

# Genome-Wide Association Mapping for Leaf Tip Necrosis and Pseudo-black Chaff in Relation to Durable Rust Resistance in Wheat

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## Abstract

The partial rust resistance genes *Lr34* and *Sr2* have been used extensively in wheat (*Triticum aestivum* L.) improvement, as they confer exceptional durability. Interestingly, the resistance of *Lr34* is associated with the expression of leaf tip necrosis (LTN) and *Sr2* with pseudo-black chaff (PBC). Genome-wide association mapping using CIMMYT's stem rust resistance screening nursery (SRRSN) wheat lines was done to identify genotyping-by-sequencing (GBS) markers linked to LTN and PBC. Phenotyping for these traits was done in Ithaca, New York (fall 2011); Njoro, Kenya (main and off-seasons, 2012), and Wellington, India (winter, 2013). Using the mixed linear model (MLM), 18 GBS markers were significantly associated with LTN. While some markers were linked to loci where the durable leaf rust resistance genes *Lr34* (7DS), *Lr46* (1BL), and *Lr68* (7BL) were mapped, significant associations were also detected with other loci on 2BL, 5B, 3BS, 4BS, and 7BS. Twelve GBS markers linked to the *Sr2* locus (3BS) and loci on 2DS, 4AL, and 7DS were significantly associated with PBC. This study provides insight into the complex genetic control of LTN and PBC. Further efforts to validate and study these loci might aid in determining the nature of their association with durable resistance.

**T**HE RUST DISEASES, which include leaf rust caused by *Puccinia triticina* Eriks., stem rust caused by *P. graminis* Pers. f. sp. *tritici*, and stripe rust caused by *P. striiformis* West. f. sp. *tritici*, are the major biotic stresses that affect global wheat production. Although several rust resistance genes have been identified, most of them are race specific and can be easily overcome by new virulent races of the pathogen (Samborski, 1985; McIntosh et al., 1995; Pretorius et al., 2000; Wanyera et al., 2006). To circumvent this challenge, rust resistance breeding has shifted its focus to achieving nonrace-specific, widespread, prolonged resistance, also known as durable resistance (Johnson, 1984). This is usually characterized by slow rusting, partial, and adult plant resistance (APR) expressed at the postseedling stage with extended latent periods that delay the spread of the disease (Caldwell, 1968; Parlevliet, 1975; Kolmer, 1996). Among the cataloged rust resistance genes, *Lr34/Yr18/Sr57/Pm38/Sb1/Ltn1* (hereafter referred to as *Lr34*) and *Sr2/Yr30/Lr27/Pbc1* (hereafter referred to as *Sr2*) have been deployed widely for several decades due to their exceptional durability.

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**Abbreviations:** ABC, adenosine triphosphate-binding cassette; APR, adult plant resistance; DArT, diversity array technology; FDR, false discovery rate; GBS, genotyping-by-sequencing; GLM, generalized linear model; LD, linkage disequilibrium; LTN, leaf tip necrosis; MLM, mixed linear model; PBC, pseudo-black chaff; PC, principal component; Q-Q, quantile-quantile; QTL, quantitative trait loci; SNP, single nucleotide polymorphisms; SRRSN, stem rust resistance screening nursery.

The gene *Lr34* is valuable, as it confers durable non-specific APR to several pathogens: leaf rust, stem rust, stripe rust, powdery mildew [*Blumeria graminis* (DC.) Speer f. sp. *tritici*], spot blotch [*Bipolaris sorokiniana* (Sacc.) Shoemaker], and barley yellow dwarf virus (Dyck and Samborski, 1982; Singh, 1992b, 1993; Kolmer, 1996; Vanegas et al., 2008; Spielmeier et al., 2005; Joshi et al., 2004). The resistance of *Lr34* is associated with a phenotype called LTN, which is characterized by postflowering necrosis of the flag leaf tips extending to a few centimeters along the leaf edges (Dyck, 1991; Singh, 1992a). Leaf tip necrosis is considered to be an innate defense mechanism and occurs spontaneously in resistant *Lr34* lines even before pathogen challenge (Rubiales and Niks, 1995; Hulbert et al., 2007). While the basis of this mechanism is not clear, Messmer et al. (2000) hypothesized that expression of *Lr34* might alter the physiology of the flag leaf, thus making it less desirable for the pathogen to grow and establish itself. Although *Lr34/Ltn1* was the first gene to be associated with LTN, the genes *Lr46/Ltn2* (Rosewarne et al., 2006), *Lr67/Ltn3* (Dyck and Samborski 1979; Hiebert et al., 2010; Herrera-Foessel et al., 2014), and *Lr68* (Herrera-Foessel et al., 2012) were also found to confer LTN in varying degrees. Wheat lines with LTN displayed an average reduction of 30.5 and 20.8% leaf and stripe rust severities, respectively (Navabi et al., 2005). However, LTN is not a favored trait for breeders due to the associated yield penalty in the *Lr34* lines (Drijpondt et al., 1990; Singh and Huerta-Espino, 1997) and its undesirable appearance (Messmer et al., 2000).

Besides *Lr34*, another important durable APR gene that has provided effective resistance for many decades is the *Sr2* gene, which, in combination with other unknown minor genes, is referred to as the *Sr2* complex (Rajaram et al., 1988; Singh et al., 2006). This gene, or closely linked genes, also confers resistance to stem rust, stripe rust, leaf rust, and powdery mildew (Singh et al., 2000; Mago et al., 2011). Drawing a parallel with the *Lr34* gene and LTN association is the association of *Sr2* with a black pigmentation called PBC (Sheen et al., 1968; Hare and McIntosh, 1979; Kota et al., 2006). Pseudo-black chaff occurs around the glumes and the internodes of the stem after anthesis (Kuspira and Unrau, 1958). It has varying degrees of expression depending on the genotype and environment (Singh et al., 2008). The genetic association between PBC and stem rust resistance is intriguing, and several mechanisms have been proposed: Waldron (1929) suggested an antagonistic relationship between stem rust resistance and susceptibility to black chaff; Goulden and Neatby (1929) and Pan (1940) suggested incomplete linkage; Bhowal and Narkhede (1986) suggested that PBC is incompletely dominant and its expression level can be modified by several genes; McFadden (1939) and Mishra et al. (2005) suggested that resistance was not invariably associated with PBC; and Kota et al. (2006) reported that the two traits were inseparable by recombination. Both monogenic (Sheen et al., 1968; Bariana et al., 2001) and digenic inheritance of PBC has been suggested (Kaur et

al., 2009). A hypothesis for PBC expression in resistant plants is that it might form physical or chemical barriers that subsequently delay the infection process (Kota et al., 2006). As higher expression of PBC might lead to shrunken kernels that reduce yield and makes it undesirable for the farmers (Sheen et al., 1968; Hare and McIntosh, 1979), breeders usually select for moderate levels of PBC while selecting for *Sr2*.

