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Research Note

Identification of a co-dominant eSTS marker linked with leaf rust resistance gene *Lr28* in wheat (*Triticum aestivum* L.)

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Abstract

Leaf rust, caused by *Puccinia triticina* (Pt), is an important disease of wheat worldwide. New leaf rust resistance loci from diverse germplasm including wild relatives of wheat are continuously being identified. Many leaf rust resistance genes produce similar infection types and therefore are difficult to combine in a single genotype using conventional techniques. Identification of molecular markers linked with resistance genes can facilitate pyramiding of two or more genes in new cultivars. Leaf rust resistance gene *Lr28* was transferred from *Aegilops speltoides* to common wheat. A doubled haploid (DH) population (192 lines) was developed from the cross PBW343+*Lr24*+*Lr28*/Lang. The parent PBW+*Lr24*+*Lr28* also carries *Lr26* and the alternate parent wheat cultivar Lang carries *Lr24*. The entire DH population was tested against *Lr24* virulent Pt pathotype 104-1,(2),3,(6),(7),11,13 and segregation at leaf rust resistance loci *Lr26* and *Lr28* was observed. These genes produce similar seedling responses against *Lr24* virulent Pt pathotype, therefore the *Lr26*-linked marker, *Iag95*, was used to identify a set of 123 DH lines that segregated monogenically for *Lr28*. This selected set was used for molecular studies. Bulked segregant analysis was carried out using 38 markers (24 SSR, 13 STS derived from EST and a gene based marker) located on chromosome 4AL, seven markers differentiated resistant and susceptible bulks. These markers were mapped on 123 DH lines. An eSTS marker *mag3092* co-segregated with *Lr28* and amplified a 200bp product in PBW343+*Lr24*+*Lr28* and 225bp in Lang. The *Ae. speltoides* translocated segment that carries *Lr28* was flanked by markers *barc343* and *wmc219*. Marker *mag3092* produced either a 225bp product or a 210+225bp doublet, when DNA templates from a set of Australian, Indian and Pakistani cultivars known not to possess *Lr28* were used. These results demonstrated the robustness of *Lr28* linked marker *mag3092* for marker assisted selection in diverse genetic backgrounds.

Keywords: Leaf rust, wheat, *Lr28*, molecular markers, marker-assisted selection, marker validation.
Abbreviations: BSA_bulked segregant analysis, DH_doubled haploid, IT_infection type, MAS_marker assisted selection, Pt_*Puccinia triticina*.

Introduction

Leaf rust, caused by *Puccinia triticina* (Pt), is a major constraint to wheat production in many wheat growing regions (Kolmer et al., 2007; Sumíková and Hanzalová 2010) and can cause up to 50% yield loss (Nayar et al., 2002). Deployment of genetic resistance is the most environment-friendly way to control this disease (Roelfs et al., 1992). More than 70 leaf rust resistance genes have been named in wheat and some of these genes were transferred from wild relatives of wheat (McIntosh et al., 1995; 2013). Leaf rust resistance gene *Lr28* located on chromosome 4AL was transferred from *Aegilops speltoides* (Tausch) Gren ex Richter (a diploid species with 2n = 14) into wheat by Riley et al., (1968). It was subsequently backcrossed into different wheat backgrounds in India (Tomar and Menon, 1998) and Australia (Bariana et al., 1996). *Lr28* is not associated with any deleterious characters and is present in the Australian wheat cultivar Sunland. Durable control of any disease relies on understanding of pathogen variation and deployment of combinations of resistance genes. An exotic Pt pathotype 104-2,3,(6),(7),11 was detected in 1984 in Australia and became widespread (Park et al., 2002). It possessed virulence for leaf rust resistance genes *Lr1*, *Lr3a*, *Lr14a*, *Lr16* and partial virulence for *Lr17a*, *Lr23* and *Lr27+Lr31*. It evolved sequentially to acquire virulence for *Lr20*, *Lr24* and *Lr37* (Park et al. 2002). Another exotic introduction of Pt pathotype 76-3,5,7,9,10+*Lr37* (with virulence for *Lr3a*, *Lr3ka*, *Lr13*, *Lr14a*, *Lr17a*, *Lr26* and *Lr37*) occurred in 2006 (http://sydney.edu.au/agriculture/plant_breeding_institute/rust_alert.shtml). It acquired virulence for *Lr20*, *Lr17b*, and *Lr24* in recent years (http://sydney.edu.au/agriculture/plant_breeding_institute/rust_alert.shtml). One or the other derivatives of these two lineages prevail in all wheat growing regions of Australia. Although virulence for *Lr28* has been reported in India (Bhardwaj et al., 2010; Kumar et al., 2010) and Australia (McIntosh et al., 1995), these pathotypes were not identified in recent annual pathogen surveys in these countries. *Lr28* is effective against currently prevailing leaf rust

