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The introgressed segment carrying rust resistance genes *Yr17*, *Lr37* and *Sr38* in wheat can be assayed by a cloned disease resistance gene-like sequence

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Abstract A cloned gene sequence (Vrga1D), with features of the nucleotide-binding-site leucine-rich repeat class of disease resistance (R) gene sequence super family, was previously shown to belong to a family of five gene members derived from a *Triticum ventricosum* Ces. (syn. *Aegilops ventricosa* Tausch) segment in wheat (*Triticum aestivum* L.). This gene family was introgressed, together with the linked rust resistance genes *Yr17, Lr37* and *Sr38* from *T. ventricosum*, to wheat chromosome 2AS. An independently derived *T. ventricosum* segment carrying a leaf rust resistance gene in a French wheat cultivar, was shown to exhibit a rust resistance response equivalent to *Lr37* as well as *Yr17* and *Sr38*. DNA probes from different regions of the Vrga1D clone consistently detected the presence of RFLPs associated with the introgressed segment carrying the resistance genes *Yr17, Lr37* and *Sr38* present in diverse wheat genotypes from Australia, Canada, France and the UK. Our results showed that the transfer of the *T. ventricosum*-derived Vrga1 gene members and the rust resistance genes were always accompanied by the loss of a corresponding set of Vrga1-related gene members in recipient wheat cultivars presumed to be of homoeoallelic origin. A PCR assay, based on sequences from the 3'-untranslated region of a Vrga1 gene member isolated from the *T. ventricosum* donor line of the introgressed segment, was developed. The PCR assay detected the presence of the introgressed rust resistance genes across the diverse wheat backgrounds and should be useful in marker-assisted selection in wheat breeding.

Keywords Wheat rust resistance · Disease resistance genes · Introgressed segment · *Aegilops ventricosa*

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Introduction

A diverse group of genes conferring resistance to diseases and pests, such as powdery mildew, three rusts (*Sr38, Lr37, Yr17*), eyespot, cereal cyst nematode and Hessian fly derived from *Triticum ventricosum* Ces. (syn. *Aegilops ventricosa* Tausch, genome designation N^vD^v), have been transferred to wheat (Delibes et al 1993; Jahier et al 1996). The *Yr17, Lr37* and *Sr38* rust resistance (R) genes which confer resistance to stripe rust (*Puccinia striiformis* West. f.sp. *tritici*), leaf rust (*Puccinia recondita* Rob. ex Desm. f.sp. *tritici* Eriks. & E. Henn.) and stem rust (caused by *Puccinia graminis* Pers.: f.sp. *tritici* Eriks. & E. Henn.), respectively, have been used by breeders in many parts of the world (McIntosh et al. 1995; Robert et al. 1999). These linked R genes were located in chromosome 2AS of VPM1 wheat (Bariana and McIntosh 1993). A wide range of wheat germplasm with rust resistance genes *Yr17, Lr37* and *Sr38* derived from crosses involving VPM1 wheat have been developed (see Table 1; McIntosh et al. 1995; Robert et al. 1999). In addition to these VPM1-derived wheats, an independent study by Dosba (1982) and Bonhomme et al. (1995) also reported introgression of a leaf rust resistance gene designated ''*Lr*'' from *T. ventricosum* into wheat. Genetic stocks carrying ''*Lr*'' in the wheat cultivar Moisson were initially produced as chromosome addition lines with the chromosome structure $6N^v$ S.$6N^vL$-$2N^v$ S (M+$6N^v$), and subsequently as $2N^vS$-2AS. 2AL translocation lines (MX12 and MX22). Furthermore, a rust-susceptible addition line of $6N^v$ S.$6N^vL$ [M+$6N^v$ (del)] in wheat further

Table 1 List and pedigrees of wheat near-isogenic line pairs (NILs; 1–13) or sib lines (14) selected for one or more of the *Sr38*, *Yr17* or *Lr37* resistance genes derived from VPM1 wheat, a *T. ventricosum* derivative