The genetic basis of the association between the durable rust resistance genes and the traits LTN and PBC is not completely understood. Despite the detection of many quantitative trait loci (QTL) for LTN (Messmer et al., 2000; Schnurbusch et al., 2004a) and PBC (Bariana et al., 2001) in several biparental mapping populations, the number and effect of these QTL vary greatly. Hence, it is important to adopt other mapping strategies that can detect all the existing allelic diversity for these traits. In this regard, an excellent alternative to traditional linkage mapping is association mapping, which has the potential to dissect complex traits (Risch and Merikangas, 1996; Thornsberry et al., 2001; Jannink and Walsh, 2002; Flint-Garcia et al., 2003; Breseghello and Sorrells, 2006). It is a powerful tool to identify significant correlations between phenotypes and the underlying sequence variations based on the principle of linkage disequilibrium (LD). It harnesses the ancestral meiotic events that have occurred at the population level in the ancestors of an existing diversity panel, thus providing a much finer resolution and reduced population development time compared with traditional linkage mapping (Risch and Merikangas, 1996; Yu and Buckler, 2006). Hence, association mapping was employed in this study to gain more insight into the genetic basis of LTN and PBC.

## Materials and Methods

### Plant Materials

As the presence of *Lr34/Ltn1* and *Sr2/Pbc* in CIMMYT's germplasm is well documented (Rajaram et al., 1988; Dyck, 1991; Singh, 1992a; Spielmeier et al., 2003; Singh et al., 2005; Kota et al., 2006), the stem rust resistance screening nursery (SRRSN) lines were used for mapping LTN and PBC. The two association panels used in this study include (i) Panel A, comprised of 504 wheat lines that were developed by crossing 14 parental lines from CIMMYT's second and fifth SRRSN in a partial diallel scheme; and (ii) Panel B, comprised of 200 elite lines from CIMMYT's second, fifth, and sixth SRRSN.

### Phenotypic Data

Phenotyping for LTN involved measuring the extent of LTN from the tip of the leaf up to a few centimeters along the edges at anthesis. A 0-to-4 scale was used where 0 = no LTN; 1 = slight LTN; 2 = medium LTN; 3 = high LTN; and 4 = very high LTN (Fig. 1). The variations in leaf length were accounted for while scoring. All the 504 lines in Panel A were evaluated for LTN in the greenhouse at Cornell University, Ithaca, NY, during fall of 2011. The

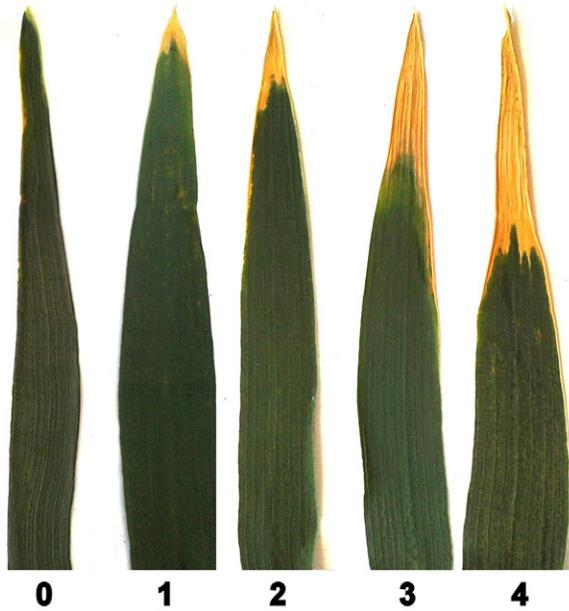


Figure 1. A 0-to-4 scale was used for phenotyping leaf tip necrosis (LTN) where 0 = no LTN; 1 = slight LTN; 2 = medium LTN; 3 = high LTN; and 4 = very high LTN.

progenies of these lines were evaluated in the field at the Kenya Agricultural Research Institute, Njoro, Kenya, during the main and off-seasons of 2012. Only 393 lines could be scored during the Njoro main season due to the simultaneous high incidence of stripe rust. The 200 lines in Panel B were also evaluated for LTN in the field at the Wheat Research Station, Wellington, India, during winter of 2013. Phenotyping for PBC was done at anthesis based on the presence of the black pigmentation around the stem internodes and glumes (Kota et al., 2006; Kaur et al., 2009). A 0 to 4 scale was used where 0 = no pigmentation; 1 = slight pigmentation; 2 = medium pigmentation; 3 = high pigmentation; and 4 = very high pigmentation (Fig. 2). Pseudo-black chaff was scored in the field at Njoro during the main and off-seasons 2012 (the progenies of the 504 lines in Panel A) and also in Wellington during winter 2013 (Panel B). In cases where the trait distributions were skewed, appropriate transformations were performed using the boxcox function in the R statistical package (R Development Core Team, 2013).

### Genotypic Data

Genotyping an adequate number of markers across the genome is essential for association mapping, such that some of them will be in LD with the causal polymorphisms (Zhu et al., 2008; Myles et al., 2009). The populations used in this study were genotyped using the GBS approach, which provides an excellent option for association mapping due to dense genome-wide coverage, robustness, reproducibility, complexity reduction in large genomes, low time and cost per sample (Elshire et al., 2011; Poland et al., 2012). Among the 3211 markers that were positioned in the Synthetic  $\times$  Oyata reference genetic map, those with missing data greater than 20%



Figure 2. A 0-to-4 scale was used for phenotyping pseudo-black chaff (PBC) where 0 = no pigmentation; 1 = slight pigmentation; 2 = medium pigmentation; 3 = high pigmentation; and 4 = very high pigmentation.

and minor allele frequency less than 5% were removed. The missing genotypic data was imputed using the Markov Chain haplotyping software (<http://www.sph.umich.edu/csg/abecasis/MACH/>) (Li et al., 2010; Marchini and Howie, 2010). The value of the rounds parameter, which specifies the number of iterations the Markov sampler has to be run, was set to 100, and the value of the states parameter, which specifies the number of haplotypes that should be considered when updating each individual, was set to 200 and 100, respectively, for the two panels. As the information from some markers within each haplotype block may be redundant, a subset of the markers that best explains the haplotype diversity existing within a block was selected by the process of haplotype tagging (Johnson et al., 2001). The tool Tagger (<http://www.broadinstitute.org/mpg/tagger/>) that was described in de Bakker et al. (2005) was used to select the tag single nucleotide polymorphisms (SNPs) in this study. Markers that had a correlation coefficient greater than 0.8, indicating high LD and possible redundancy, were excluded. The pairwise LD between the markers was calculated based on their allele frequency correlations ( $r^2$ ) using the R statistical package, and the LD decay plot was obtained.

