecodes.com). SNP identified in STS sequences were used to design KASP markers using Batchprimer3 software (http://probes.pw.usda.gov/batchprimer3/). STS and KASP markers were named with the prefix *sun* and *sunKASP*, respectively (sun = Sydney University), followed by consecutive numbers. STS and KASP markers showing polymorphism between the parental genotypes were genotyped in both populations.

STS markers were amplified by PCR in 25-µl reaction volumes containing 40 ng of genomic DNA, 200 mM dNTPs, 1× Immolase PCR buffer containing 1.5 mM $MgCl_2$ and 100 nM each forward and reverse primers, and 0.02 U of Immolase DNA polymerase (Bioline). Thermal cycling conditions comprised a touchdown profile consisting of an initial denaturation at 95°C for 10 min; followed by 92°C for 30 s, 65°C (with a 1°C drop per cycle) for 30 s, and 72°C for 30 s for 5 cycles; then, 35 cycles of 30 s at 92°C, 40 s at 60°C, and 50 s at 72°C; and a final extension step of 72°C for 10 min. PCR product (3 µl of each marker) was visualized on a 2% agarose gel stained with GelRed (Biotium).

Statistical analysis and mapping. A χ^2 analysis was performed to test goodness-of-fit of the observed segregation among the DH and RIL populations with the expected genetic ratios for hypothesized genetic models. Genetic linkage maps were constructed using MapManager software, version QTXb20 (Manly et al. 2001), and the Kosambi mapping function for conversion of recombination fractions to centimorgans (Kosambi 1943). The final linkage map was drawn using MapChart software (Voorrips 2002).

Results

Assessment of rust responses. WAWHT2046 expressed IT 23C-33C against *P. striiformis* f. sp. *tritici* pathotype 134 E16+Yr17+Yr27+, whereas the susceptible genotypes Carnamah and Avocet S produced IT 3^+ (Fig. 1A). When tested in the greenhouse against this pathotype, 61 WAWHT2046/Carnamah DH lines were moderately resistant (IT 23C-3C) and 45 lines were susceptible (IT 3^+), whereas 45 WAWHT2046/Avocet S RIL displayed IT 12C-23C and 23 lines expressed IT 3^+ (Table 1). A χ^2 analysis of the segregation data for the DH population conformed to monogenic segregation, whereas the WAWHT2046/Avocet S RIL population showed a significant deviation from a 1:1 ratio (Table 1). The skewness observed in the RIL population presumably resulted from differential loss of susceptible lines during generation advancement of the RIL population.

When scored under field conditions, WAWHT2046 and Carnamah produced stripe rust responses of 2 and 6, respectively, on the 1 to 9 scale and Avocet S was scored 9. Stripe rust responses among the DH lines varied from 2 to 8, and from 2 to 9 among the RIL population. Adult plant stripe rust response variation in both populations indicated the involvement of adult plant resistance genes in addition to the *Yr34* scored at the seedling stage. Stripe rust responses for DH lines and RIL carrying *Yr34* ranged from 2 to 5, indicating that this gene alone expressed an intermediate level of resistance.

WAWHT2046 carries the awn inhibitor allele *B1* and its awns are less than 2 cm long (tip-awned), whereas Carnamah is fully awned.

Assessment of both populations for segregation at the *B1/b1* locus showed that the tip-awned phenotype was associated with resistance. Among the DH population, 60 lines were tip-awned and 45 lines were awned. One awned DH line showed a resistant response. The RIL population showed segregation that was skewed toward tip-awned; 45 RIL were tip-awned and 22 awned. One tip-awned and susceptible recombinant was also observed in this population (Table 1).

Genetic mapping. Selective genotyping of eight resistant and eight susceptible WAWHT2046/Carnamah DH lines identified 33 SNP linked with *Yr34*. The linked SNP were converted into KASP markers and tested on the parental genotypes and three resistant and three susceptible DH lines. KASP assays showing scorable cluster patterns were tested on the entire DH population. Of the 20 KASP markers listed in Table 2, 19 were incorporated into the WAWHT2046/Carnamah map (Fig. 2). Only four KASP markers (*KASP_2837*, *KASP_2838*, *KASP_47624*, and *KASP_56756*) were polymorphic when tested on the WAWHT2046/Avocet S RIL population. These markers were tested on the entire RIL population.

