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Leaf tip necrosis, molecular markers and β1-proteasome subunits associated with the slow rusting resistance genes *Lr46/Yr29*

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Abstract Resistance based on slow-rusting genes has proven to be a useful strategy to develop wheat cultivars with durable resistance to rust diseases in wheat. However this type of resistance is often difficult to incorporate into a single genetic background due to the polygenic and additive nature of the genes involved. Therefore, markers, both molecular and phenotypic, are useful tools to facilitate the use of this type of resistance in wheat breeding programs. We have used field assays to score for both leaf and yellow rust in an Avocet-*YrA* × Attila population that segregates for several slow-rusting leaf and yellow rust resistance genes. This population was analyzed with the AFLP technique and the slow-rusting resistance locus *Lr46/Yr29* was identified. A common set of AFLP and SSR markers linked to the *Lr46/Yr29* locus was identified and validated in other recombinant inbred families developed from single chromosome recombinant populations that segregated for *Lr46*. These populations segregated for leaf tip necrosis (LTN) in the field, a trait that had previously been associated with *Lr34/Yr18*. We show that LTN is also pleiotropic or closely linked to the *Lr46/Yr29* locus and suggest that a new *Ltn* gene designation should be given to this locus, in addition to the one that already exists for *Lr34/Yr18*. Coincidentally, members of a small gene family encoding *β*-1 proteasome subunits located on group 1L and 7S chromosomes implicated in plant defense were linked to the *Lr34/Yr18* and *Lr46/Yr29* loci.

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Introduction

Rust diseases of wheat (*Triticum aestivum* L.) are major constraints to production in most wheat growing regions of the world. Control of these diseases has often been achieved through the use of race-specific resistance genes that can result in a hypersensitive response to inhibit the growth of invading pathogens. However, these types of genes have been quickly overcome by new virulent pathotypes (races) of the fungi. When cultivars containing the same resistance genes are deployed over large areas, resistance breakdown can lead to large scale epidemics. A more durable resistance (Johnson 1988) to rusts involves slow rusting (Caldwell 1968) genes that affect fungal growth through a number of mechanisms including longer latent periods, production of fewer uredinia and smaller uredinia sizes (Kolmer 1996). High, or near-immune, levels of resistance in wheat to leaf (or brown) rust caused by *Puccinia triticina* and yellow (or stripe) rust caused by *Puccinia striiformis* f. sp. *tritici* were achieved by pyramiding between 4 and 5 slow-rusting genes that have small to intermediate additive effects (Singh et al. 2000). However, the genetic location of most of the genes that contributed to resistance is not known. Phenotypic or molecular markers for these genes will help to identify the number and genetic location of the genes involved in slow-rusting resistance to the rusts.

To date, only three slow-rusting loci have been identified with gene designations and established genomic locations. Two loci show effects against leaf and

yellow rust and are conferred by the *Lr34/Yr18* complex on chromosome 7DS (Suenaga et al. 2003) and the *Lr46/Yr29* complex on 1BL (William et al. 2003a). It is unknown whether these loci contain genes with pleiotropic effects against both pathogens, or whether they contain closely linked genes to confer the dual resistance. A third locus on 3BS contains the stem rust (caused by *P. graminis* f. sp. *tritici*) resistance gene *Sr2* and a closely linked yellow rust gene *Yr30* (Singh et al. 2005). Amplified fragment length polymorphisms (AFLPs) and Sequence tagged microsatellites (STM) were mapped to the resistance locus of *Sr2*, (Spielmeyer et al. 2003; Hayden et al. 2004). Some phenotypic markers are also closely associated with the *Sr2* locus. These are pseudo black chaff (*Pbc*), a pigmentation which occurs around the glumes and stem internodes (Hare and McIntosh 1979), and a high-temperature-induced seedling chlorosis (HTISC) (Brown 1997).

Both phenotypic traits are difficult to use as markers in breeding programs as *Pbc* is partially dominant and expression varies due to genetic background, while HTISC is recessively inherited. Similarly the trait, leaf tip necrosis (LTN), associated with *Lr34/Yr18* (Singh 1992) has been shown in other studies to be quantitatively expressed, controlled by several QTLs and is variable in different backgrounds and environmental conditions (Messmer et al. 2000). The LTN trait described by Singh (1992) to be associated with *Lr34/Yr18* locus was observed by investigating a number of crosses between *Lr34/Yr18*/LTN positive lines and *Lr34/Yr18*/LTN negative lines. LTN is also associated with resistance to spot blotch disease of wheat caused by *Bipolaris sorokiniana* (Joshi et al. 2004) and the gene *Bdv1* that confers slow yellowing to barley yellow dwarf virus (Singh 1993). Marker studies have yet to be conducted to determine the location of the spot blotch resistance although the lack of recombination between this resistance and LTN in near-isogenic reselections indicates that the phenotypes are from very closely linked genes or are pleiotropic effects of a single gene.