### Association Analysis for Leaf Tip Necrosis and Pseudo-Black Chaff using Generalized and Mixed Linear Models

Association analysis was done using both the generalized linear model (GLM) and the MLM in TASSEL 4.0 (Bradbury et al., 2007). As the structure resulting from subgroups within the population with an unequal distribution of alleles can result in spurious associations (Knowler et al., 1988; Lander and Schork, 1994; Ewens and Spielman,

## Linkage Disequilibrium decay

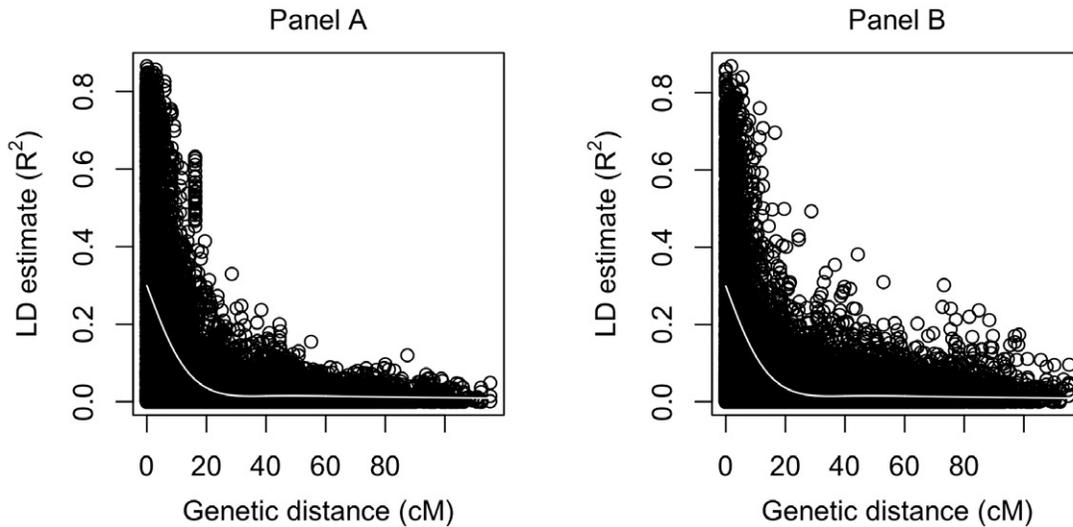


Figure 3. Scatter plot showing the linkage disequilibrium (LD) decay across the chromosomes for both the panels. The genetic distance in cM is plotted against the LD estimate ( $R^2$ ) for pairs of markers.

1995; Pritchard and Rosenberg, 1999), population structure (Q matrix) was used as a fixed-effect covariate in both the models. It was taken into account by using the first two principal components (PC1 and PC2) (Patterson et al., 2006; Price et al., 2006; Zhao et al., 2007) calculated in TASSEL using the correlation matrix. The GLM was run using marker effects and population structure as fixed effects with 1000 permutations. As the unified MLM (Yu et al., 2006) includes kinship (familial relatedness) as a random effect component, the scaled identity-by-state method (Endelman and Jannink, 2012) in TASSEL was used to calculate the kinship matrix (K matrix). The MLM was run with the optimum level of compression along with the population-parameters-previously-determined (P3D) method (Zhang et al., 2010). An  $\alpha$ -level of 0.001 was used to declare significance of the markers. To correct for multiple comparisons, the step-up procedure of Benjamini and Hochberg (1995), which controls the false discovery rate (FDR; the expected proportion of false discoveries among the rejected null hypotheses) was used with a cut-off value of 0.1. The positions of the significant markers in the Synthetic  $\times$  Opatá map (Poland et al., 2012) were compared with the markers detected in previous studies using map information from GrainGenes (<http://wheat.pw.usda.gov/GG2/index.shtml>).

## Results and Discussion

### Phenotypic Data Analysis

The phenotypic distributions of LTN and PBC evaluated at different locations and seasons and their normal quantile–quantile (Q-Q) plots comparing the sample quantiles to the theoretical quantiles is shown in Supplemental Fig. S1a,b. The LTN score was  $2.13 \pm 1.09$  in Ithaca (2011),  $1.66 \pm 0.96$  in Njoro (main season 2012),  $0.77 \pm 0.84$  in Njoro (off-season 2012), and  $2.08 \pm 0.97$  in Wellington (2013). The correlation between LTN and

stem rust severity was  $-0.19$  and  $-0.15$  in the Njoro, main and off-seasons, respectively. Similarly, a negative correlation of  $-0.16$  was obtained between LTN and leaf rust severity in Wellington. This indicates the inverse relationship between LTN and rust severity. A linear model for the LTN scores with families, locations (Ithaca and Kenya combined data for two seasons) and families  $\times$  locations as fixed effects was fitted. A high significance for all these sources of variation at the 0.001 level (Supplemental Table S1) indicates that the environment and the family  $\times$  environmental interactions are important in the expression of LTN. The mean PBC score was  $1.32 \pm 1.12$  in Wellington (2013),  $1.7 \pm 0.93$  in Njoro (main season 2012), and  $1.11 \pm 0.93$  in Njoro (off-season 2012). Negative correlations of  $-0.34$ ,  $-0.35$ , and  $-0.23$  were obtained between stem rust severity and PBC in the Njoro main season, Njoro off-season, and Wellington, respectively. This adds evidence to their inverse relationship, indicating that increase in PBC levels is associated with reduction in stem rust severity and vice versa.

### Genotypic Data and Linkage Disequilibrium Analysis

After filtering, 673 tag SNP's were obtained for Panel A. The majority of these were distributed across the wheat A and B genomes (40 and 55%, respectively), while the D genome had the fewest (5%). Similarly, 815 tag SNPs were obtained for the Panel B and the marker coverage was 43, 50, and 7% for the A, B, and D genomes, respectively. The scatter plots of the allele frequency correlations ( $r^2$ ) between the GBS markers across the chromosomes against the genetic distance (in cM) for both the panels are shown in Fig. 3. Linkage disequilibrium decayed to  $<0.1$  at about 20 cM in both the mapping panels.