Eighteen STS markers were designed (Table 3) from scaffold-77836, which contained the DArT marker *wPt-7061* previously reported to be linked to *Yr48* (Lowe et al. 2011). Nine of the STS markers (*sun713*, *sun715*, *sun717*, *sun719*, *sun720*, *sun721*, *sun723*, *sun724*, and *sun726*) were monomorphic between the parental genotypes on an agarose gel and were subsequently Sanger sequenced. Markers *sun710* and *sun718* amplified multiple fragments in the parental genotypes, whereas *sun714*, *sun716*, *sun722*, and *sun727* failed to amplify in WAWHT2046. Markers *sun711*, *sun712*, and *sun725* were polymorphic between the parents (WAWHT2046 versus Carnamah and Avocet S) and were tested on both the DH and RIL populations.

Sanger sequencing of the nine STS markers not revealing amplicon size variation among the parental genotypes identified SNP variation in the amplicons produced by markers *sun713*, *sun717*, *sun719*, *sun724*, and *sun726*, whereas no sequence variation was observed for markers *sun715*, *sun720*, *sun721*, and *sun723*. Of the five KASP markers developed for SNP within the STS markers, four (*sunKASP_109*, *sunKASP_110*, *sunKASP_112*, and *sunKASP_113*) produced scorable polymorphic clusters among the parental genotypes WAWHT2046, Carnamah, and Avocet S and were tested on both populations (Table 4).

The WAWHT2046/Carnamah and WAWHT2046/Avocet S populations were also tested with markers *gwm410*, *cfa2149*, *gpw2181*, *wPt-7061*, *gwm291*, *SNF-A2*, *gwm595*, and *gwm6*, which were previously mapped to the distal part of chromosome 5AL (Lowe et al. 2011).

The final genetic linkage map for the WAWHT2046/Carnamah DH population comprised 34 markers (19 KASP markers derived from the 90K SNP map, 4 *sunKAS*P markers derived from Sanger sequencing, 7 SSR, and 4 STS), and the map of the WAWHT2046/Avocet S RIL population comprised 16 markers (4 SSR, 4 STS, 4 KASP markers derived from linked 90K SNP, and 4 sunKASP markers derived from Sanger sequencing) (Fig. 2). Eleven markers (*sun711*, *sun712*, *sun725*, *sunKASP_109*, *sunKASP_110*, *sunKASP_112*, *sunKASP_113*, *gwm410*, *cfa2149*, *gpw2181*, and *wPt-7061*) cosegregated with *Yr34* in both populations. Marker *KASP_6988* cosegregated with *Yr34* only in the DH population. The awn inhibitor locus *B1* mapped 1 and 0.7 cM proximal to *Yr34*, whereas SSR marker *gwm291* mapped 1.5 and 2.3 cM proximal to *B1* in WAWHT2046/Carnamah and WAWHT2046/Avocet S, respectively (Fig. 2). All cosegregating markers, except *cfa2149*, were located in scaffold-77836 (Fig. 3).

Validation of *Yr34*-linked markers. *Yr34*-linked markers were tested on 71 Australian cultivars (Fig. 3). Markers *sun712*, *cfa2149*,

Table 1. Frequency distribution of lines in the WAWHT2046/Carnamah doubled-haploid (DH) and WAWHT2046/Avocet S recombinant inbred line (RIL) populations when tested against *Puccinia striiformis* f. sp. *tritici* pathotype 134 E16A+Yr17+Yr27+ and for segregation at the *B1* awn inhibitor locus

Population	IT[a]	Genotype frequency	*B1B1*	*b1b1*	Total
Carnamah/ WAWHT2046[b]	23C-3C	*Yr34Yr34*	60	1	61
	3+	*yr34yr34*	0	45	45
	Total		60	46	106
WAWHT2046/Avocet S[c]	12C-23C	*Yr34Yr34*	45	0	45
	3+	*yr34yr34*	1	22	23
	Total	Total	46	22	68

[a] Infection type.
[b] For χ^2: $\chi^2_{1:1\ (B1\ \text{versus}\ b1)} = 1.85$ ($P_{1\text{d.f}} > 0.05$), $\chi^2_{1:1\ (Yr34\ \text{versus}\ yr34)} = 2.42$ ($P_{1\text{d.f}} > 0.05$), and $\chi^2_{1:1:1:1\ (B1\ \text{versus}\ Yr34)} = 106.30$ ($P_{3\text{d.f}} > 0.05$).
[c] For χ^2: $\chi^2_{1:1\ (B1\ \text{versus}\ b1)} = 8.47$ ($P_{1\text{d.f}} < 0.05$), $\chi^2_{1:1\ (Yr34\ \text{versus}\ yr34)} = 7.11$ ($P_{1\text{d.f}} < 0.05$), and $\chi^2_{1:1:1:1\ (B1\ \text{versus}\ Yr34)} = 79.65$ ($P_{3\text{d.f}} < 0.05$).