The slow rusting loci *Lr34/Yr18* and *Lr46/Yr29* are intriguing as they confer similar slow rusting phenotypes to both leaf rust and yellow rust. This paper describes further similarities between these loci. We initially identified an LTN phenotype segregating in a population not containing the *Lr34/Yr18* locus. The genomic location of this phenotypic marker is described and a new *Ltn* gene designation is suggested. Additional molecular mapping studies around the *Lr46/Yr29* locus has lead to new AFLP markers, and along with existing AFLP (William et al. 2003a, b) and microsattelite markers, finer maps of this slow-rusting locus is described. Given the parallels between these pairs of slow-rusting genes located at non-homoeologous loci, it remains unknown how many genes are shared in common between the respective loci. We also report the identification of homologs of genes encoding β-1 proteasome subunits that are located in genomic intervals that contain the *Lr46/Yr29* and *Lr34/Yr18* loci. These genes are of particular interest as this family has been reported to be involved in plant defense (Suty et al. 2003).

Materials and methods

Development and Evaluation of Avocet-*YrA* × Attila F_5 lines

A F_2 plant derived F_5 single seed descent (SSD) population containing 148 lines was developed from the cross of Avocet-*YrA*, susceptible to both leaf and yellow rusts and Attila that shows a moderate to high level of slow-rusting to both rusts. The SSD lines were evaluated in the field for reaction to yellow and leaf rusts at CIMMYT research stations in Toluca near Mexico City and Ciudad Obregon in Sonora State, Mexico, respectively. Approximately 80 seeds of the parents and SSD lines were sown on top of the 75 cm wide raised beds in paired row plots, 1 m in length, with 20 cm between rows and a 50 cm pathway. Rust epidemics were initiated about 4 and 8 weeks after planting (yellow rust and leaf rust, respectively) by inoculating the susceptible spreader rows of cv. Morocco planted as hills on one side of the plots in the pathway. To initiate the epidemics, Morocco was sprayed with a suspension of rust urediniospores in the lightweight mineral oil, Soltrol 170 (Chevron Phillips Chemical Company, The Woodlands, TX, USA). The percent rust severity for each plot was evaluated for three crop seasons (2000, 2002 and 2003 for yellow rust and 1999–2000, 2001–2002 and 2003–2004 for leaf rust) according to the modified Cobb Scale (Peterson et al. 1948). Leaf rust severity was estimated visually initially when the susceptible parent Avocet-*YrA* showed between 80 and 100% rust severity on flag leaves and a second time about 10 days later. Yellow rust severity was estimated once each season, when the susceptible parent showed between 70 and 90% severity. The population was also scored for leaf tip necrosis in the 2003–2004 season at Ciudad Obregon.

Development and evaluation of single chromosome recombinant lines Lalbahadur × Lalb. (Pavon 1B) and Lalbahadur × Lalb. (Parula 1B)

Singh et al. (1998) developed partially backcrossed chromosome substitution lines where chromosome 1B from two cultivars, Pavon 76 and Parula, was substituted for the 1B chromosome of the leaf rust susceptible cultivar Lalbahadur (Lalb.). These substitution lines were crossed with Lalb. and F_5 or F_6 populations developed. The Lalb./(Pavon 1B) population consisted 181 F_5 lines and the Lalb./(Parula 1B) population was comprised of 170 F_6 lines. Both populations were scored for leaf rust reaction and leaf tip necrosis in the field at Ciudad Obregon during 2003–2004 crop season.

DNA Extraction, AFLP, SSR and RFLP analysis

All three populations were grown in the greenhouse in 10 cm pots (each pot contained 10 seeds from one line, leaf material from individual pots was pooled for DNA extraction) for 5 weeks for DNA extraction using a CTAB (alkyltrimethyl–ammonium bromide) based extraction procedure (Hoisington et al. 1994). The AFLP technique (Vos et al. 1995) was used to identify polymorphic markers associated with resistance. A total of 208 AFLP primer combinations using *Pst1*/*Mse1* restriction enzyme sites were applied to bulks (pooled DNA from 12 leaf rust resistant lines, 10 yellow rust resistant lines and 10 lines susceptible to both diseases) from the Avocet-*YrA* × Attila population. Adaptor and primer sequences are described in William et al. (2003a). Sequences of primers that identified new polymorphisms were: GACTGCGTAGGTGCAGACA (P35), GACTG CGTAGGTGCAGTCC (P84) and GACTGCGTAGG TGCAGTCG (P85) combined respectively with GATG AGTCCTGAGTAACGA (M55), GATGAGTCCTGA GTAAGTT (M78), and GATGAGTCCTGAGTAAG CA (M67). All amplification reactions were completed under the conditions described by William et al. (2003a) except for the selective amplifications using the *Pst1* primers ending in GCN. These primers required the application of FailSafe PCR Premix Buffer F from Epicentre (Madison WI, USA) and were used according to the manufacturers instructions. Fragment detection utilized a chemiluminescent technique as described by Hoisington et al. (1994). Primer sequences from the simple sequence repeat (SSR) markers located in the distal region of wheat chromosome 1BL (*barc081, barc188, gwm140, gwm259, gwm268, gwm274, wmc44, wmc367, wmc719*) were accessed from the Graingenes website http://www.wheat.pw.usda.gov/GG2/index.shtml. Additional SSR markers TG 818 and 1719 were kindly supplied by Dr. Martin Ganal, TraitGenetics, Gatersleben, Germany. Approximately 50 ng of genomic DNA was used in PCR amplification of the SSR using the recommended annealing temperatures for the respective SSR markers. Visualization of the amplified SSR products was by using agarose gel electrophoresis (3%) coupled with ethidium bromide staining.