## Principal Component Analysis

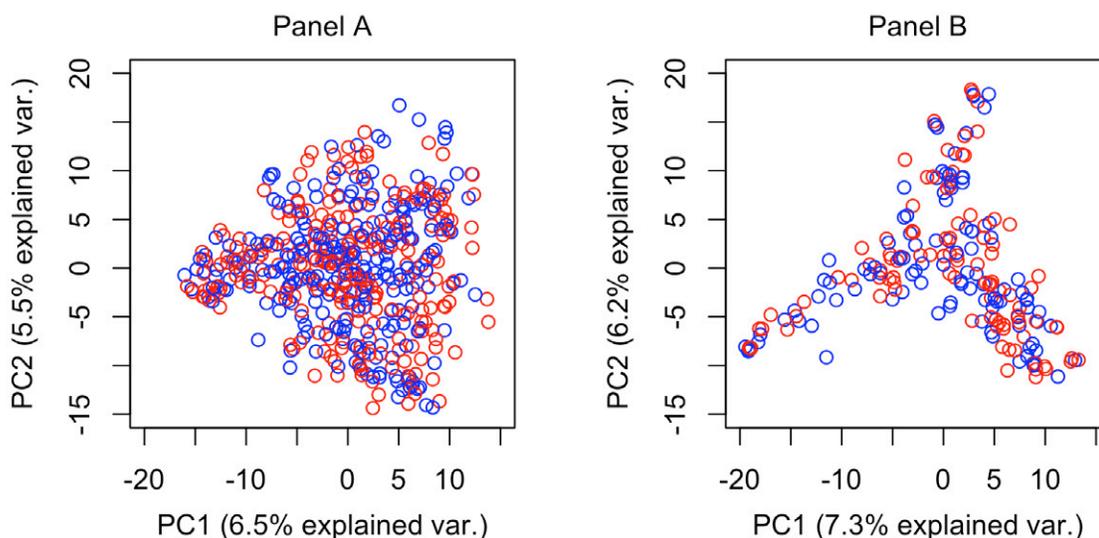


Figure 4. Principal component (PC) analysis for illustrating population structure in both the panels. The blue circles represent PC1 and the red circles represent PC2. Panel A shows no population structure and Panel B shows weak population structure.

### Population Structure and Kinship Analysis

The plot of the first two principal components (PCs) for both panels is shown in Fig. 4. Panel A had almost no population structure as it was comprised of offspring derived from 14 randomly intermated parents. The first PC explained 6.5% of the variance and the second PC explained 5.5% of the variance. Panel B, had weak population structure, with the first PC explaining 7.3% of the variance and the second PC explaining 6.2% of the variance. Population structure in this panel was inferred using the STRUCTURE software (Pritchard et al., 2000) and individuals were assigned to two subpopulations ( $K = 2$ ). The pairwise  $F$ -statistic for population structure ( $F_{ST}$ ) for the pair of subpopulations was calculated using the method of Weir and Cockerham (1984) and was found to be 0.04. This adds evidence to the weak genetic differentiation of the subpopulations in Panel B. Kinship between the individuals was calculated and a heat map of the marker-based kinship matrix for both the panels is shown in Supplemental Fig. S2. While Panel A had intermediate familial relationships, Panel B had weak relationships except for a few hotspots with related lines.

### Analysis of Marker–Trait Associations for Leaf Tip Necrosis

In the association analysis, MLM was considered to be more reliable than GLM considering the presence of some familial relatedness in both the panels. The 18 markers that were significantly associated with LTN in the different datasets are reported in Table 1. The Q-Q plot of  $p$ -values comparing the uniform distribution of the expected  $-\log_{10}(p)$  to the observed  $-\log_{10}(p)$  and the Manhattan plot of  $-\log_{10}(p)$  by chromosomes for the different LTN datasets is shown in Supplemental Fig. S3a and S4a. In the Ithaca data set, the most significant marker was GBS\_3714 on chromosome 5B. This marker, along with the other significant

markers on chromosomes 1BL, 2BL, 4B, and 7DS, explains 12.32% of the total phenotypic variance for LTN. In the Njoro main season, the most significant marker was GBS\_11149 on chromosome 3BS along with significant markers on chromosomes 5B and 7BS explain 22.2% of the total phenotypic variance for LTN. In the Njoro off-season, the most significant marker was GBS\_16379 on chromosome 2BL, which, along with markers on chromosomes 2BL, 3BS, and 7BS, explain 18.91% of the LTN variability. Finally, in the Wellington data set, GBS\_2248, the most significant marker on chromosome 1BL, in addition to markers on chromosome 2BL and 7BL, explain 14.05% of the total variance for LTN.

On chromosome 1BL, GBS\_9433 (88 cM) and GBS\_2248 (87.4 cM) were significant in the Ithaca and Wellington data sets, respectively. The *Lr46/Ltn2* gene, which is pleiotropic to LTN, is also located on chromosome 1BL. The marker *wmc44*, which flanks the *Lr46* gene, was approximately 16 cM proximal in a study by Mateos-Hernandez et al. (2006) using  $F_3$  families of Lalbahadur  $\times$  Lalbahadur (Pavon1B) and 10.9 cM proximal in another study by Rosewarne et al. (2006) using  $F_5$  families of the same population. This marker is 11.8 cM from GBS\_9433 and 11.2 cM from GBS\_2248 in the Synthetic  $\times$  Opatata map (Fig. 5, chromosome 1BL). This indicates that our significant markers might correspond to the exact predicted interval for the *Lr46* locus. On chromosome 2BL, GBS\_16379 (49.2 cM), GBS\_11423 (49.8 cM), and GBS\_15442 (53.1 cM) were significant in the Njoro off-season data set; GBS\_302 (48.9 cM) in the Ithaca data set; and GBS\_19244 (51 cM) in the Wellington data set. These markers are proximal to wPt-4199 (46.8 cM), the marker identified to flank a stem rust QTL enhanced by *Lr34* (Kolmer et al., 2011). They are also close to the marker *wmc474* (53.1 cM), which is linked to the *Lr13* gene (Cakir et al., 2008). The positions of wPt-4199 and