Table 1. Distribution of 192 DH lines when tested against *Lr24*-virulent Pt pathotype 104-1,(2),3,(6),(7),11,13 at the seedling stage and isolation of a set of 123 DH lines segregating only for *Lr28* through *Lr26*-linked marker genotyping.

Phenotype	Number of DH lines		$\chi^2_{3:1}$
	Observed	Expected	
Lr26+Lr28			
Non-segregating resistant (*Lr26Lr26Lr28Lr28*)	141	144	0.06
Non-segregating susceptible (*lr26lr26lr28lr28*)	51	48	0.19
Total	192	192	0.25
Lr28			$\chi^2_{3:1}$
Non-segregating resistant (*Lr28Lr28*)	72	61.5	1.79
Non-segregating susceptible (*lr28lr28*)	51	61.5	1.79
Total	123	123	3.58

pathotypes and, therefore, represents a good candidate for gene pyramiding.

Marker-assisted selection (MAS) is a very useful technique to pyramid two or more genes in wheat cultivars, especially when specialised isolates are not available and/or genes to be pyramided produce similar responses. Markers closely linked with leaf rust resistance genes *Lr24*, *Lr26*, *Lr34*, *Lr37* and *Lr47* have been reported and primer and allele size details are listed in Bariana et al., (2007). *Lr28*, *Lr34* and *Lr47* are currently effective against prevalent Pt pathotypes in Australia. Four markers were reported to be linked with *Lr28; gwm160* (Vikal et al., 2004), $SCAR421_{625}$ (Prabhu et al., 2003), $SCAR421_{570}$ (Cherukuri et al., 2005) and *wmc313* (Bipinraj et al., 2011). All these markers are dominant. Co-dominant markers are preferred over dominant markers for their ability to differentiate heterozygotes from homozygotes in early stages of breeding. This study aimed to develop and validate a closely linked co-dominant marker for *Lr28* and to establish the break-points of *Ae. speltoides* translocation in wheat.

Results

Seedling leaf rust tests on DH population

PBW343+*Lr24*+*Lr28* and Lang produced infection type (IT) 0 and IT3+ against Pt pathotype 104-1,(2),3,(6),(7),11,13, respectively (Fig. 1). This pathotype was avirulent on *Lr26* and hence digenic segregation was observed (Table 1). Genotyping with *Lr26*-linked scar marker *Iag95* allowed identification of *Lr26* carrying genotypes. DH lines carrying *Lr26* were removed to constitute a population that showed segregation only at the *Lr28* locus and this population was used for identification of markers closely linked with this gene (Table 1).

Bulked segregant and linkage analysis

Of 39 markers, 29 were polymorphic and seven showed linkage among the bulks. These markers were genotyped on 123 DH lines. A genetic map of chromosome 4AL representing seven markers and *Lr28* was generated (Fig. 2a). Markers *mag3092*, *Owm45RF2R2* and $SCS421_{570}$ co-segregated with *Lr28*. Markers *wmc497*, *wmc776* and *wmc219* mapped distal to *Lr28* and *barc343* was proximal (Fig. 2a). Of these six closely linked markers, *mag3092* and *barc343* were co-dominant and all other markers were dominant. Marker *mag3092* amplified 200 bp and 225 bp products in PBW343+*Lr24*+*Lr28* and Lang, respectively.