No.	Resistant (R) and susceptible (S) pairs of NILs	Pedigree of R NIL	*Specificity for selected wheat[a]
(1)	Trident (R) Spear (S)	VPM 1/5[a] Cook//4[a] Spear	*Yr17*
(2)	Sunstate (R) Hartog (S)	VPM 1/5[a] Cook//4[a] Hartog	*Yr17*
(3)	T66837 (R) Tatiara (S)	VPM 1/5[a] Cook//3[a] Tatiara	*Yr17*
(4)	Sunvale (R) Cook (S)	VPM 1/7[a] Cook	*Yr17*
(5)	T67036 (R) Dagger (S)	VPM 1/5[a] Cook//4[a] Dagger	*Yr17*
(6)	T66992 (R) Bayonet (S)	VPM 1/5[a] Cook//4[a] Bayonet	*Yr17*
(7)	T66962 (R) Bindawarra (S)	VPM 1/5[a] Cook//4[a] Bindawarra	*Yr17*
(8)	HR429 (R) Sunstar (S)	VPM 1/5[a] Sunstar	*Yr17*
(9)	195764 (R) 195776 (S)	VPM 1/6[a] Cook//3[a] K441 VPM 1/6[a] Cook//3[a] K441	*Yr17*
(10)	195792 (R) 195805 (S)	VPM 1/4[a] Cook//3[a] QT4118 VPM 1/4[a] Cook//3[a] QT4118	*Yr17*
(11)	195822 (R) 195835 (S)	VPM 1/2[a] K1056//3[a] K2745 VPM 1/2[a] K1056//3[a] K2745	*Yr17*
(12)	Avocet + *Yr17* (R) Avocet (S)	VPM1/6[a] Avocet	*Yr17*
(13)	Thatcher/VPM1 (R) Thatcher (S)	VPM1/6[a] Thatcher	*Yr17*, *Lr37*
(14)	E30.82 (R) E30.57 (S)	B41/Rendezvous (selfed F_7) B41/Rendezvous (selfed F_7)	*Sr38*

[a] Although the NILs were initially selected for the rust resistance genes shown, all the resistant members of the NIL pair carry all three introgressed resistance genes

delineated the 2N^v S translocated chromosomal segment as the source of the ''*Lr*'' gene (Bonhomme et al. 1995). Similar to the VPM1-derived rust resistance genes, the ''*Lr*'' gene in MX12 and MX22 was also introgressed to chromosome 2AS. However, the precise relationship between the ''*Lr*'' gene and *Lr37* from VPM1 wheat had not been fully established until the work reported in the present study.

A gene sequence (Vrga1D) containing a nucleotide binding-site leucine-rich repeat (NBS-LRR), a characteristic feature of several cloned plant disease resistance genes, was recently isolated from *T. ventricosum* (Seah et al. 2000). Vrga1D homologues in *T. ventricosum* accession no. 10 was shown to be located in the introgressed segment containing the ''*Lr*'' gene as well as the independently derived group of *Yr17*, *Lr37* and *Sr38* genes in two wheat isolines. In this study, the objectives were firstly to examine the stem, stripe and leaf rust resistance reaction between the two independently derived introgressed segments in wheat in order to establish the relatedness of *Lr37* to ''*Lr*'', as well as *Yr17* and *Sr38* to any corresponding genes in the MX lines. Secondly, the Vrga1D sequence was used as a probe on a diverse range of wheat backgrounds from Australia, UK, Canada and France in order to ascertain its diagnostic capability. Thirdly a PCR assay, based on a homologue of the Vrga1D sequence capable of unequivocal detection of the presence of the introgressed segment carrying this group of rust resistance genes, was developed for marker-assisted selection in wheat improvement.

Materials and methods

Plant genetic stocks and rust response analysis

A set of 13 wheat near-isogenic line pairs (NILs) derived from VPM1 wheat (see Table 1) produced by the National Cereal Rust Control Program (Plant Breeding Institute, Cobbitty, Australia), and a 'Thatcher' wheat cultivar NIL pair by P.L. Dyck (Agriculture Canada, Winnipeg), were used. Two F_7 sib lines differing for the presence of *Sr38* introduced from the UK wheat cultivar, Rendezvous, were also kindly supplied by J. Davidson and S. Kleven (CSIRO, Canberra, A.C.T., Australia). A set of rust-resistant 'Moisson' (French wheat cultivar) chromosome addition (M+6N^v) and translocation lines (MX12 and MX22) and a rust susceptible addition wheat line [M+6N^v (del)], as described in Bonhomme et al. (1995), were used in rust response tests.