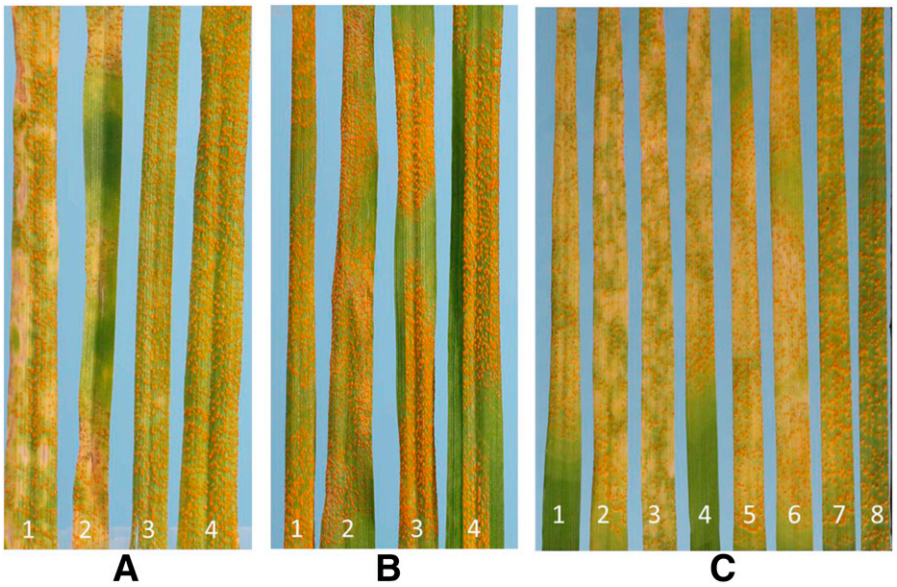

Fig. 1. Infection types at the two-leaf stage produced by 1: WAWHT2046, 2: *Yr48* carrying RIL143, 3: Avocet S, and 4: Carnamah when tested with **A,** *Puccinia striiformis* f. sp. *tritici* pathotype 134 E16+Yr17+Yr27+; **B,** *P. striiformis* f. sp. *tritici* pathotype 110 E143A+; and **C,** F_2 plants (examples 1, 2, 3, 4), WAWHT2046 (5), RIL143 (6), Carnamah (7), and Morocco (8) inoculated with *P. striiformis* f. sp. *tritici* pathotype 134 E16A+Yr17+Yr27+.

and *wPt-7061* were codominant and *cfa2149* and *wPt-7061* amplified additional fragments in the parental genotypes and test cultivars (Fig. 4). Markers *sun711* and *sun725* amplified a monomorphic product in all lines tested and a second product in the parental lines carrying *Yr34* and *Yr48*. Markers *gwm410* and *gpw2181* behaved in the opposite manner, amplifying a monomorphic fragment in all lines tested but absent in the parental lines carrying *Yr34* and *Yr48* (Table 5). Of the KASP markers, only *sunKASP_109* and *sunKASP_112* gave clear genotyping results across the Australian cultivars, with both markers producing the *Yr34*-linked (A:A) alleles in cultivar Orion (Table 5). These results indicated that codominant markers *sun712*, *cfa2149*, *sunKASP_109*, and *sunKASP_112* can be used for MAS of *Yr34*.

Relationship of *Yr34* with *Yr48*. Adult plant stripe rust resistance gene *Yr48* was mapped on the distal region of chromosome 5AL (Lowe et al. 2011). When tested on the WAWHT2046/Carnamah DH and WAWHT2046/Avocet S RIL populations, all DNA markers reported to be linked to *Yr48* cosegregated with *Yr34*. To assess the relationship between these genes, WAWHT2046 (*Yr34*) and RIL143 (carrying only *Yr48*) were tested at the two-leaf stage with *P. striiformis* f. sp. *tritici* pathotypes 134 E16+Yr17+Yr27+ (post-2002) and 110 E143A+ (pre-2002) (Fig. 1A and B). RIL143 produced responses similar to WAWHT2046 with both pathotypes, suggesting that *Yr34* and *Yr48* are the same gene.

