ORIGINAL ARTICLE

Fine mapping of high-temperature adult-plant resistance to stripe rust in wheat cultivar Louise

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Abstract

Bread wheat is a major food crop on a global scale. Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, has become one of the largest biotic stresses and limitations for wheat production in the 21st century. Post 2000 races of the pathogen are more virulent and able to overcome the defense of previously resistant cultivars. Despite the availability of effective fungicides, genetic resistance is the most economical, effective, and environmentally friendly way to control the disease. There are two major types of resistance to stripe rust: all-stage seedling resistance (ASR) and adult-plant resistance (APR). Although both resistance types have negative and positive attributes, ASR generally is race-specific and frequently is defeated by new races, while APR has been shown to be race non-specific and durable over time. Finding genes with high levels of APR has been a major goal for wheat improvement over the past few decades. Recent advancements in molecular mapping and sequencing technologies provide a valuable framework for the discovery and validation of new sources of resistance. Here we report the discovery of a precise molecular marker for a highly durable type of APR – high-temperature adult-plant (HTAP) resistance locus in the wheat cultivar Louise. Using a Louise × Penawawa mapping population, coupled with data from survey sequences of the wheat genome, linkage mapping, and synteny analysis techniques, we developed an amplified polymorphic sequence (CAPS) marker LPHTAP2B on the short arm of wheat chromosome 2B, which cosegregates with the resistant phenotype. LPHTAP2B accounted for 62 and 58% of phenotypic variance of disease severity and infection type data, respectively. Although cloning of the LPHTAP2B region is needed to further understand its role in durable resistance, this marker will greatly facilitate incorporation of the HTAP gene into new wheat cultivars with durable resistance to stripe rust.

Keywords: durable resistance, expressed sequence tag, molecular marker, *Puccinia striiformis*, *Triticum aestivum*

Introduction

Global food security largely relies on the production of wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.), which are the three major cereals. Wheat holds the top position in the world by the amount of land used for its production, 218 million hectares, and the second by total weight, 761 million metric tons (USDA 2019). Approximately one third of wheat production is lost annually due to biotic and abiotic stresses with more than 20% of total losses attributed to fungal, bacterial, viral, and nematodal diseases (Savary *et al.* 2012). The cereal rusts (*Puccinia* spp.), caused by Basidiomycete fungi including stem rust (*P. graminis* Pers.:Pers. f. sp. *tritici* Erikss. & E. Henn.), stripe rust (*P. striiformis* Westend. f. sp. *tritici* Erikss.), and leaf rust (*P. triticina* Erikss.) of wheat, are the major contributors to yield loss. An

assessment of historical and contemporary occurrence of the diseases with crop loss magnitude in major wheat-producing regions, estimated that stripe rust is the most significant biotic threat to sustainable wheat production worldwide (Wellings 2011; Chen 2014; Schwessinger 2016). Stripe rust can reduce wheat grain yield up to 100% in susceptible cultivars (Chen 2005). Fungicide treatment and genetic resistance are the two major ways to control stripe rust. Due to the high cost of chemical treatment, soil contamination, and the possibility for rust pathogens to develop fungicide resistance, genetic resistance is considered a more economical and environmentally friendly approach to control stripe rust (Line 2002; Chen 2005; Ellis *et al.* 2014; Oliver 2014).

There are multiple classifications of genetic resistance to stripe rust based on separation criteria: range of infection types, influence of plant developmental stage, race-specificity, rust development pattern and types of phenotypic reactions, influence of temperature, and the use of greenhouse or field tests (Line 2002; Chen 2013). Although many of these classifications intersect and comprise a similar type of resistance, two broad definitions are the most common: all-stage (seedling) resistance (ASR) and adult-plant resistance (APR) (Chen 2005). Most permanently named stripe rust resistance genes belong to ASR (McIntosh *et al.* 2017). All-stage resistance genes are also commonly referred to as seedling, vertical, and race-specific resistance. Usually a single major *R* gene confers a high level of resistance to specific *P. striiformis* f. sp. *tritici* races during the whole life cycle of a plant. ASR often triggers a hypersensitive response via programed death of infected cells, which in turn limits pathogen hyphal expansion in host tissue. A majority of the ASR genes code for intracellular receptor proteins with a common nucleotide binding site and leucine rich repeat domain (NBS-LRR) (Jones and Dangl 2006).