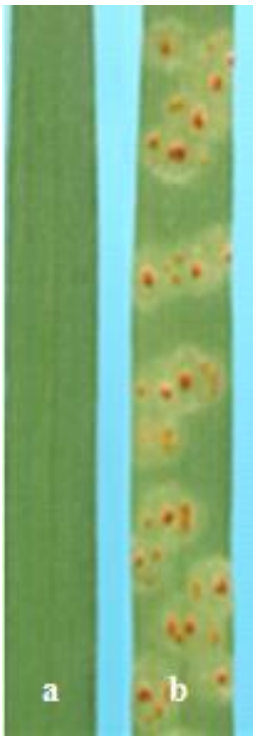

Fig 1. Infection types produced by parental genotypes a) PBW343+Lr28 and b) Lang against Lr24-virulent pathotype 104-1,2,3,(6),(7),11,13.

Validation of closely linked markers on diverse germplasm

A set of 77 genotypes including Australian, Indian and Pakistani cultivars and controls was used to validate co-segregating markers *mag3092*, *Owm45F3R3* and $SCS421_{570}$. Marker *mag3092* produced a 200bp amplicon in *Lr28* carrying genotypes Tc+*Lr28*, CS 2D/2M 3/8, CS 2A/2M 4/2 and Sunland, whereas genotypes lacking *Lr28* produced either 225bp or 210+225 bp products. The SCAR marker $SCS421_{570}$ and *Owm45F3R3* did not produce any amplicon in genotypes known to lack *Lr28*. These results demonstrated the robustness of co-dominant marker *mag3092* for marker assisted selection of *Lr28* in diverse genetic backgrounds.

Translocation break-point on 4AL

Three markers; *mag3092*, *Owm45F3R3* and $SCS421_{570}$ co-segregated with *Lr28*, whereas flanking markers *wmc219* and *barc343* showed 1.2 and 7.7% recombination, respectively.

Table 2. List of markers linked with *Lr28* and parental amplicon details.

Marker[a]	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	PBW343+*Lr28*	Lang
barc343	ggcctaattacaagtccaaaag	gctcaaagtaaagttcacgaatat	154	144
Owm45F3R3	ggctcgtctacaccaacgac	ttggggtctttaggcatgag	275	-
SCS421	acaaggtaagtctccaacca	agtcgaccgagattttaacc	570	-
mag3092	atatcggacccagtcattgc	acgtgccgaagaggaactta	200	225
wmc219	tgctagtttgtcatccgggcga	caatcccgttctacaagttcca	-	204
wmc776	ccatgacgtgacaacgcag	attgcaggcgcgttggta	-	180+194
wmc497	cccgtggttttctttccttct	aacgacagggatgaaaagcaa	270	-

[a]sources of these primers are mentioned in the text and are listed in the reference section