Tests on 600 F_2 plants from a cross between WAWHT2046 (*Yr34*) and RIL143 (*Yr48*) with pathotype 134 E16A+Yr17+Yr27+ failed to detect variation for stripe rust response, indicating close linkage or allelism between these two genes. All plants produced IT 23C-3C (Fig. 1C). Furthermore, all *Yr34*-linked markers in the WAWHT2046/Carnamah and WAWHT2046/Avocet S populations produced the same alleles in the lines carrying *Yr34* and *Yr48* (RIL143) (Table 5). Genotyping of random KASP markers for different chromosomes on WAWHT2046, RIL143, and 48 randomly

Table 2. Kompetitive allele-specific polymerase chain reaction (KASP) markers developed for *Yr34*-linked single-nucleotide polymorphism (SNP) detected by iSelect 90K Infinium array selective genotyping

KASP marker	SNP	Allele 1 primer[a]	Allele 2 primer[b]	Common primer
KASP_6988	[T/C]	catgggaatgctccatgaatttgct	tatgggaatgctccatgaatttgcc	cttgatcagagaatcagatgttggaagat
KASP_26751	[A/G]	tctcctaccctaatcctatca	tcttctcctaccctaatcctatcg	ttcggatttcggcttctgcgtcaa
KASP_5003	[A/C]	tatgaagatgtactgcccactcct	tgaagatgtactgcccactccg	tatatgtccaagccacctggtttcaaata
KASP_3334	[A/G]	atgaaagccgtagcggat	tgctatgaaagccgtagcggac	cggtttcaaagtacttgagagatctctta
KASP_48690	[T/C]	atagtatatataattagaaattctccttgtga	tatagtatatataattagaaattctccttgtgg	cacccacaatacaaacacaaagtttgactt
KASP_38700	[A/C]	gaaagcttggacgaggctcca	tgaaagcttggacgaggctccc	gcagggatttcatattgccaatcccat
KASP_2837	[A/G]	acggtaagaagtgatagcgctatga	tggtaagaagtgatagcgctatgg	ctcaacaccaccacaatgatcaccaa
KASP_71385	[A/G]	ccagaagagaaaaccatgtgatctgt	tcagaagagaaaaccatgtgatctgc	tccattttcggaattgtctcctgtctt
KASP_56756	[T/C]	ccatgcaaaataatcttatcattcgatca	tccatgcaaaataatcttatcattcgatcg	actaacttttctcatggggatcatcgttt
KASP_2838	[T/C]	cgtcggtccatccgcga	tcgtcggtccatccgcgg	atttgtctacgcggttagggttaatacat
KASP_14661	[A/G]	aagtattgtagttttctagttctacatttctat	tgtattgtagttttctagttctacatttctac	tggccaagctcaaacaattgaaacttcaa
KASP_7282	[A/G]	caggggtctctgcttcctcct	tggggtctctgcttcctccc	gtagacaaactactgctcgagccaa
KASP_7014	[A/C]	cctcggcggcctgtacgtt	tctcggcggcctgtacgtg	cgatgccgagaatggcactgctt
KASP_23336	[T/C]	agacaaggctaacgttgtcgagat	tgacaaggctaacgttgtcgagac	gcctctggtttcccgatggtgat
KASP_14680	[T/C]	gaaactgtggcagaagtaccgca	taaactgtggcagaagtaccgcg	ggacgtggccgaagtaggcaat
KASP_65128	[T/C]	gatgtcgtattgcttgtagtcctgt	tatgtcgtattgcttgtagtcctgc	ccgagcttcccaaaggtgctgat
KASP_3323	[T/C]	ccattctttaattcaacaaacattggtgatt	tcattctttaattcaacaaacattggtgatc	gcaattccaggctgatttgacaatatcat
KASP_48095	[T/G]	caaggaggtggtggtggccta	taggaggtggtggtggcctc	gcgtctaaggacatgaaagaacatcattt
KASP_58284	[T/C]	ccattctttaattcaacaaacattggtgatt	tcattctttaattcaacaaacattggtgatc	gcaattccaggctgatttgacaatatcat
KASP_47624	[A/C]	gcgcattctcgtgtcccct	tgcgcattctcgtgtccccg	cacagccaggacatcgtcggta

[a] A1 primer labeled with FAM: GAAGGTGACCAAGTTCATGCT.
[b] A2 primer labeled with HEX: GAAGGTCGGAGTCAACGGATT.