Rust tests

The following pathotypes (PBI accession no.) were used: *P. striiformis* f.sp *tritici* pt. 110 E 143A+ (444); *P. recondita* f.sp *tritici*

Table 2 Infection types produced by different genetic stocks and control lines when tested against three rust pathogens. Seedlings from the plant genotypes were inoculated separately with each rust culture described in the Materials and methods section, and infection type (IT) was scored according to Bariana and McIntosh (1993)

Genotype	*P. striiformis* f.sp *tritici* Infection type[a]	*P. recondita* f.sp *tritici* Infection type[a]	*P. graminis* f.sp *tritici* Infection type[a]
Mx12	;CN	;12=	;
Mx22	;CN	;1	;
Moisson + 6N^{V}	;CN	;12=	;
Moisson + 6N^{V}del	3+	3	2=
Moisson	3+N	3+	2=
VPM1	;CN	;12=	X=
Trident	;CN	;12=	X=
Spear	3+	3+	3+

[a] = Hypersensitive fleck; 1 = small rust uredinia, scattered, not erupting through the epidermis and enclosed by chlorotic and/or necrotic tissue; 2 = larger rust uredinia, erupting through the epidermis and often associated with chlorotic and/or necrotic tissue; 3 = large, freely sporulating uredinia associated with varying levels of chlorosis. The symbols ''+'' and ''-''were appended to the major infection type classes to denote more or less disease, respectively. The presence of additional chlorosis and necrosis was noted using ''C'' and ''N'', respectively. These infection types result from host-pathogen interaction. Infection types 3+ indicates susceptible responses. X = range of infection types from resistant to susceptible scattered randomly on a single leaf; caused by a single pathotype and not a mixture

pt. 104–2,3,5,6,(7),11 (472) and *P. graminis* f.sp *tritici* pt. 34–1,2,3,4,5,6,7 (103). Seedlings of experimental material were grown and inoculated, and infection types (IT) scored according to Bariana and McIntosh (1993). Incubation conditions for each rust are described in Bariana and McIntosh (1994). VPM1, Trident and Spear were used as controls when Moisson-derivatives were tested with the three rust pathogens.

RFLP and PCR-based assay

RFLP analysis on *Dra*I-digested genomic DNA from all the wheat genetic stocks, using subclones from *Vrga1D* as probes, was performed. Two subclones were used; one was from a *Bam*HI- *Pst*I fragment that spans the entire nucleotide binding sites to the middle of the leucine-rich region (Genbank accession no. AF158634) and was named Vrga1DNBS. The second subclone, csVrga13', contained the 3'untranslated region in addition to 100 bp of the 3' end of the predicted coding region of Vrga1D. DNA filter hybridisation and washing conditions were as described in Seah et al. (1998).

The oligonucleotide primers Vlr2.6–3': 5'-TCTGTATTCT CCTTGGGTAC-3' (forward-primer 1) and Vlr2.4–5': 5'-TTTTCA CCTGCTTGCAGCAC-3' (reverse-primer 1), were designed to amplify a 886-bp fragment from the LRR and the 3'-untranslated region of the Vrga1D gene sequence (Genbank accession no. AF158634), and the corresponding region in *T. ventricosum* # 10 the donor line for the introgressed segment in wheat. Primer sequences specific to *T. ventricosum* #10, csVrgal3'F and csVrgal3'R, were then employed in a PCR-assay to detect the alien segment in wheat for marker-assisted-selection. The PCR reaction-mix and amplification conditions are described in Seah et al. (1998) and used 100 ng of total genomic DNA as a template.

Results

Rust response tests

Translocation lines MX12 and 22, chromosome addition lines Moisson+ 6N^{v} and Moisson + 6N^{v}del, Moisson, and VPM1 were tested against the three rusts. The infection types produced by these genotypes and their description, along with positive and negative controls, are presented in Table 2.