Analysis of β-1 proteasomes

Triticeae EST clones were identified from genomic sequence similarities to the rice BAC clone OSJNBa004F13 [chosen on the basis that it forms part of the collinear region between rice chromosome 6 and wheat markers associated with *Lr34*/*Yr18* (Nelson et al. 1997; Kudrna et al. 2000) on wheat 7S]. Chromosomal location of the Triticeae ESTs were verified by RFLP analysis using nulli-tetrasomic Chinese Spring cytogenetic stocks (Lagudah et al. 1991). EST BE495774 was used as a probe in DNA hybridization to screen cDNA and BAC libraries from the diploid D genome progenitor (Lagudah et al. 1997; Moullet et al. 1999) and a BAC library of hexaploid wheat (Nilmalgoda et al. 2003). Conditions for BAC library screening were as described in Moullet et al. (1999). Low copy sequences isolated from the BE495774 hybridizing BACs were used to confirm the chromosomal origin of the BAC clones. A 300 bp fragment located downstream of the 3′UTR of the β1-proteasome gene found in a chromosome 7D BAC clone isolated from the D genome progenitor was used in RFLP analysis and mapped relative to the markers located in the *Lr34*/*Yr18*/LTN QTL present in the Opata × Synthetic mapping family (Nelson et al. 1997). RFLP analysis was also conducted with EST BE495774 on pooled DNA restricted with the endonuclease *DraI* from 10 homozygous resistant and 10 homozygous susceptible lines from the Avocet-*YrA* × Pavon 76 family (William et al. 2003a) segregating at the *Lr46*/*Yr29* locus. Sequences isolated from two BAC clones of chromosome 1B origin (utilizing the hexploid wheat BAC library) that hybridized to BE495774 were used in PCR analysis to identify STS markers that were mapped in the Lalb. × Lalb.(Pavon1B) and Lalb. × Lalb.(Parula1B), single chromosome recombinant families segregating for *Lr46*. Hotstar Taq (QIAGEN) was used in PCR analysis of the STS markers under the recommended manufacturer's conditions. Primer sequences for the STS markers were identified from low copy regions of the respective BAC clones and were not from the proteosome gene region. Sequences of primers are as follows: BAC17R-F- CCCATGCTGACATGGC CACAT, BAC17R-R- CTCTGCTCTTTAGTAGTTG CC and the PCR reannealing temperature was at 55°C. Primer sequences for the second STS marker are 24-1PromF- ACACACTGTGTCCTACCAACC and 24-1PromR- CTCTGCCTGCGGCGGCTTTGG and the PCR reannealing temperature was at 64°C.

Statistical analysis

The *r* value of the Pearson Correlation Coefficient was calculated for leaf and yellow rust data between years by using the corresponding function in a Microsoft Excel worksheet and significance determined by a SAS computer program (SAS Institute, Cary, NC, USA). Linkage analysis was completed using MAPMAKER 2.0 (Lander et al. 1987) and linkage groups established with a LOD score of 3.0. The coefficient of determination (r^2) was calculated as a measure of the proportion of the phenotypic variation explained by the markers using simple linear regression with Q-GENE (Nelson 1997).

Results and discussion

Field characterization of Avocet-*YrA* × Attila F_5 population

Scoring twice for leaf rust severity facilitated the discrimination initially between susceptible and highly

susceptible lines and subsequently between resistant and highly resistant lines. Over the three seasons, the susceptible Avocet-*YrA* parent ranged between 80 and 100% of the leaf area covered by the rust in the initial observations. In the second scoring, Attila ranged between 5 and 10% of the leaf area covered. Notes for yellow rust were usually only taken once a season, with Avocet-*YrA* ranging from 70 and 90% of the leaf area covered by the rust and Attila ranging from 5 to 15%. The level of rust infection was consistent for the lines over years as indicated by high Pearson correlation coefficients (Table 1). The rust data was therefore averaged over the 3 years for further analysis.