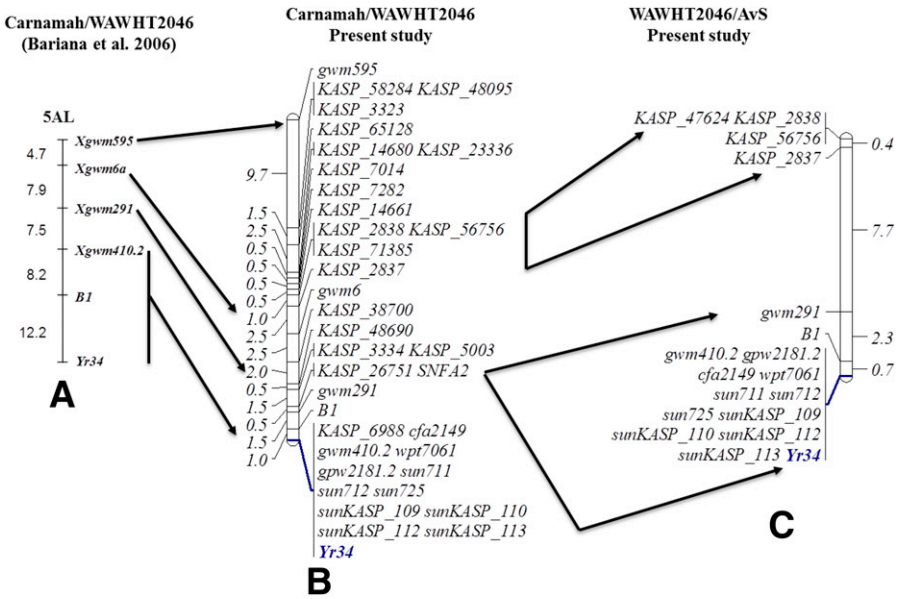

Fig. 2. Genetic linkage maps of chromosome 5AL. **A,** WAWHT2046/Carnamah doubled-haploid (DH) population; **B,** WAWHT2046/Carnamah DH population; and **C,** WAWHT2046/Avocet S recombinant inbred line population.

selected F_2 plants confirmed the hybrid nature of the population. These results indicated that *Yr34* and *Yr48* are the same gene.

Discussion

The deployment of combinations of resistance genes is a strategy recommended to extend the durability of rust resistance genes deployed in commercial wheat varieties. Gene combinations help to enhance the durability of resistance by requiring multiple virulence changes in the pathogen. Breeding genotypes carrying resistance gene pyramids is greatly facilitated by the development of tightly linked molecular markers that can be used to select for the presence of multiple resistance genes in early generations. The purpose of this study was to develop and validate tightly linked, breeder-friendly molecular markers for MAS of *Yr34*.

Selective genotyping performed on the WAWHT2046/Carnamah DH population identified multiple linked SNP on the 90K iSelect array, many of which could be successfully converted into single-locus KASP markers. The combination of genetic mapping of these KASP markers on the DH and RIL populations, and localization of flanking sequences for the linked 90K SNP markers in the IWGSC genome sequence assembly of cultivar Chinese Spring, positioned *Yr34* near the telomere of 5AL. Indeed, marker *KASP_6988* cosegregating with *Yr34* was in the most distal scaffold (scaffold-77836) of 5AL. We utilized the sequence of scaffold-77836 to develop a series of markers that also cosegregated with *Yr34* in both mapping populations. Of these markers, *sun712* was shown to be the best gel-based codominant marker for detecting and tracking the inheritance of *Yr34*, because the 200-bp difference in alternate allele product size is easily differentiated (Fig. 4). KASP markers *sunKASP109* and *sunKASP112* would be equally suitable for MAS, except for the transfer of *Yr34* to cultivar Orion (Table 5).