Stripe rust

VPM1 and its derivative Trident, translocation lines MX12 and MX22 and addition line Moisson+ 6N^{v} produced infection type (IT) ';CN' in response to infection with *P. striiformis* f. sp *tritici*. Cultivar Moisson, Moisson + 6N^{v}del and the susceptible control, Spear, exhibited IT '3+' (see Table 2). The similar infection type produced by the *T. ventricosum*-derived translocation lines MX12 and MX22, VPM1 and addition line Moisson + 6N^{v}, and susceptibility of the addition line with deleted 2N^{v} S (Moisson + 6N^{v}del) and recurrent parents Moisson and Marne (Bariana and McIntosh 1993), indicated that the stripe rust resistance gene carried by these lines was *Yr17*.

Leaf rust

VPM1, Trident, MX12, and Moisson+ 6N^{v} produced a similar infection type (IT ';12=') when tested with *P. recondita tritici*, whereas line MX22 produced a slightly lower but non-distinct IT ';1'. The parental *T. ventricosum* line produces an infection type IT ';12=' (Bariana and McIntosh 1994). Cultivars Spear, Moisson and the addition line with deleted 2N^{v} S (Moisson + 6N^{v}del) were susceptible with IT '3+' (see Table 2). These results indicated that the leaf rust resistance gene in VPM1 and that present in the Moisson-derivatives are the same. These results further imply that '*Lr*' is in fact *Lr37*. The slightly lower infection type produced by line MX22 may be due to subtle background differences because no other triple rust-resistant material with infection types similar to that produced by *T. ventricosum* derivatives is known.

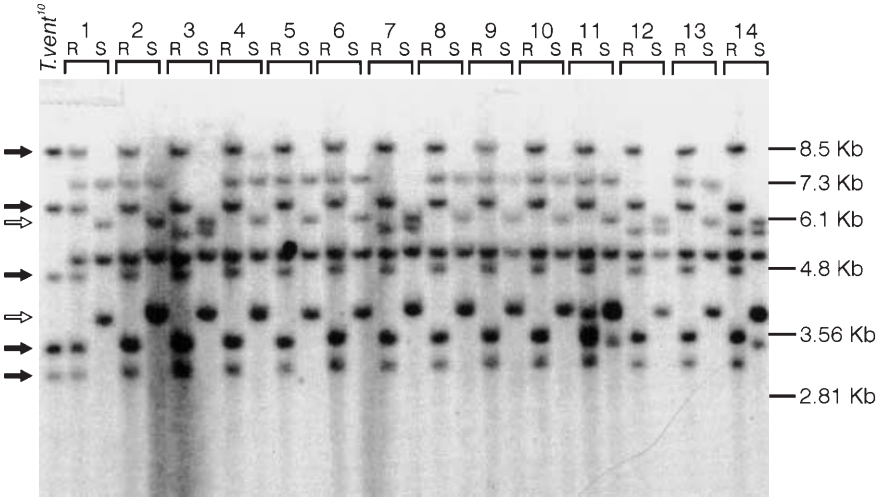

Fig. 1 DNA hybridisation patterns of the Vrga1DNBS probe on *Dra*I-digested genomic DNA from a set of resistant (*R*) and susceptible (*S*) wheat isolines taken from Table 1: (*1*) Trident (*R*)/Spear (*S*); (*2*) Sunstate (*R*)/Hartog (*S*); (*3*) 66837 (*R*)/Tatiara (*S*); (*4*) Sunvale (*R*)/Cook (*S*); (*5*) 67036 (*R*)/Dagger (*S*); (*6*) 66992 (*R*)/Bayonet (*S*); (*7*) 66962 (*R*)/Bindawarra (*S*); (*8*) HR429 (*R*)/Sunstar (*S*); (*9*) 195764 (*R*)/195776 (*S*); (*10*) 195792 (*R*)/195805 (*S*); (*11*) 195822 (*R*)/195835 (*S*); (*12*) Avocet +Yr17 (*R*)/ Avocet (*S*); (*13*) Thatcher/VPM1 (*R*)/ Thatcher (*S*); (*14*) E30.82 (*R*)/E30.57 (*S*). *T. vent 10* refers to *T. ventricosum* #10. *Arrows* (*dark*) indicate the diagnostic RFLP fragments associated with the presence of the rust genes from the 2 N^v S segment of *T. ventricosum*. The open arrows are fragments derived from chromosome 2 A of the recipient wheat background. Size markers in kilobases (kb) are indicated to the left of the figure