The average leaf rust severity showed a uniform distribution while the yellow rust severity showed a normal distribution (Fig. 1). The number of genes that conferred resistance was estimated by comparing the observed frequencies of lines that resembled each of the parents, with frequencies that would be expected in an F_5 generation if 2, 3 or 4 genes conferred resistance in the resistant parent (Singh and Rajaram 1994). To calculate the most likely number of genes involved in resistance, lines were classified into three categories, homozygous parental type resistant (HPTR) lines, homozygous parental type susceptible (HPTS) lines and others (intermediate severity levels). The frequency of observed lines in each category, along with expected values for 2, 3 and 4 genes, are summarized in Table 2. The χ^2 values indicate that there are at least two and three genes that conferred resistance to leaf rust and yellow rust, respectively. These data are similar to those of William et al. (2003a), where the resistant Pavon 76 was shown to contain the same expected number of resistance genes for each of the two rusts. Both Pavon 76 and Attila show moderate to high levels of rust resistance in the field, suggesting that durable resistance can be obtained by combining only a few slow rusting genes that have additive effects.

A highly significant ($r = 0.76$, $P < 0.01$) correlation between the three years averages for leaf rust and yellow rust severities indicated that common, or closely linked genes, were involved in resistance to the two rusts. This is not surprising as a number of loci are known to contain multiple slow rusting resistance genes, e.g., *Lr34/Yr18* (Singh 1992, Suenaga et al. 2003), *Lr46/Yr29* (William et al. 2003a). These loci could either contain multiple, closely linked genes, or just a single gene with multiple pleitropic effects. It is interesting that such

Table 1 Comparison of leaf rust and yellow rust severity data for different seasons using the Pearson correlation coefficient (r)

Years compared	Coefficient for leaf rust	Coefficient for yellow rust
Year 1 and 2	0.81*	0.57*
Year 1 and 3	0.81*	0.86*
Year 2 and 3	0.85*	0.66*

* Significant at $P = 0.01$

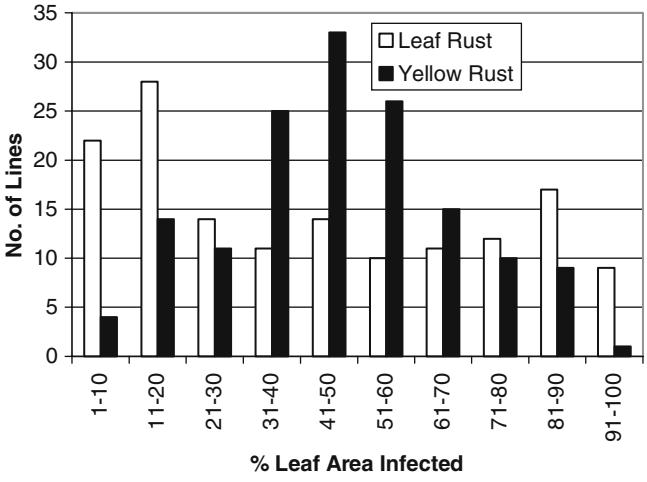

Fig. 1 Percentage of leaf area infected by leaf and yellow rust on individual lines from the Avocet-*YrA* × Attila population averaged over 3 years of field data

complex loci exist. It could be that the resistance genes are involved in some mechanism that is essential to components of the infection process that it is highly conserved across not only pathotypes, but also across different fungal species. Such a mechanism, when completely elucidated, may provide a clue as to how one could genetically engineer broad-spectrum durable resistance.

Association of LTN with resistance

The three populations (Lalb. × Lalb.(Pavon 1B), Lalb. × Lalb.(Parula 1B) and Avocet-*YrA* × Attila) were scored for flag leaf LTN when close to maturity in the field. In the single chromosome recombinant line populations, it was observed that LTN co-segregated with the resistance to leaf rust. Lines were therefore grouped into three categories, LTN+ were homozygous for both LTN and resistance, LTN± were segregating for LTN and resistance and LTN− did not contain LTN and were susceptible. There were no lines that showed recombination between LTN and resistance. These populations were not screened for yellow rust since Lalb. demonstrates moderate levels of resistance to this pathogen. Table 3 shows the χ^2 analyses for the inheritance of LTN (and therefore resistance) in the single chromosome recombinant line populations. Both populations were in agreement with a single gene segregating for resistance and LTN ($P > 0.01$). In the Avocet-*YrA* × Attila population, LTN was associated with low levels of disease severity (Fig. 2). This was particularly obvious for the leaf rust data, and is a reflection that there is probably only one other gene for resistance segregating in this population (Fig. 2a). There were more lines of an intermediate resistance level for yellow rust, both with and without LTN (Fig. 2b), and this would be expected when there are three or more genes conferring resistance,

Table 2 Estimation of the number of additive, slow-rusting genes for leaf rust and yellow rust through the identification of homozygous parental type resistant (HPTR), homozygous parental type susceptible (HPTS) and intermediate (Other) lines in the Avocet-*YrA* × Attila F_5 population

	Frequency of responses				
	HPTR	HPTS	Other	χ^2 leaf rust	χ^2 yellow rust
Observed frequency (leaf rust)	21	22	105		
Observed frequency (yellow rust)	18	10	120		
Expected frequency, 2 genes	28.3	28.3	91.3	5.4	24.6*
Expected frequency, 3 genes	12.4	12.4	123.2	16.1*	3.1
Expected frequency, 4 genes	5.4	5.4	137.2	103.0*	35.5*

* Significantly different from expected ratios at $P > 0.01$

one of which is linked to LTN. The absence of any lines carrying LTN and having high rust scores further underline the linkage to resistance and the importance of this phenotypic marker in the Avocet-*YrA* × Attila population.