Several markers previously reported to be linked with stripe rust resistance gene *Yr48* (Lowe et al. 2011) were also physically located in the same region of chromosome 5AL as *Yr34*. Four KASP and

Table 3. Primer sequences and parental amplicon sizes for sequence-tagged site markers designed from International Wheat Genome Sequencing Consortium Refseq scaffold-77836

Number	Marker	Primer sequence (5′–3′)	WAWHT2046 (bp)	Carnamah/Avocet S (bp)
1	*sun710-F*	gaacgtacggctcttcttgc	1,137 + 700 + 250	1,137 + 700 + 250
	sun710-R	cgaactacatgcgaagcaaa		
2	*sun711-F*	cgaaacctgtcggtttgttt	1,135 + 950	1,135
	sun711-R	ccctcaccaaatccaatgtc		
3	*sun712-F*	agagaagcgagcaactgagg	900	1,150
	sun712-R	gggtggggagtattggaaat		
4	*sun713-F*	aaacccttggctggaacttt	1,072	1,072
	sun713-R	ggagccaaatggataagcaa		
5	*sun714-F*	tcacaaccaagacgagttgc	Null	1,166
	sun714-R	ttgggaaagcaaggaaaatg		
6	*sun715-F*	gtgtgttgccaacccttctt	1,014	1,014
	sun715-R	gttgccactttggttggagt		
7	*sun716-F*	atttttgcagatggctttgg	Null	1,187
	sun716-R	gttgccactttggttggagt		
8	*sun717-F*	tgtgatggctcagcgtagtc	1,056	1,056
	sun717-R	ttgttgaaattggggctttc		
9	*sun718-F*	ccccaatttcaacaatgtcc	1,030 + 600 + 200	1,030 + 600 + 200
	sun718-R	atgcgagctgcttcttcttc		
10	*sun719-F*	agaagcagctcgcatcagtt	1,194	1,194
	sun719-R	ttttgggcgtgccttatatc		
11	*sun720-F*	atcggataaaaggcagcgta	1,053	1,053
	sun720-R	cctaggttgaccccgttttt		
12	*sun721-F*	taagggcatctagggaccaa	1,003	1,003
	sun721-R	cctaggttgaccccgttttt		
13	*sun722-F*	tgtcaaagacttctgaagaaaattg	Null	1,003
	sun722-R	caccgccttcctctactcc		
14	*sun723-F*	cagagcatggacaaaaagca	1,022	1,022
	sun723-R	agctagtgcctttgctctcg		
15	*sun724-F*	atatgagccgtccattctcg	1,045	1,045
	sun724-R	actcctggttacgccaacac		
16	*sun725-F*	tggtaccacacgctaagca	1,035 + 750	1,035
	sun725-R	gggtggggagtattggaaat		
17	*sun726-F*	ggcctggtcaagggtacg	1,012	1,012
	sun726-R	gggtggggagtattggaaat		
18	*sun727-F*	gatgcgtgcattcagttttg	Null	1,051
	sun727-R	cgttcgtagctgttagtagatgtagg		

Table 4. Kompetitive allele-specific polymerase chain reaction (KASP) markers designed for single-nucleotide polymorphism (SNP) detected in sequenced parental sequence-tagged site (STS) amplicons

STS marker	KASP marker	Allele 1 primer[a]	Allele 2 primer[b]	Common primer	SNP
sun713	*sunKASP_109*	ggatgtagtttgtcaccagcc	aggatgtagtttgtcaccagca	ggattaacatattcctcgaatgc	[C/A]
sun717	*sunKASP_110*	cgcatttccccttgacc	cgcatttccccttgacg	gcttcccaccccttatttcac	[C/G]
sun719	*sunKASP_111*	gccagccaacaagcaac	gccagccaacaagcaag	ttgacatccaaaaccagtgc	[C/G]
sun726	*sunKASP_112*	agcgcgctctcttagcag	agcgcgctctcttagcaa	aaagaggtaatgtgctgacctg	[G/A]
sun724	*sunKASP_113*	ttgctagcttctacggcctg	ttgctagcttctacggcctc	tgtgagcagacacaatgcaa	[G/C]

[a] A1 primer labeled with FAM: GAAGGTGACCAAGTTCATGCT.
[b] A2 primer labeled with HEX: GAAGGTCGGAGTCAACGGATT.

three STS markers derived from the scaffold-77836 cosegregated with *Yr34*. The awn inhibitor allele *B1* is not completely linked to *Yr34* but is sufficiently close (<1 cM) to be used as an effective morphological marker to select for *Yr34* in the field. In areas where awned cultivars are preferred, prebreeding might be used to produce awned recombinants between *B1* and *Yr34* for use in breeding. Alternatively, RIL143 is awned and could be used as an awned source of *Yr34/Yr48*.