Stem rust

VPM1 and its derivative, Trident, produced IT 'X='. The susceptible control cultivar, Spear, produced IT '3+'. Translocation lines Mx12 and Mx22, and the addition line Moisson+ 6N^v, produced IT ';', whereas cultivar Moisson and the addition line with a deleted 2N^v S (Moisson + 6N^vdel) produced IT '2=' (see Table 2). The resistance response, IT '2=', of cultivar Moisson was attributed to the gene *Sr29*, which is common in European wheats. It is known that the *T. ventricosum*-derived gene *Sr38* interacts with other stem rust resistance genes to produce a lower IT ';' (Bariana and McIntosh 1993), and presumably that has also occurred in this instance. The IT ';' expressed by lines MX12, MX22 and Moisson+ 6N^v is due to a combination of *Sr38* (*T. ventricosum*) and an additional gene (s) from Moisson.

Comparison of the results from the three rusts demonstrated the likelihood that the linked rust resistance genes *Yr17*, *Lr37* and *Sr38* had been introgressed to chromosome 2AS in two independent introgression events. This indicated either the translocation-prone nature of chromosome 2 A or an homoeologous relationship between the N^v and A genomes of *T. ventricosum* and wheat, respectively.

Vrga1D homologues are present on the introgressed segment carrying the rust resistance genes

Both the Vrga1DNBS and csVrga1D3' subclones from the Vrga1D sequence (Genbank accession #AF158634; Seah et al. 2000) used as RFLP markers, detected the introgressed segment carrying *Yr17*, *Lr37* and *Sr38* (Figs. 1 and 2). Five gene members from the donor, *T. ventricosum* accession #10, detected by Vrga1DNBS, were present in all isolines carrying the introgressed segment(VPM1-derived rust resistance genes) in all 14 wheat backgrounds. These five gene members were previously shown to be present in MX12, MX22 and Moisson +6N^v, but were absent in Moisson and the Moisson + 6N^v del line (Seah et al. 2000). These results confirmed the 2N^v origin of the five gene members. All the VPM1 derivatives were accompanied by a corresponding loss of two Vrga1 gene members (approximately 6.1-kb and 4-kb RFLPs; Fig. 1). These two fragments were present in all 14 wheat backgrounds, including Moisson, that lack the *Yr17*, *Lr37* and *Sr38* genes. Vrga1-related sequences have previously been mapped to the short arms of homoeologous group-2 chromosomes (Spielmeyer et al. 1998). The 6.1-kb and 4-kb restriction fragments (Fig. 1, see open arrows) derived from chromosome 2 A (Seah 1999) were invariant in all the wheat backgrounds, whereas polymorphic fragments associated with other Vrga1 members (7.3 kb, 5.8 kb, 3.3 kb) were from either homoeologous chromosome 2B or 2D.

Three polymorphic regions, designated A, B and C (Fig. 2), were identified using csVrga13' as a probe between each pair of isolines differing for the presence or absence of the introgressed segments harbouring the resistant genes. Regions A and C (Fig. 2) carry diagnostic RFLPs indicative of a homoeoallelic pattern between each member of the wheat iso-lines. In region C (Fig. 2), an approximately 0.4-kb RFLP was associated with the presence of the rust resistance genes on the introgressed segment. A wheat-derived diagnostic fragment (approxi-