A complete lack of recombination between LTN and resistance in the single chromosome recombinant line populations suggests that LTN is a pleiotropic effect of *Lr46*/*Yr29*. Pleiotropism could be confirmed by either creating point mutations in the resistance gene and making observations if the loss of resistance is also associated with the loss of LTN or through transformation experiments where the gene of interest is inserted and expressed in a non-gene carrying genetic background. Research efforts are currently underway to attempt to clone slow rusting resistance genes such as *Lr34* and *Lr46* (in laboratories in Australia, North America and Europe). Once these efforts are successful, it should be relatively easy to test for pleiotropism. To facilitate the molecular cloning of *Lr46*, fine mapping to saturate the genomic region containing *Lr46* is needed. The previously identified linked or pleiotropic slow rusting resistance genes *Lr34*/*Yr18* are also associated with LTN, where the phenotype is expressed close to flowering (Singh 1992). Within the three populations we used, LTN appeared to be less severe than the LTN associated with *Lr34*/*Yr18*. However, conclusions regarding the level of LTN expression associated with *Lr46*/*Yr29* can not be drawn in this study as expression of this phenotypic marker has previously been shown to be modified by the genetic background or the environment (Messmer et al. 2000).

Table 3 χ^2 values for the populations segregating for leaf tip necrosis (LTN)

	Frequency of responses		
	Observed	Expected (single gene)	χ^2
Lalbahadur × Lalb.(Pavon 1B) (F5)			
LTN +	66	79.6	
LTN ±	30	22.75	
LTN −	85	79.6	5.03 NS
Lalbahadur × Lalb.(Parula 1B) (F6)			
LTN +	67	79.7	
LTN ±	16	10.6	
LTN −	87	79.7	5.41 NS
Avocet-*YrA* × Attila (F5)			
LTN +	67	63.875	
LTN ±	22	18.25	
LTN −	59	63.875	1.30 NS

The ratios used in the tests for a single gene were: F5 population 0.4375 Resistant : 0.125 Heterozygous : 0.4375 Susceptible. F6 population 0.4688 Resistant : 0.0625 Heterozygous : 0.4688 Susceptible
NS, not significant at $P > 0.01$

AFLP and SSR analysis defines and validates the *Lr46*/*Yr29* locus

The AFLP markers *Pst* AAG *Mse* CTA (P33/M59) and *Pst* AAG *Mse* CGA (P33/M55) have previously been mapped to the long arm of Chromosome 1B are known to be associated with the slow-rusting locus *Lr46*/*Yr29* (William et al. 2003a, 2003b). Three new AFLP markers, *Pst* TCC *Mse* GTT (P84/M78), *Pst* TCG *Mse* GCA (P85/M67) and *Pst* ACA *Mse* CGA (P35/M55) mapped to the same linkage group as the two aforementioned markers in the Avocet-*YrA* × Attila population. Furthermore, the phenotypic marker LTN also mapped to the same chromosomal region and all markers accounted for significant levels of phenotypic variance for leaf rust and yellow rust severities (Fig. 3a). With leaf rust, LTN accounted for 84% of the phenotypic variation. As there is at least one additional gene contributing to leaf rust resistance in the Avocet-*YrA* × Attila population, it is expected that this second gene(s) would make a minor contribution to resistance. The closest molecular marker associated with *Lr46* was from the AFLP marker P84/M78. This marker accounted for 52% of the phenotypic variance of leaf rust and was mapped 5.8 cM from LTN. It was previously shown (above) that yellow rust resistance was conferred by a minimum of three minor additive genes. Correspondingly, LTN accounted for 53% of the phenotypic variance observed with yellow rust resistance. Again this was by far the most significant marker making it most closely associated with *Yr29*.

These markers were also mapped in the two chromosome 1B substitution populations that segregated only for the *Lr46*/*Yr29* genes. However, not all of these