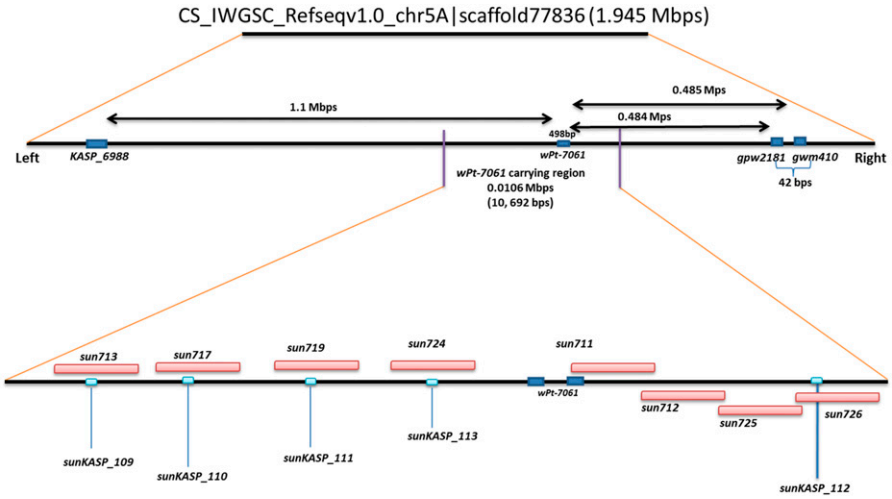

Fig. 3. Diagrammatic representation of positions of *Yr34*-linked markers in International Wheat Genome Sequencing Consortium scaffold-77386.

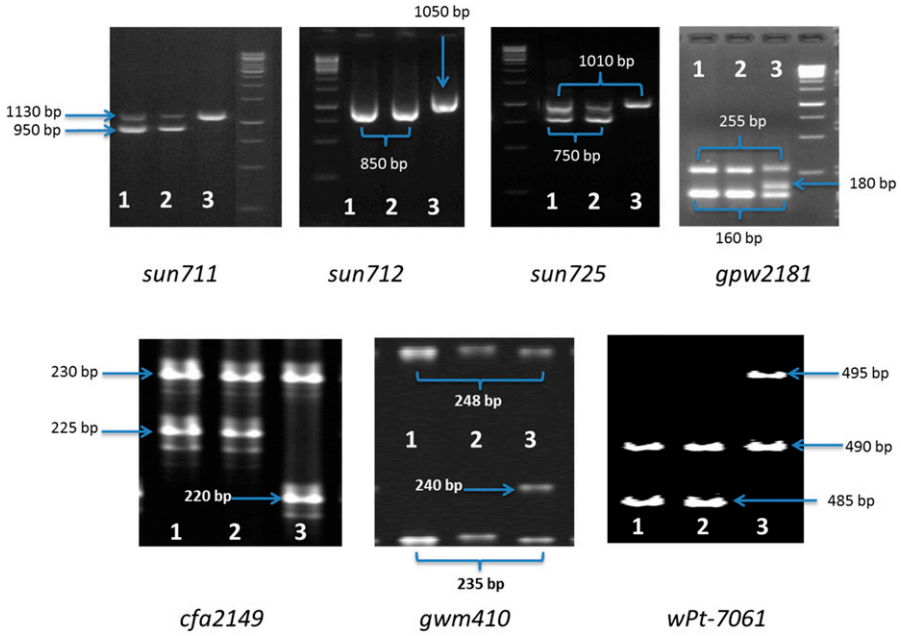

Fig. 4. Polymorphism of *Yr34*-linked markers among genotypes WAWHT2046-*Yr34* (lane 1), RIL143-*Yr48* (lane 2), and Carnamah (lane 3, susceptible control).

Table 5. Haplotype diversity of *Yr34*-linked markers in Australian cultivars and the *Yr48* stock RIL143

	Allele		Base pairs						
Genotypes	***sunKASP_109***	***sunKASP_112***	***sun712***	***wPt-7061***	***cfa2149***	***sun711***	***sun725***	***gwm410***	***gpw2181***
WAWHT2046 (*Yr34*)	A:A	A:A	850	485 + 490	225 + 230	1,130 + 950	1,010 + 750	235 + null + 248	160 + null + 255
RIL143 (*Yr48*)	A:A	A:A	850	485 + 490	225 + 230	1,130 + 950	1,010 + 750	235 + null + 248	160 + null + 255
Carnamah	C:C	G:G	1,050	490 + 495	220 + 230	1,130	1,010	235 + 240 + 248	160 + 180 + 255
Avocet S	C:C	G:G	1,050	490 + 495	220 + 230	1,130	1,010	235 + 240 + 248	160 + 180 + 255
Orion	A:A	A:A	1,050	490 + 495	220 + 230	1,130	1,010	235 + 240 + 248	160 + 180 + 255
AGT Katana + 69 others[a]	C:C	G:G	1,050	490 + 495	220 + 230	1,130	1,010	235 + 240 + 248	160 + 180 + 255