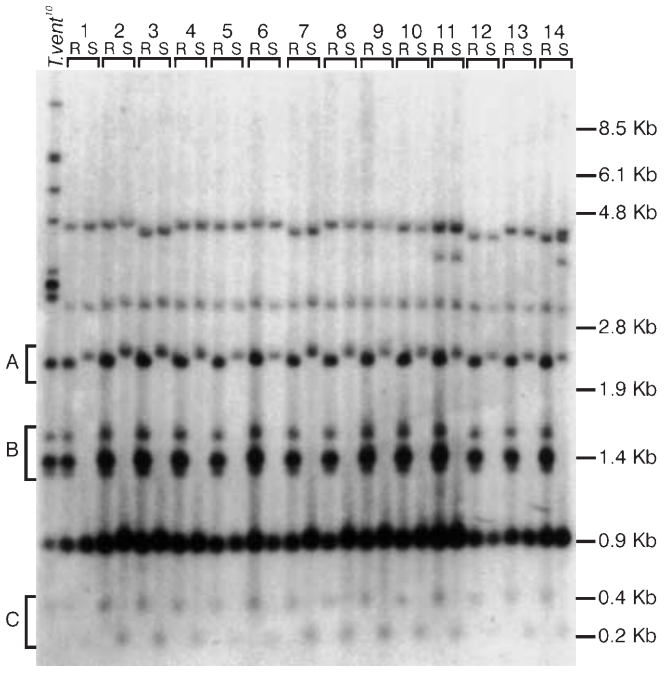

Fig. 2 DNA hybridisation patterns of the csVrga1D3'probe on *Dra*I-digested genomic DNA from a set of resistant (*R*) and susceptible (*S*) wheat isolines taken from Table 1: (*1*) Trident (*R*)/Spear (*S*); (*2*) Sunstate (*R*)/Hartog (*S*); (*3*) 66837 (*R*)/Tatiara (*S*); (*4*) Sunvale (*R*)/Cook (*S*); (*5*) 67036 (*R*)/Dagger (*S*); (*6*) 66992 (*R*)/Bayonet (*S*); (*7*) 66962 (*R*)/Bindawarra (*S*); (8) HR429 (*R*)/Sunstar (*S*); (*9*) 195764 (*R*)/195776 (*S*); (*10*) 195792 (*R*)/195805 (*S*); (*11*) 195822 (*R*)/195835 (*S*); (*12*) Avocet +Yr17 (*R*)/ Avocet (*S*); (*13*) Thatcher/VPM1 (*R*)/ Thatcher (*S*); (*14*) E30.82 (*R*)/E30.57 (*S*). *T. vent10* refers to *T. ventricosum* #10. Three of the major polymorphic regions between each pair of isolines are designated as A, B *and C*, with regions *A and C* revealing the alternative homoeoallelic fragments. Size markers in kilobases (kb) are indicated to the left of the figure

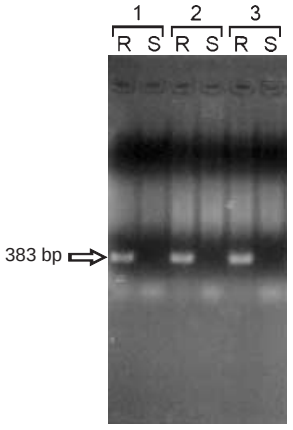

Fig. 3 PCR amplification profile of (*1*) Trident (*R*)/ Spear(*S*), (*2*) Sunstate (*R*)/Hartog (*S*), (*3*) 66837 (*R*)/Tatiara (*S*), amplified with the primers csVrga13'F and csVrga13'R at an annealing temperature of 55°C. The *arrow* indicates the expected product size of 383 bp from the 3'-untranslated region of the Vrga1 homologue present within the 2N^v S segment from *T. ventricosum* carrying the group of rust resistant genes. *R* = resistant line; *S*-susceptible line

mately 0.2 kb) associated with the absence of the resistance genes was also consistently detected by the csVrga1D3' marker in the rust-susceptible lines (Fig. 2). Seah (1999) observed the 'homoeo-allelic' behaviour of the diagnostic bands detected by the csVrga1D3' marker among the progeny of heterozygous individuals. The ability of the csVrga1D3' marker to identify either 'homoeo-allelic' variant demonstrates the added usefulness of this marker in marker-assisted selection.