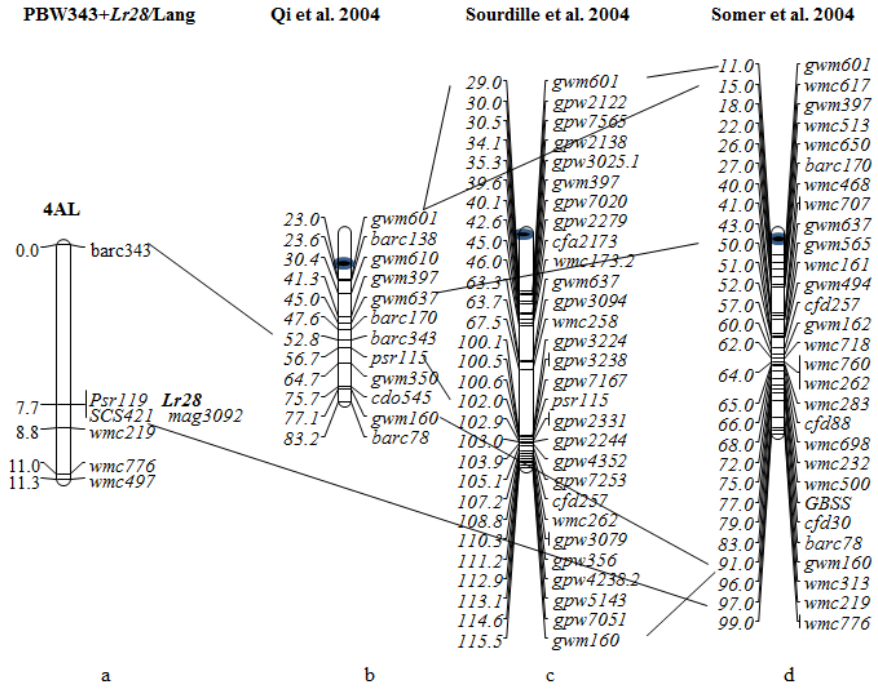

Fig 2. Comparative location of markers (*barc343* and *wmc219*) in 4AL in current study and published maps: a. PBW343+*Lr28*/Lang map b. Qi et al., (2004) c. Sourdille et al., (2004) and d. Somers et al., (2004) flanking the *Ae. speltoides* translocation.

Markers *SCS421*$_{570}$ (Cherukuri et al., 2005) and *Owm45F3R3* (Randhawa et al., 2014) produced 570bp and 275bp amplicons in PBW343+*Lr28*, respectively, and both failed to amplify any product in Lang (Table 3). These results indicated that the *Ae. speltoides* translocated segment on chromosome 4AL carries alternate functional allele at the *mag3092* locus in contrast to all other marker loci. The genetic distance between markers *barc343* and *gwm219* that flanked the *Ae. speltoides* translocation was observed to be 8.8 cM (Fig 2a). In contrast, these markers appeared to be approximately 40 cM apart based on their location in other bi-parental crosses where the *Ae. speltoides* translocation is absent (Fig. 2b; 2c; 2d). The *Ae. speltoides* translocation appears to cover a region of approximately 30 cM on chromosome 4AL.

Discussion

The donor source for *Lr28* used in this study carried additional leaf rust resistance genes *Lr24* and *Lr26*. As no Pt pathotype with combined virulence for *Lr24* and *Lr26* was available, a set of 123 DH lines segregating only at the *Lr28* locus was identified by using the *Lr24*-virulent Pt pathotype and *Lr26*-linked molecular marker *Iag95*. Molecular mapping identified a co-dominant eSTS marker, *mag3092*, which showed co-segregation with *Lr28*. A gene based dominant marker *Owm45F3R3* also co-segregated with *Lr28*. *Owm45F3R3* was derived from the 4AL Zipper (Randhawa et al., 2014) and *mag3092* from the *Fusarium* infection inducible EST BQ903300 from cDNA libraries of Sumai No. 3 spikes (Xue et al., 2008). Markers *barc343* and *wmc219* (Fig. 2a) determined the size of *Ae. speltoides* translocation.

Mclntosh et al., (1982) reported a recombination fraction of 39 cM between *Lr28* and the chromosome 4A centromere. Chromosome 4A of modern wheat has undergone cyclical translocations with chromosomes 5A and 7B (Naranjo et al., 1987; 1988; Liu et al., 1992; Devos et al., 1995; Mickelson-Young et al., 1995). The distal region of the 4AL arm was derived from 7BS. The 4AL2.3 C-band in chromosome 4A of Chinese Spring (Gill et al., 1991) was shown to be smaller than that in the *Lr28* carrying stocks 2D/2M 3/8 and 2A/2M 4/2 (Friebe et al., 1996). Friebe et al., (1996) referred to the unpublished results of L. Mickelson-Young, who observed the presence of RFLP probes pTk-suG10 and pCDOI400 representing 4AL and 7S, respectively, in 2A/2M 4/2 and the absence of probe pPSR160 representing the distal part of 7S in 2D/2M 3/8. These workers concluded that the *Lr28* carrying segment was most likely derived from the short arm of the *Ae. speltoides* chromosome 7S#2 through homoeologous recombination. Markers *barc343* and *wmc219* were mapped 8.8 cM apart in the current study. According to the maps of Qi et al., (2004) and Somers et al., (2004) the markers *barc343* and *wmc219* are located at 52 cM and 94 cM positions, respectively (Fig 2b; 2d). Based on recombin-

Table 3. Validation of *Lr28*-linked marker *mag3092* on wheat cultivars and breeding lines.