[a] Other genotypes: Axe, Baxter, Bolac, Calingiri, Catalina, Chara, Cobra, Corack, Correll, Crusader, Dart, Derrimut, Diamondbird, EGA Bonnie Rock, EGA Bounty, EGA Burke, EGA Gregory, EGA Wedgetail, EGA Wylie, Elmore CL Plus, Emu Rock, Envoy, Espada, Estoc, Forrest, Fortune, Gauntlet, Gazelle, GBA Sapphire, Giles, Gladius, Grenade CL Plus, Impala, Impose CL Plus, Janz, Justica CL Plus, King Rock, Kord CL Plus, Kunjin, Lang, Lincoln, Livingston, Mace, Magenta, Merinda, Merlin, Phantom, Preston, Scout, Sentinel, Shield, Spitfire, Strezlecki, Sunco, Sunguard, Suntop, Sunvale, Sunvex, Sunzell, Ventura, Waagan, Wallup, Wedin, Westonia, Wyalkatchem, Wylah, Yandanooka, Yitpi, and Young.

Yr48 was originally assigned a different name from *Yr34* based on the complete linkage of *Yr48* with marker *gwm410* and the initial mapping of *Yr34* 20.4 cM proximal to *gwm410* (Bariana et al. 2006) (Fig. 2A). Marker *gwm410* amplifies alleles on other group 5 homeologs and it is possible that genotypic misclassification caused the higher estimated genetic linkage with *Yr34* in the original study. In this study, *gwm410* was completely linked to *Yr34* (Fig. 2B and C), similarly to what was reported for *Yr48* (Lowe et al. 2011). Based on the complete linkage with the same subset of molecular markers, the identical alleles displayed by these markers in wheat genotypes carrying *Yr34* and *Yr48*, the absence of susceptible segregants among 600 F_2 plants from a cross between WAWHT2046 (*Yr34*) and RIL143 (*Yr48*), and the identical resistance response (IT 23C-3C) to post-2000 *P. striiformis* f. sp. *tritici* pathotypes exhibited by the two genes at the two-leaf stage (Fig. 1), we concluded that *Yr34* and *Yr48* represent the same resistance gene. Because the *Yr34* name has precedence over *Yr48*, we propose to designate *Yr48* as a synonym of *Yr34*.

The markers developed in this study can be used for efficient marker-assisted pyramiding of *Yr34* or *Yr48* with other marker-tagged genes such as *Yr51* (Randhawa et al. 2014), *Yr57* (Randhawa et al. 2015), *Yr58* (Chhetri et al. 2016), *Yr71* (Bariana et al. 2016), *Lr48* (Nsabiyera et al. 2016), *Sr2* (Mago et al. 2011), *Sr22* (Periyannan et al. 2011), *Sr45* (Periyannan et al. 2014), *Sr56* (Bansal et al. 2014), *Yr47/Lr52* (Qureshi et al. 2017), *Lr34/Yr18/Pm38* (Lagudah et al. 2006), *Lr57/Yr40* (Kuraparthy et al. 2007), *Lr67/Yr46* (Moore et al. 2015), *Lr76/Yr40* (Bansal et al. 2017), and *Sr39/Lr35* (Mago et al. 2009) to produce triple rust-resistant cultivars carrying combinations of seedling and adult-plant resistance genes.

Continual improvements to the assembly quality and annotation of the bread wheat reference genome is accelerating the rate at which markers closely linked to a trait of interest can be developed and reducing the effort required to clone the underlying gene. An interesting candidate gene identified in the region linked to *Yr34* is bHLH-MYC_N (pfam14215), a member of superfamily cl16716 (PSSM ID: 327206) located in scaffold-77836. This gene is known to regulate the biosynthesis of phenylpropanoids (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), which influence plant responses to a number of stresses (Vogt 2010) and play a key role in promoting plant resistance to pests (La Camera et al. 2004). Further experimental work will be required to elucidate the causal gene for *Yr34*.

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