PCR diagnostic assay for the introgressed segment carrying rust resistance

In addition to the RFLP markers used in distinguishing between the rust-resistant and -susceptible wheat lines (Table 2), a PCR assay was also developed. Oligonucleotide primers taken directly from the Vrga1D sequence could only be used in a diagnostic assay for the introgressed genes by introducing an internal primer sequence in a nested PCR reaction. This was considered to lack robustness for marker-assisted-selection as it required two rounds of PCR analysis. The corresponding 3'untranslated region of Vrga1D in the *T. ventricosum* accession #10 donor line of the introgressed segment was sequenced and shown to be 88% identical to Vrga1D with a number of base-substitution differences. Sequences specific to *T. ventricosum* accession #10, were then used to design the primers csVrga13'F and csVrga13'R to amplify a 383-bp product in a single PCR reaction. The 383-bp amplification product was present only in isolines with the introgressed segment carrying the rust resistance genes and absent in all the recurrent wheat parents that lacked *Yr17*, *Lr37* and *Sr38* (Fig. 3). A gradient PCR experiment using different annealing temperatures showed that the 383-bp fragment was consistently amplified across annealing temperatures ranging from 50 to 61°C only in the isolines or cultivars with the triple rust resistance genes, and thus demonstrates the utility of the PCR assay in marker-assisted selection.

Discussion

The subclone Vrga1DNBS, which contains all the conserved motifs found in all members of the NBS-LRR class of cloned disease resistance genes, did not detect related sequences from the D^v genome component of the donor line *T. ventricosum* accession #10. This was evident from the consistent transfer of all five Vrga1 gene members on the introgressed 2N^v segment derived from *T. ventricosum* accession #10 into wheat (Fig. 1). Variation among diploid D-genome accessions, where some accessions were found to be lacking Vrga1-related sequences (Seah and Lagudah, unpublished), provides further support for the absence of Vrga1-related members in the D^v genome component of *T. ventricosum* accession #10. Unlike the Vrga1DNBS subclone, the use of the csVrga1D3' subclone as a probe revealed

the presence of additional fragments (Fig. 2; four fragments > 4.8 kb and three other fragments between 2.8 and 4.8 kb) from the donor *T. ventricosum* accession. These fragments were not transferred along with the $2N^v$ introgressed segment into wheat and may be located elsewhere in the N^v genome component. However, if they are from the $2D^v$ homoeoloci, this may suggest that portions of the corresponding Vrga1DNBS coding region may have diverged from the Vrga1D sequences, or are deleted, since they were undetected by the Vrga1DNBS probe.

The possibility that the Vrga1D gene, or one of the other four closely related gene members from *T. ventricosum* introgressed into the wheat lines (Table 2), represents a candidate R gene for one or more of the rust resistance genes group was raised earlier (Seah 1999). To-date, the R genes *L6*, *M* and *Rp1*-D which confer resistance to rust pathogens in flax (*Linum usitatissimum* L.) and maize (*Zea mays* L.) have been isolated (Lawrence et al. 1995; Anderson et al. 1997; Collins et al. 1999). These cloned resistance genes also belong to the NBS-LRR class of R genes. It thus remains to be shown whether the R gene(s) encoding *Yr17*, *Lr37* and *Sr38* may also belong to the NBS-LRR class of R genes. If this were shown to be the case, the advantage of the use of the NBS-LRR gene sequences as markers increases the probability that they do assay candidate R genes or their homologues and hence serve as 'perfect markers' for selection. Alternatively, the Vrga1D gene-family members may be closely linked to the genes encoding *Yr17*, *Lr37* and *Sr38* due to suppressed recombination in the introgressed segment and, therefore, would not constitute 'perfect markers' but simply completely linked markers. Relatively simple hybridisation patterns can also be obtained (Fig. 1) in contrast to the use of the generic *Xpsr150–2M*^{v} marker which was previously shown to be linked to this group of resistance genes (Bonhomme et al. 1995; Robert et al. 1999). Therefore, in addition to *Xpsr150–2M*^{v} and the SCAR SC-Y15 marker, which were shown to be linked with the *Yr17* gene in wheat (Robert et al. 1999), there are now two other RFLP markers and a PCR assay based on the Vrga1D and related sequences. In this study we have shown that sequences or probes from Vrga1D and its homologues reliably detect the introduced segment from *T. venricosum* carrying *Yr17*, *Lr37* and *Sr38* across 14 wheat backgrounds.

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