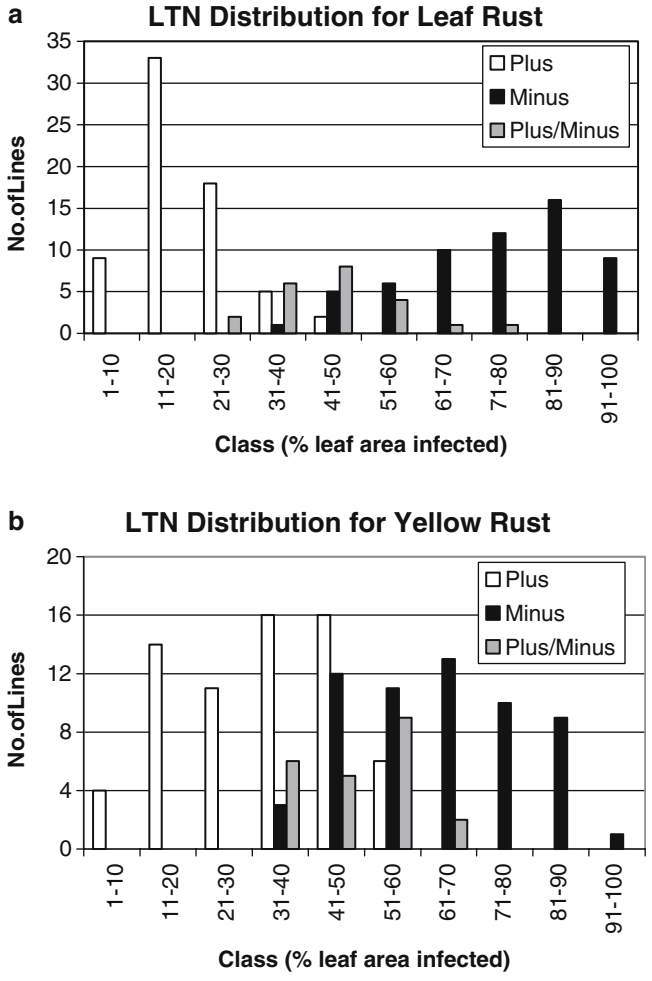

Fig. 2 Distribution of LTN in the Avocet-*YrA* × Attila population in relation to % leaf area infected by leaf rust (**a**) and yellow rust (**b**). Lines containing LTN are always in the more resistant classes, while lines without LTN are generally more susceptible

markers were polymorphic in the single chromosome recombinant populations (Fig. 3b, c). Those that were polymorphic and mapped with a significant LOD score (≥3.0), were also associated with LTN and leaf rust resistance. SSR markers when compared to AFLP markers provide for cross referencing of loci across different populations. Accordingly the position of the AFLP markers common to all three populations was validated relative to the SSR markers linked to the *Lr46*/*Yr29*/LTN locus in the single chromosome recombinant families (Fig. 3b, c). These data confirm that a second LTN phenotypic marker is associated with resistance other than *Lr34*/*Yr18*.

β1-proteasome subunits are located in genomic regions carrying *Lr46*/*Yr29*/LTN and *Lr34*/*Yr18*/LTN

In view of the overall similarities in phenotype between the *Lr46*/*Yr29*/LTN and *Lr34*/*Yr18*/LTN traits, any gene family involved in plant defense and located in regions close to, or at both of these loci, was noted for further comparative analysis. From among the Triticeae ESTs that correspond to their orthologs in rice chromosome 6 and their collinear region with wheat chromosome 7S that contain markers in the vicinity of the *Lr34*/*Yr18* QTL (Nelson et al. 1997, Kudrna et al 2002), the ESTs BE492474, BE495774 and BE517331, were chosen for further analysis. These ESTs encode part of the exons of β1 proteasome subunits (Fig. 4) and were closely related to orthologs in rice and tobacco. We isolated a full length cDNA clone from the D genome progenitor (Genbank #DQ023305) and comparisons using the deduced amino acids showed sequence identities and similarities of 86 and 90% respectively with rice and 73 and 81% with tobacco. The tobacco orthologs have been inferred to be involved in plant defense as they were identified in a differential display analysis from pathogen infected and salicylic acid treatments (Etienne et al. 2000). RFLP analysis using BE495774 as a probe (identical patterns as BE492474 and 517331 on euploid wheat) on nullitetrasomic cytogenetic stocks in the wheat cultivar, Chinese Spring showed the β1 proteasome subunits constituted a small gene family located on homoeologous group 1 and 7 chromosomes (Fig. 5a). DNA hybridization patterns of BAC clones (probed with BE495774) isolated from the D genome progenitor containing the β1 proteasome genes grouped into two classes and corresponded with the chromosome 1D and 7D fragments of Chinese spring. Sequence analysis of the β1 proteasome gene region from the 1D (GenBank #DQ023303) and 7D BACs (Genbank #DQ023304) used as representatives of the group 1 and 7 gene members showed that each class contained a full length gene which consisted of eight exons but differed in the sizes of the introns, in particular intron 1 (Fig. 4). Further differentiation of the β1 proteasome gene members occurred in exon 1, where insertions of two triplet nucleotides in the chromosome 7D member resulted in the addition of serine (nucleotide position 13 from initiation codon) and glycine (position 25) residues (Fig. 4). These differences in exon 1 may be a distinguishing feature of the group 1 and 7 encoded β1 proteasomes in the Triticeae, as either of the two haplotypes were found among all Triticeae ESTs that possess exon 1 in GenBank.