Cultivars/lines	*Lr28*	Base pair		
		mag3092	*SCS421*	*Owm45F3R3*
PBW343+ *Lr28*, Tc+*Lr28*, CS 2D/2M 3/8, CS 2A/2M 4/2, Sunland	+	200	570	275
Lang, Thatcher, PBW343	-	225	Null	Null
Australian cultivars				
Calingiri, Derrimut, Frame, Goldmark	-	225	Null	Null
Bonnie Rock, Braewood, Camm, Carinya, Carnamah, Diamondbird, EGA Gregory, Ellison, Frame, Giles, Gladius, H45, Halberd, Kelalac, Kukri, Qal2000, Rubric, Sunlin, Sunsoft 98, Sunvale, Sunzell, Tatiara, Ventura, Westonia, Wyalkatchem,	-	210+225	Null	Null
Indian cultivars/lines				
C273, C518, C591, DBW17, FLW2, FLW6, HD2402, HD2733, K9107, PBW343, PBW502, PBW533, PBW550, WH542	-	210+225	Null	Null
Pakistan cultivars/lines				
Auqab 2000, Bhakkar-02, BWP-2000, BWP-97, FSD-08, GA-2002, Iqbal-2000, Lasani-2008, Manthar-2003, Margalla-99, Marvi-2000, Marwat-2001, Millat-11, Moomal-2002, Pasban-90, Pirsabak-2004, Punjab-11, Seher-2006, SH-2003, SH-88, Shafaq- 2006	-	225	Null	Null
AS-2003, AS-2002, Bhittai, Blue Silver-71, Chenab-2000, Fakhre-Sarhad, Fareed-2006, Inqalab-91, Pak-81, Saleem-2000, Tatara, Wafaq-2001, Zarlashta-99	-	210+225	Null	Null

ation between these markers in the present study and those reported in Qi et al., (2004) and Somers et al., (2004), the translocation appears to span a 30 cM interval.

The absence of trait-linked marker allele and/or presence of an alternate allele in potential recurrent parents determine the success of a marker assisted selection program. Therefore, it is important to validate marker-trait associations across different genetic backgrounds. The co-dominant marker *mag3092* and dominant makers *Owm45F3R3* and $SCS421_{570}$ (Cherukuri et al., 2005) were positively validated on stocks carrying *Lr28* and negatively on wheat genotypes from Australia, India and Pakistan that lacked *Lr28*. Leaf rust resistance genes *Lr24* and *Lr37* are effective against *Lr28*-virulent pathotypes and *vice-versa*. Markers linked with *Lr24* and *Lr37* are also available (Bariana et al., 2007) and can be used to select combinations of these genes in breeding populations. In addition, adult plant resistance genes *Lr34*, *Lr35* and *Lr46* can also be combined as markers linked with these resistance genes are available. Strategic pyramiding of resistance genes where robust marker-trait associations are known is required to achieve durable rust control.

Materials and methods

Plant material

A doubled haploid (DH) population was developed from the cross PBW343+*Lr24*+*Lr28* (PBW343+*Lr28*) / Lang (*Lr24*). Thatcher, Tc+*Lr28* (C96.20), CS 2D/2M 3/8 (C77.1), CS 2A/2M 4/2 (C77.2), PBW343 and Sunland were used as controls in leaf rust tests and to validate linked markers. In addition to the DH population, a set of 29 Australian, 14 Indian and 34 Pakistani wheat cultivars/lines were included in this study.

Seedling leaf rust tests

PBW343+*Lr28*/Lang DH lines were sown in 9 cm diameter pots containing pine bark (8 parts) and coarse sand (2 parts). Four DH lines per pot were sown and parental lines were included as controls. The greenhouse management procedure is described in Bariana and McIntosh (1994). Ten to 12 day-old seedlings were inoculated with *Puccinia triticina* (Pt) pathotype 104-1,2,3,(6),(7),11,13 (PBI culture no. 547). Inoculated pots were placed in an incubation room fitted with an ultrasonic humidifier to provide 100% humidity for 24 hours. Pots were then moved to a temperature-controlled microclimate room set at 25°C. Leaf rust infection was assessed 12-14 days after inoculation following a scale described in McIntosh et al., (1995).