**Table 1. Markers significantly associated with leaf tip necrosis in the different data sets.**

Marker	Chromosome	Position	<i>p</i> -value ( <i>Q</i> ) <sup>†</sup>	FDR <sup>‡</sup> -adjusted <i>p</i> -value ( <i>Q</i> )	<i>p</i> -value ( <i>Q</i> + <i>K</i> ) <sup>†</sup>	<i>R</i> <sup>2</sup> ( <i>Q</i> + <i>K</i> )	Data set <sup>§</sup>
GBS_9433	1BL	88	$1.75 \times 10^{-3}$	$5.53 \times 10^{-2}$	$3.29 \times 10^{-3}$	$2.51 \times 10^{-2}$	Ithaca 2011
GBS_2248	1BL	87.4	$1.90 \times 10^{-4}$	$8.19 \times 10^{-2}$	$5.39 \times 10^{-4}$	$4.25 \times 10^{-2}$	Wellington 2013
GBS_302	2B	48.9	$1.35 \times 10^{-3}$	$2.77 \times 10^{-2}$	$7.35 \times 10^{-3}$	$2.06 \times 10^{-2}$	Ithaca 2011
GBS_16379	2B	49.2	$3.57 \times 10^{-4}$	$1.67 \times 10^{-2}$	$4.68 \times 10^{-4}$	$3.36 \times 10^{-2}$	Njoro 2012 OS
GBS_11423	2B	49.8	$1.21 \times 10^{-4}$	$1.38 \times 10^{-2}$	$3.44 \times 10^{-3}$	$2.47 \times 10^{-2}$	Njoro 2012 OS
GBS_19244	2B	51	$3.21 \times 10^{-3}$	$8.10 \times 10^{-2}$	$4.64 \times 10^{-3}$	$3.42 \times 10^{-2}$	Wellington 2013
GBS_15442	2B	53.1	$2.58 \times 10^{-4}$	$1.67 \times 10^{-2}$	$6.41 \times 10^{-4}$	$3.22 \times 10^{-2}$	Njoro 2012 OS
GBS_11149	3BS	10.2	$6.72 \times 10^{-11}$	$4.52 \times 10^{-8}$	$1.57 \times 10^{-3}$	$4.52 \times 10^{-2}$	Njoro 2012 MS
			$7.39 \times 10^{-7}$	$4.97 \times 10^{-4}$	$1.57 \times 10^{-3}$	$2.82 \times 10^{-2}$	Njoro 2012 OS
GBS_11653	4B	25.3	$3.08 \times 10^{-4}$	$3.14 \times 10^{-2}$	$4.68 \times 10^{-3}$	$2.35 \times 10^{-2}$	Ithaca 2011
GBS_22182	5B	70.3	$5.42 \times 10^{-3}$	$8.10 \times 10^{-2}$	$6.26 \times 10^{-3}$	$3.21 \times 10^{-2}$	Wellington 2013
GBS_15485	5B	73.8	$2.28 \times 10^{-3}$	$7.67 \times 10^{-2}$	$2.19 \times 10^{-3}$	$3.47 \times 10^{-2}$	Njoro 2012 MS
GBS_3714	5B	73.8	$1.19 \times 10^{-4}$	$2.77 \times 10^{-2}$	$6.88 \times 10^{-4}$	$3.21 \times 10^{-2}$	Ithaca 2011
GBS_18258	7BL	6.8	$1.64 \times 10^{-3}$	$8.10 \times 10^{-2}$	$2.32 \times 10^{-3}$	$3.17 \times 10^{-2}$	Wellington 2013
GBS_1203	7BS	68.9	$1.65 \times 10^{-6}$	$5.55 \times 10^{-4}$	$5.18 \times 10^{-3}$	$2.29 \times 10^{-2}$	Njoro 2012 OS
			$1.73 \times 10^{-5}$	$5.82 \times 10^{-3}$	$5.86 \times 10^{-3}$	$2.90 \times 10^{-2}$	Njoro 2012 MS
GBS_15572	7BS	68.9	$6.72 \times 10^{-5}$	$1.19 \times 10^{-2}$	$1.11 \times 10^{-3}$	$3.86 \times 10^{-2}$	Njoro 2012 OS
GBS_18119	7BS	68.9	$6.44 \times 10^{-5}$	$7.92 \times 10^{-3}$	$1.55 \times 10^{-3}$	$3.67 \times 10^{-2}$	Njoro 2012 MS
			$6.73 \times 10^{-4}$	$3.02 \times 10^{-2}$	$4.03 \times 10^{-3}$	$2.41 \times 10^{-2}$	Njoro 2012 OS
GBS_23290	7BS	68.9	$1.37 \times 10^{-4}$	$1.38 \times 10^{-2}$	$4.67 \times 10^{-3}$	$2.34 \times 10^{-2}$	Njoro 2012 OS
			$3.09 \times 10^{-3}$	$9.90 \times 10^{-2}$	$2.46 \times 10^{-3}$	$3.78 \times 10^{-2}$	Njoro 2012 MS
GBS_11611	7DS	94.3	$6.41 \times 10^{-3}$	$3.22 \times 10^{-2}$	$6.70 \times 10^{-3}$	$2.19 \times 10^{-2}$	Ithaca 2011

<sup>†</sup> *Q*, population structure matrix; *K*, kinship matrix.

<sup>‡</sup> FDR, false discovery rate.

<sup>§</sup> OS, off-season; MS, main season.

*wmc474* relative to the significant markers in the Synthetic × Oyata map is shown in Fig. 5, chromosome 2BL.

On chromosome 3BS, the marker GBS\_11149 (10.2 cM) was significant in the Njoro main and off-seasons. Previously, Messmer et al. (2000) identified a LTN QTL in 3B flanked by markers *Lrk10b* and *Xpsr1196*. The positions of these markers could not be located in the Synthetic × Oyata map, but the location of *Xpsr1196b* in the telomeric region of chromosome 3BS (Forno × Oberkulmer map; Messmer et al., 1999) indicates that it is located proximal to the *Sr2* locus. Schnurbusch et al. (2004b) have also reported a putative QTL for LTN on 3BS (QLtn. sfr.3BS) in one environment flanked by the markers *Xcfd79b* and *tam61B*. The marker *Xcfd79b* is 5 cM proximal to the *Sr2* locus on the wheat composite map (2004). Similarly, GBS\_11149, the marker significant in this study, is 3.1 cM away from the diversity array technology (DArT) marker, *wPt8446* (Fig. 5, chromosome 3BS), which is approximately 4.8 cM from the *Sr2* locus (Yu et al., 2014). Hence, GBS\_11149 is approximately 7.9 cM from the *Sr2* locus.

On chromosome 4B, the marker GBS\_11653 was significantly associated with LTN only in the Ithaca data set. There were two previous reports of minor QTL for LTN on 4B: a QTL flanked by markers *Xpsr921* and *Xpsr953b* (Messmer et al., 2000) and a QTL flanked by markers *gwm368* and *gwm540* (Schnurbusch et al., 2004b). The position of the markers identified by Messmer et al. (2000) could not be compared, as they are available only in the Forno × Oberkulmer map. The marker *gwm368*,

identified by Schnurbusch et al. (2004b), is approximately 11.3 cM away from *wmc710* that is 2 cM away from the significant marker GBS\_11653 (Fig. 5, chromosome 4B). Hence, GBS\_11653 is identifying a different unknown locus and it also does not coincide with any leaf rust resistance gene in chromosome 4B. On chromosome 5B, the markers GBS\_15485 (73.8 cM), GBS\_3714 (73.8 cM), and GBS\_22182 (70.3 cM) were associated with LTN in Njoro main season, Ithaca, and Wellington, respectively. Messmer et al. (2000) reported a region in chromosome 5BL significant for LTN and flanked by markers *Xgwk163b* and *Xpsr426*; however, the position of these markers is only available in the Forno × Oberkulmer map and could not be compared. However, this significant region is proximal to the *Lr34* interacting DArT marker locus (*wPt9103*) reported by Yu et al. (2011) for stem rust resistance (Fig. 5, chromosome 5B).