Using a 300 bp fragment downstream from the 3′UTR of a β1 proteasome subunit isolated from a BAC clone on chromosome 7D, a single copy RFLP (Fig. 5b) was mapped 3 cM distal to the wheat marker wg834 located within the *Lr34*/*Yr18*/LTN QTL (Nelson et al. 1995). Localization of the 7D β1 proteasome subunit gene in the *Lr34*/*Yr18* QTL is consistent with recent findings of Schnurbusch et al. (2004) who delineated a collinear region of three rice BAC clones (AP003708 = OSJNBa004F13 containing β1 proteasome, AP000399, AP003487) to span the major QTLs for leaf rust and LTN inferred to be the *Lr34*/*Yr18*/LTN locus on chromosome 7DS present in the winter wheat cultivar Forno.

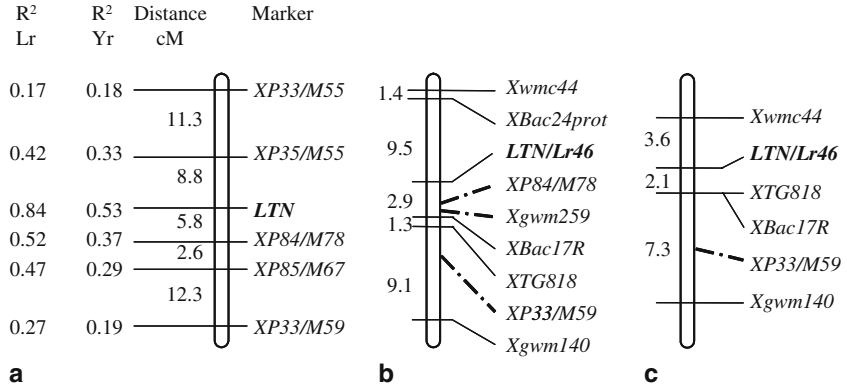

Fig. 3 Maps of molecular markers and the phenotypic markers of LTN and resistance conferred by *Lr46/Yr29* in the F_5 Avocet-*YrA* × Attila population (**a**), Lalbahadur × Lalb.(Pavon 1B) (**b**) and Lalbahadur × Lalb.(Parula 1B) (**c**). R^2 values are shown for the markers in map 3a as the Avocet-*YrA* × Attila population contained more than one QTL for resistance. In **b** and **c** the framework map was constructed from codominant markers and the location of dominant AFLP and SSR markers in the respective maps are shown by a *dotted line*

The wheat EST BE495774 when used as a probe also revealed RFLP differences between the parental lines Avocet-*YrA* and Pavon (carrier of *Lr46/Yr29*) and the same RFLPs differentiated pooled DNA from each of 10 homozygous resistant and susceptible lines (data not shown) previously employed in bulk segregant analysis for the *Lr46/Yr29* locus from the progeny of Avocet-*YrA* × Pavon (William et al. 2003a). A larger sample size was required to estimate the linkage between *β*1 proteasomes of chromosome 1B origin and qualitative phenotypes for *Lr46/Yr29* such as the discrete traits scored in the single chromosome recombinant lines. Sequences from two non-overlapping BAC clones (BAC17 and BAC24 both with sequence identities of 90% relative to corresponding exons from the gene member on chromosome 7D) of chromosome 1B origin that hybridized to BE495774 were used to generate PCR fragments to develop STS markers that could differentiate between Lalb. and the *Lr46/Yr29* carrying Lalb.(Pavon 1B) and Lalb.(Parula1B). Polymorphic STS markers [approximately 500 bp in Lalb. and 1.7 kb in Lalb.(Pavon1B/Parula1B)] from one of the BAC ends of BAC 17 (Bac17R) were mapped in the single chromosome recombinant families and were located approximately 2–3 cM distal to the *Lr46/Yr29*/LTN locus (Fig. 3b, c). An STS marker (XBac24prot., a 150 bp fragment in Lalb.) derived from the 5′UTR of the proteasome gene present in BAC24 amplified a major fragment in Lalb. and was mapped 9.5 cM proximal to *Lr46/Yr29*/LTN and close to the SSR wmc44 (Fig. 3b). The distinctiveness of the sequences in the 5′UTR of the proteasome in BAC24 was characteristic of the proteasome gene members derived from chromosomes 1B, 1D and 7D where they showed high sequence identities (≥90%) in their exons, but their promoter regions were completely unrelated.

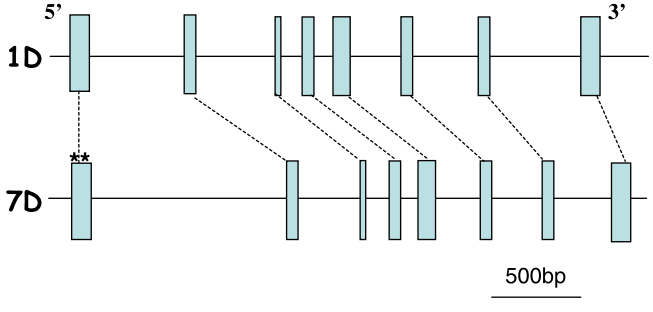

Fig. 4 The structure of *β*1 proteasome gene members from group1 and 7 chromosomes isolated from D genome BACs. The *boxed objects* are the exons. * refers to the position of insertion codons (nucleotide position 13 and 25) relative to the start of the initiation codon in exon 1 of the chromosome 7D gene member. The complete nucleotide sequence for each gene has been deposited in Genbank# DQ023303 (chromosome 1D member) and DQ023304 (7D member). Triticeae ESTs BE495774 and BE517331 spans exons 1–5 and BE492474 spans exons 1–6