Molecular mapping of Lr28

Genomic DNA was extracted from leaf tissue using the modified CTAB protocol described in Bansal et al., (2014). DNA was quantified with a NanoDrop 3300 Fluorospectrometer and diluted to a final concentration of 30 ng/µl. *Lr26*-linked marker *Iag95* (Mago et al., 2002) was used to identify the presence of this gene in DH lines and lines carrying *Lr26* were discarded.

Bulked segregant analysis (BSA) was performed to identify molecular markers linked with *Lr28*. DNA from 16 homozygous resistant (HR) and 16 homozygous susceptible (HS) DH lines were mixed in equal amounts to form resistant and susceptible bulks. Based on available wheat genetic maps of chromosome 4AL (Somers et al., 2004; Qi et al., 2004: Song et al., 2005; Sourdille et al., 2004; Xue et al., 2008), 37 markers were tested on both parents and bulks. Of the 37 markers, 24 were SSRs (*gpw356*, *gpw3030*, *gpw7051*, *gpw2139*, *gpw5095*, *gpw4153*, *gpw1142*, *gwm350*, *gwm160*, *wpt4487*, *wpt5172*, *wpt763*, *wpt150*, *wpt4620*, *wpt4238*,

wpt6966, *barc78*, *bar343*, *cfd31*, *wmc219*, *wmc313*, *wmc497*, *wmc722* and *wmc776*) and 13 were EST based STS markers (*BE444404-1*, *BE444404-2*, *BF483646-1*, *BF483646-2*, *mag974*, *mag1159*, *mag987*, *mag1576*, *mag1604*, *mag3092*, *mag3273*, *mag787* and *mag1367*). We also used previously reported *Lr28* linked SCAR marker *SCS421$_{570}$* (Cherukuri et al., 2005) and a gene based marker *Owm45F3R3* reported to be linked with *Yr51* in chromosome 4AL (Randhawa et al., 2014). Ten µl PCR mixture contained 2 µl DNA template (30ng/µl), 1.0 µl PCR 10x buffer (containing 15 mM $Mgcl_2$), 0.75 µl of each dNTP (2.5 mmol/µl), 0.4 µl M13 labelled forward primer (1.25 µM), 0.4 µl (5 µM) reverse primer, 0.04 µl Taq DNA polymerase (0.2 U), 0.1 µl of (0.5 µM) M13 labelled with IRD700 or IRD800 and 5.31 µl ddH_2O. The forward primer was tailed at the 5' end with the M13 forward sequence to serve as a template for the IRD700 or IRD800-labeled M13 primer. PCR amplification was carried out in T100™ Thermal Cycler (BIO-RAD, USA) by following the conditions listed at the GrainGenes web site (http://wheat.pw.usda.gov) for 30 cycles. PCR product was checked on 2% agarose gel for amplification. Subsequently, the IRD700 or IRD800-labelled PCR products were size-separated on 6% polyacrylamide gels with the Li-COR 4300 system (LI-COR® Biosciences 4647 Superior Street Lincoln, NE 68504, USA).

Statistical analyses

Chi squared (χ^2) test was used to evaluate the goodness-of-fit of observed and expected segregation ratios. The banding patterns of the resistant parent, susceptible parent and heterozygotes were denoted as A, B and H, respectively. Linkage analysis was performed using MAPMANAGER QTXb20 (Manly et al., 2001) and recombination values were converted to genetic distances using the Kosambi mapping function (Kosambi 1943). The linkage map was drawn using MAPCHART software version 2.2 (Voorrips 2002).

Conclusion

An eSTS marker, *mag3092*, showed complete linkage with *Lr28*. In addition to the identification of a robust co-dominant *Lr28*-linked marker, this study also delineated the size of translocated *Ae. speltoides* segment. Validation studies involving *Lr28*-carrying and *Lr28*-lacking genotypes demonstrated the diagnostic nature of *mag3092* for marker assisted selection of this gene in wheat breeding programs.

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