On chromosome 7BL, the marker GBS\_18258 (6.8 cM) was significant only in the Wellington data set. The gene *Lr68* that was associated with slight LTN is also located at chromosome 7BL. Hence, the marker *Xgwm146*, which was estimated to be 0.6 cM (Herrera-Foessel et al., 2012) from *Lr68*, was used to verify if the marker significant in this study corresponded to this gene. As this marker was not present in the Synthetic × Oyata map, the marker *barc182*, which is 1.6 cM from *Xgwm146* (Wheat Synthetic × Oyata BARC map), was used to find the approximate position of *Xgwm146*. The marker *barc182* is at the same position (6.8 cM) as GBS\_18258 that was significant in this study (Fig. 5,

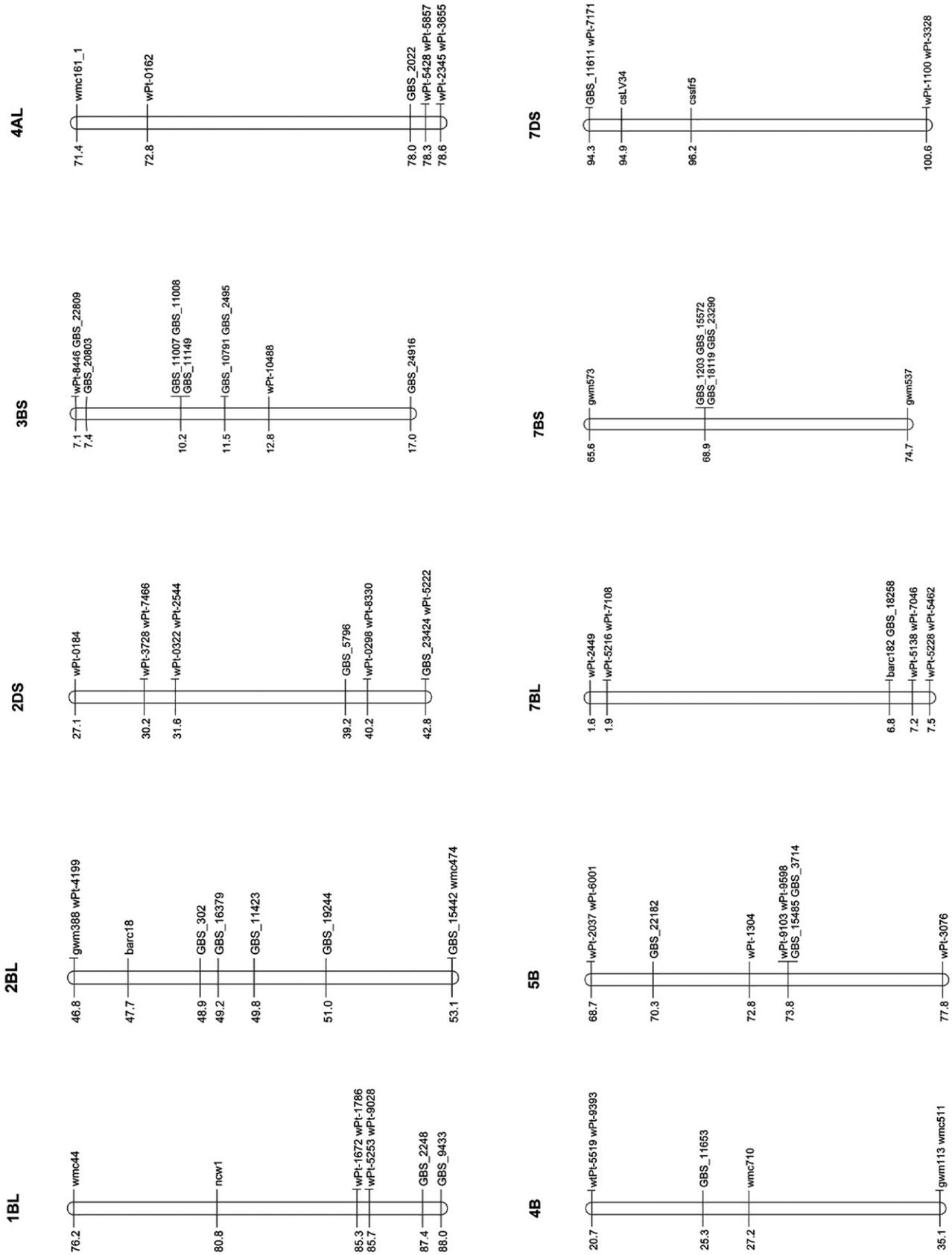


Figure 5. Map positions of the genotyping-by-sequencing (GBS) markers significant for leaf tip necrosis (LTN) and pseudo-black chaff (PBC) in the Synthetic x Opata map.