In this paper we have identified molecular and phenotypic markers that are linked to the slow rusting genes *Lr46/Yr29*. The closet AFLP/STS/SSR markers are within 2–3 cM of the *Lr46/Yr29* loci. The phenotypic marker LTN was inseparable from the resistant phenotype in two single chromosome recombinant line populations, suggesting very tight linkage or pleiotropism. This makes LTN a useful phenotypic marker for this slow-rusting gene, especially when these types of genes are used in conjunction with other similar genes. However, it must be recognized that the slow rusting resistance gene complex *Lr34/Yr18* is also tightly linked to LTN. Individually they are useful field based markers if the parents only contain one or the other of the associated slow-rusting genes and no other QTLs for LTN are present. For crosses that contain both *Lr34/Yr18* and *Lr46/Yr29* genes, molecular markers would be useful additions to differentiate lines containing both resistances. Moreover the variable nature of LTN expression found in different wheat growing regions, points to the need for developing more tightly linked and diagnostic markers for these traits. While ß-1 proteasome gene members have been linked to these loci, the occurrence

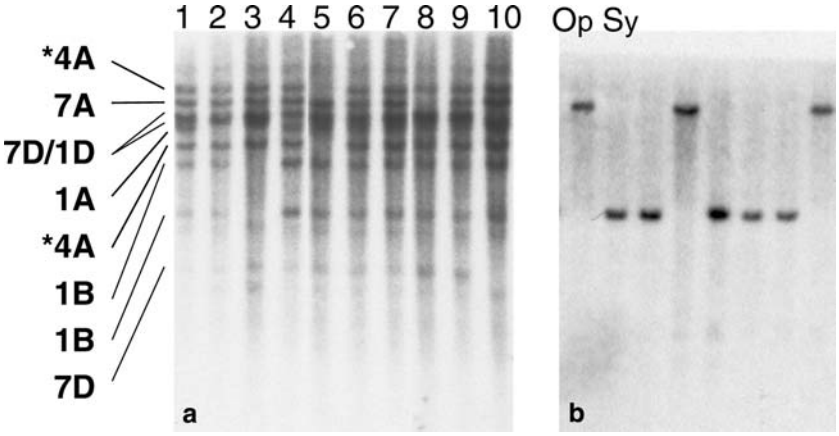

Fig. 5 a) The location of β1 proteasome gene members on group 1 and 7 chromosomes based on RFLP analysis from the wheat cv. Chinese Spring (*lane 1*) and a subset of nulli-tetrasomic cytogenetic stocks (*lanes 2–10*) restricted with the endonuclease *Dra1* and probed with the wheat EST BE495774 containing β1 proteasome sequence. (*lane 2*) N1AT1B, (*3*) N1BT1A, (*4*) N1DT1A, (*5*) N4AT4B, (*6*) N4BT4A, (*7*) N4DT4A, (*8*) N7AT7B, (*9*) N7BT7A, (*10*) N7DT7A. Note *4A fragments represent sequences located on 7BS because of the ancient translocation between chromosme 4A and 7B. **b** Hybridization of a 300 bp fragment downstream of the 3′UTR β1 proteasome gene from chromosome 7D BAC to *Eco*R1 restricted DNA from the wheat cv Opata (*Op*) and Synthetic (*Sy*) parents and a subset of their recombinant inbred progeny

of recombination between chromosome 1B ß-1 proteasome genes and *Lr46/Yr29*/LTN locus discounts any suggestion of large linkage blocks with suppressed recombination clustering the LTN and non-race specific *Lr* and *Yr* genes located in 1BL and 7DS. A reciprocal interchromosomal translocated wheat chromosome that involved *Lr34* on 7DS has previously been reported (Dyck et al. 1994); however it remains to be shown if the translocated segment also involves chrosome 1BL.

It is interesting that two different genetic loci present on non-homoeologous chromosomes 1BL and 7DS containing *Lr/Yr* slow-rusting genes also confer LTN in what could be a pleiotropic manner. If the assumption of pleiotropism is correct, then it would seem that these genes may function in similar ways, not only in how they recognize pathogens, but also in the effect their presence has within the plant. The gene symbol *Ltn* was previously assigned to a gene linked to *Lr34/Yr18* genes. We have identified a second leaf tip necrosis gene linked to *Lr46/Yr29* and suggest a new gene symbol *Ltn2* be designated in association with *Lr46/Yr29* located on chromosome 1BL while *Ltn1* be used in association with *Lr34/Yr18* located on 7DS.

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