**Table 2. Markers significantly associated with pseudo-black chaff.**

Marker	Chromosome	Position	$p$ -value ( $Q$ ) <sup>†</sup>	FDR <sup>‡</sup> -adjusted $p$ -value ( $Q$ )	$p$ -value ( $Q + K$ ) <sup>†</sup>	Marker $R^2$ ( $Q + K$ )	Data set <sup>§</sup>
GBS_5796	2D	39.2	$1.28 \times 10^{-3}$	$1.34 \times 10^{-2}$	$6.04 \times 10^{-3}$	$5.26 \times 10^{-2}$	Wellington 2013
GBS_23424	2D	42.8	$5.39 \times 10^{-4}$	$2.44 \times 10^{-2}$	$1.84 \times 10^{-3}$	$2.54 \times 10^{-2}$	Njoro 2012 OS
			$1.27 \times 10^{-21}$	$1.04 \times 10^{-18}$	$1.91 \times 10^{-7}$	$1.46 \times 10^{-1}$	Wellington 2013
GBS_22809	3B	7.1	$5.41 \times 10^{-35}$	$3.76 \times 10^{-32}$	$3.62 \times 10^{-18}$	$1.93 \times 10^{-1}$	Njoro 2012 MS
			$1.55 \times 10^{-13}$	$1.05 \times 10^{-10}$	$2.89 \times 10^{-7}$	$6.18 \times 10^{-2}$	Njoro 2012 OS
GBS_20803	3B	7.4	$9.66 \times 10^{-11}$	$2.62 \times 10^{-8}$	$6.04 \times 10^{-4}$	$7.72 \times 10^{-2}$	Wellington 2013
GBS_11007	3B	10.2	$4.60 \times 10^{-21}$	$1.87 \times 10^{-18}$	$2.38 \times 10^{-8}$	$1.93 \times 10^{-1}$	Wellington 2013
			$5.88 \times 10^{-23}$	$1.33 \times 10^{-20}$	$8.24 \times 10^{-9}$	$8.51 \times 10^{-2}$	Njoro 2012 MS
GBS_11008	3B	10.2	$4.01 \times 10^{-23}$	$1.33 \times 10^{-20}$	$4.93 \times 10^{-9}$	$8.74 \times 10^{-2}$	Njoro 2012 MS
GBS_11149	3B	10.2	$3.85 \times 10^{-11}$	$3.69 \times 10^{-9}$	$2.90 \times 10^{-3}$	$2.59 \times 10^{-2}$	Njoro 2012 MS
GBS_10791	3B	11.5	$3.00 \times 10^{-16}$	$4.09 \times 10^{-14}$	$1.66 \times 10^{-6}$	$6.01 \times 10^{-2}$	Njoro 2012 MS
			$1.14 \times 10^{-18}$	$1.95 \times 10^{-16}$	$1.20 \times 10^{-8}$	$8.32 \times 10^{-2}$	Njoro 2012 MS
GBS_2495	3B	11.5	$2.87 \times 10^{-10}$	$9.72 \times 10^{-8}$	$6.75 \times 10^{-4}$	$2.95 \times 10^{-2}$	Njoro 2012 OS
			$3.18 \times 10^{-14}$	$3.60 \times 10^{-12}$	$3.92 \times 10^{-7}$	$5.79 \times 10^{-2}$	Njoro 2012 MS
GBS_24916	3B	17	$1.54 \times 10^{-6}$	$2.07 \times 10^{-4}$	$6.63 \times 10^{-3}$	$2.02 \times 10^{-2}$	Njoro 2012 OS
GBS_2022	4A	78	$5.39 \times 10^{-10}$	$1.10 \times 10^{-7}$	$3.57 \times 10^{-6}$	$1.34 \times 10^{-1}$	Wellington 2013
GBS_11611	7D	94.3	$1.41 \times 10^{-4}$	$9.62 \times 10^{-3}$	$2.99 \times 10^{-3}$	$2.57 \times 10^{-2}$	Njoro 2012 MS

<sup>†</sup>  $Q$ , population structure matrix;  $K$ , kinship matrix.

<sup>‡</sup> FDR, false discovery rate.

<sup>§</sup> OS, off-season; MS, main season.

chromosome 7BL) and hence, GBS\_18258 might be just proximal to *Lr68*, indicating its association with LTN.

On chromosome 7BS, markers GBS\_1203 (68.9 cM), GBS\_18119 (68.9 cM), and GBS\_23290 (68.9 cM) were significant in the Njoro main and off-seasons, while GBS\_15572 (68.9 cM) was significant only in the Njoro main season. There has been only one previous study (Li et al., 2012) that detected a marker, *Xgwm361.2* on chromosome 7BS, that was positively correlated to the length of LTN in a population developed from Ning 7840/Chokwang. However, the position of this marker was not available in the Synthetic × Opatá map, for comparison. Finally, the marker GBS\_11611, located on chromosome 7DS (94.3 cM), was associated with LTN only in the Ithaca dataset. This marker is 0.6 and 1.9 cM from the *Lr34* associated markers *csLV34* and *cssfr5*, respectively (Fig. 5, chromosome 7DS). While marker *csLV34* is 0.31 cM proximal to *Lr34* (Kolmer et al., 2008; Lagudah et al., 2009), *cssfr5* is a gene-specific marker for *Lr34* (Lagudah et al., 2009). The chromosomal location of GBS\_11611 indicates that it is closely linked to *Lr34/Ltn1*, the major gene associated with LTN.

### Analysis of Marker–Trait Associations for Pseudo-Black Chaff

Twelve GBS markers were significantly associated with PBC in the different data sets (Table 2). The Q-Q plot of  $p$ -values and the Manhattan plot of  $-\log_{10}(p)$  by chromosomes for the different PBC datasets is shown in Supplemental Fig. S3b and S4b. In the Njoro 2012 main season, the most significant marker was GBS\_22809 on chromosome 3BS followed by several markers on chromosome 3BS and one marker on chromosome 7DS that explain 61.78% of the total PBC variance. In the Njoro 2012 off-season, GBS\_22809 on chromosome 3BS was the most

significant marker followed by markers on chromosome 3BS and 2DS that only explain 13.69% of the total PBC variance. In the Wellington dataset, GBS\_11007 was the most significant marker. This marker, along with other markers on chromosomes 3BS, 4AL, and 2DS, explain 60.31% of the variation in PBC. The markers significantly associated with PBC on chromosome 3BS span an interval of ~10 cM (7.1 to 17 cM). GBS\_22809, the most significant marker among them, is at the same location (7.1 cM) as the DArT marker *wPt8446* in the Synthetic × Opatá map (Fig. 5, chromosome 3BS). *wPt8446* is approximately 4.8 cM proximal to the *Sr2* locus (Yu et al., 2014). This indicates that the markers on chromosome 3BS significantly associated with PBC are at the same location as the *Sr2* gene. The markers GBS\_22809, GBS\_10791, GBS\_11149, and GBS\_11008 were significantly associated with stem rust severity in both the Njoro seasons, while GBS\_11007, GBS\_2495, and GBS\_24916 were significant only in the main season. This strongly suggests the involvement of a single gene in expressing both stem rust resistance and PBC.

On chromosome 2DS, GBS\_5796 (39.2 cM) and GBS\_23424 (42.8 cM) were significant in the Wellington and Njoro off-season data sets, respectively. There has been only one previous report (Bariana et al., 2001) of a region on chromosome 2D that enhanced PBC expression in one season. Among the known stem rust resistance genes on chromosome 2DS, *Sr6* was found to be 7 cM proximal to the markers significant in this study. This gene has been mapped close to the DArT marker *XwPt\_0330* within a distance of 2.8 cM (Tsilo et al., 2010). Although this marker was not present in the Synthetic × Opatá map, the marker *wPt-3728* which is about 1 cM proximal to this marker (CIMMYT integrated DArT map; Crossa et al., 2007) is found in the

Synthetic × Opata map about 9 cM away from the markers significant in the present study (Fig. 5, chromosome 2DS). Since *Sr6* has not been observed in association with PBC previously, the significant markers might be indicating another unknown gene in that locus.

Chromosome 4AL had only one significant marker (GBS\_2022; 78 cM) associated with PBC in the Wellington dataset. Previous studies by Yu et al. (2011) identified two loci on chromosome 4AL significantly associated with PBC. One of the markers, *wPt-5857*, identified by them is 0.3 cM proximal to the markers significant in this study (Fig. 5, chromosome 4A), and hence, it may be identifying the same locus. However, the role of this locus in PBC expression is unknown, and no stem rust resistance genes are located here. On chromosome 7DS, the marker GBS\_11611 was significant for PBC only in the Njoro main season. This marker is proximal to the *Lr34* locus as mentioned previously (Fig. 5, chromosome 7DS). Kaur et al. (2009) have reported a QTL called QPbc.sun-7DS close to the *Lr34* locus that enhanced PBC. The same marker, GBS\_11611, was also significantly associated with LTN in the Ithaca dataset.

## Conclusions

The chromosomal locations that were significantly associated with LTN in the different datasets include 1BL, 2BL, 3BS, 4B, 5B, 7BS, 7BL, and 7DS. Among these, the locus on chromosome 7DS corresponding to the *Lr34* gene confirms the well-documented pleiotropic association with LTN (Messmer et al., 2000; Suenaga et al., 2003; Schnurbusch et al., 2004b, 2004a; Krattinger et al. 2009; Risk et al., 2012). *Lr34* has been cloned and found to be an adenosine triphosphate-binding cassette (ABC) transporter (Krattinger et al., 2009). The locus on chromosome 1BL corresponds to the *Lr46/Sr58/Yr29/Pm39/Ltn2* gene, which confers some LTN. Like *Lr34*, it also confers multipathogen resistance and is pleiotropic or closely linked to the stripe rust resistance gene *Yr29* (William et al., 2003; Rosewarne et al., 2006), the powdery mildew resistance gene *Pm39* (Lillemo et al., 2008), the stem rust resistance gene *Sr58* (Singh et al., 2013), and spot blotch resistance (Lillemo et al., 2013). Finally, the slow-rusting gene *Lr68*, was associated with LTN only in the Wellington dataset. All three genes that are slow rusting and confer APR are known to be associated with LTN. The only gene previously associated with LTN but not detected in this study was *Lr67*. This could be due to the very poor marker coverage of chromosome 4D or the low frequency of the gene in the population.

Besides these genes, a locus on chromosomes 2BL previously identified to interact with the *Lr34* locus was also associated with LTN in this study. This is suspected to be the seedling resistance gene *Lr13*. Interactions between *Lr13* and *Lr34* have been reported previously in several studies (Roelfs, 1988; Kloppers and Pretorius, 1997; Oelke and Kolmer, 2005). In fact, the interaction

between *Lr34* and other seedling resistance genes has been suggested to be a mechanism contributing to durable rust resistance (Sawhney, 1992; German and Kolmer, 1992). This nature of *Lr34* brought in the concept of the *Lr34* complex, which is defined as the product of additive interactions involving *Lr34* and two or three additional slow-rusting genes (Singh and Rajaram, 1992). Can this locus be part of the durable rust resistance *Lr34* complex enhancing LTN? This is a question to be answered. In addition to these major genes and interacting loci, a locus on chromosome 3BS, which could correspond to the *Sr2* gene or novel loci linked to it, was observed only in the Njoro seasons. Finally, three unknown loci on chromosome 5B (identified in Ithaca, Njoro main season, and Wellington datasets), on chromosome 7BS (identified in both the Njoro seasons), and on chromosome 4BS (Ithaca dataset) were also associated with LTN in this study.

The loci detected for LTN differ between locations and seasons, suggesting the high environmental dependence of this trait. Although *Lr34* was the major gene associated with LTN, a marker linked to this region on 7DS was detected only in the Ithaca greenhouse dataset. This could be due to the fact that the gene *Lr34* is highly environment specific, requiring optimum combinations of environmental factors for expression (Drijepontd and Pretorius, 1989). A similar observation was made by Risk et al. (2012) with the *Lr34* transgenics that were exposed to a cold temperature treatment at the seedling stage. These exhibited a much stronger LTN than the plants that were not treated, thus suggesting the increase in LTN to be a consequence of elevated *Lr34* expression levels at low temperatures. Besides temperature, other environmental factors could also influence the expression of the *Lr34* gene. While the *Lr34* resistance is detected by the production of fewer or smaller uredinia in the greenhouse (Drijepontd and Pretorius, 1989), it expresses variable pustule size of a typical moderate reaction in the field (Dyck, 1987). This could be the reason why this gene was significantly associated with LTN only in the greenhouse at Ithaca, NY. Similar to *Lr34* are the LTN loci linked to genes *Lr46* (significant only in the Wellington–Cornell data sets), and *Lr68* (significant only in the Wellington data set). None of these loci were associated with LTN in the population evaluated at Njoro, which could be due to the previously mentioned genotype-by-environment interactions. On the other hand, a locus on chromosome 3BS and 7BS was associated with LTN only in the warmer temperatures of Njoro. Hence, the expression of LTN associated with the *Lr34*, *Lr46*, *Lr67*, and *Lr68* genes and the modified expression due to the combinations or interactions of other genes is suggested to be a result of interaction with the environment.

In the case of PBC, loci on four chromosomal locations (2DS, 3BS, 4AL, and 7DS) were significantly associated in the different datasets. Among these, the locus on chromosome 3BS, corresponding to the *Sr2* locus, was consistent in all the analyses, thus confirming its association with PBC. The involvement of additional loci and a common underlying mechanism contributing to

both PBC and stem rust resistance was suggested by Yu et al. (2011). In our study, the other region detected in both the Njoro off-season and the Wellington dataset was 2DS. Although this region could not be precisely assigned to the location of a major stem rust resistance gene, it might be close to the gene *Sr6*. This gene has a slow-rusting nature, as reported by Cox and Wilcoxson (1982), and virulence has been observed in several studies (Huerta-Espino, 1992). Nevertheless, *Sr6* was effective in defeating the race15B epidemics during the 1950s (Kolmer, 2001) and is known to confer good resistance against most North America stem rust races (Leonard, 2001). The fact that it has never been observed in association with PBC suggests that the region significant in this study might correspond to an unknown locus linked to *Sr6*. In addition to these loci, a region on chromosome 4A was significant for PBC only in the Wellington population. It coincided with the locus identified by Yu et al. (2011), but it is not in the vicinity of a reported stem rust resistance gene. Finally, a locus on chromosome 7DS, proximal to the *Lr34* region, was also associated with the expression of PBC in the Njoro main season. Although the role of *Lr34* in PBC is unknown, given that *Lr34* is an ABC transporter, it might play a role in transporting a substrate that causes the black pigmentation.

An interesting observation in this study is that the *Lr34* locus on chromosome 7DS and the *Sr2* locus on chromosome 3BS were sometimes associated with both LTN and PBC. This indicates that there might be an association between the genetic basis of these traits. Further studies are required to examine the role of genes conferring LTN and PBC and what level of resistance could be achieved when both these traits co-occur. In conclusion, these association-mapping panels identified many genetic loci associated with LTN and PBC and their relationship to previously mapped rust resistance genes. Further efforts to characterize the molecular basis of these traits will provide a better insight of their association with durable resistance.

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