Despite being relatively easy to deploy and combine, along with high resistance levels, all-stage *R* genes lack durability manifested in effective resistance over prolonged periods of time and widespread use (Johnson 1981). Broad incorporation of strong ASR resistance leads to increased selective pressure on the pathogen. Successive rapid evolution of highly virulent races have made a majority of known ASR genes ineffective in multiple wheat growing regions of the world (Milus *et al.* 2009; Wellings 2011; Ren *et al.* 2012; Hou *et al.* 2016). On the other hand, adult-plant resistance, often referred to as race non-specific, horizontal, or slow rusting, usually provides broad spectrum defense, with a reduced degree of initial response and slower disease development (Chen 2013).

Plants carrying APR genes or quantitative trait loci (QTL) are susceptible in seedling tests with the possibility of any infection stage occurring, but

express varying levels of resistance in post-seedling stages, in both field and greenhouse environments (Chen 2005). High-temperature adult-plant resistance (HTAP), which is triggered in late developmental stages, when average daily temperatures rise above 21°C, is considered to be durable and nonrace-specific (Qayoum and Line 1985; Chen 2013). Plants with only HTAP resistance show susceptibility to all stripe rust races at the seedling stage, but as the plant matures, infection progression slows down, reducing the number of new infection sites and inoculum spread. The greatest degree of HTAP resistance can completely inhibit sporulation; however, in most cases HTAP resistance alone is incomplete, and is affected by environmental conditions, plant growth stage, and disease pressure (Chen 2005).

Currently, a combination of all-stage and HTAP resistance is considered to be the optimal breeding strategy for the control of stripe rust (Chen 2014; Ellis *et al.* 2014). Stripe rust resistance, and HTAP resistance in particular, has been one of the top priorities for wheat breeding in the last decade (Chen 2013; Schwessinger 2016). Despite such importance, the discovery and field testing of HTAP resistance is a long and laborious process since APR does not manifest itself until advanced developmental stages and higher temperatures, and can be masked by all-stage resistance genes present in the source germplasms (Ellis *et al.* 2014). Along with the search for new sources of HTAP resistance, there is a need for the identification and development of precise molecular markers, for easy implementation in breeding programs.

Here we report the discovery of a molecular marker for the HTAP resistance locus from the spring wheat cultivar Louise (Kidwell *et al.* 2006; Carter *et al.* 2009). Using a combination of next generation sequencing (NGS), linkage mapping, and synteny approaches, we were able to develop a cleaved amplified polymorphic sequence (CAPS) marker on the short arm of wheat chromosome 2B, which co-segregates with the resistant phenotype in the Louise × Penawawa mapping population. This marker can be used in breeding programs to facilitate introgression of the 2B HTAP gene into new cultivars.

Materials and Methods

Plant materials

A population of 188 $F_{5:6}$ spring wheat recombinant inbred lines (RILs) was used as host material. RILs were made through a single seed descent from F_2 seed harvested from a single F_1 plant of a cross between Louise (PI 634865) and Penawawa (PI 495916). Penawawa is a cultivar susceptible to *P. striiformis* f. sp. *tritici*,

whereas Louise carries a potentially novel HTAP stripe resistance gene.

RILs and parental lines were evaluated for resistance in four field locations. Seeds were planted in 50 cm rows with 30 cm spacing in a randomized complete block design with three replications per location. Experimental trials were evaluated under natural *P. striiformis* infection. Disease symptoms were assessed as infection type and disease severity. Infection type (IT) was rated on a 0–9 scale; disease severity (DS) was based on the percentage of plants infected in the row. Greenhouse experiments were performed with the same set of 188 RILs and parental lines using a randomized complete block design with three replicates. Temperatures were set to 21–24°C (day) and 15–18°C (night) with 16 hours photoperiod. At the beginning of heading (Feekes 10.2) plants were moved to a dew chamber (10°C; 100% humidity) and inoculated with fresh *P. striiformis* f. sp. *tritici* urediniospores, race PST-100 using spore-talc mixture (1 in 20 parts). After 24 hours plants were moved back to the greenhouse with 16 hours photoperiod at 28°C during the day and 15°C at night. Infection data was collected as described previously 18–20 days post-inoculation.

For the DNA collection, seedlings were planted in 15 cm round 3 liter pots and grown in a greenhouse with a diurnal cycle: 16 h light at 25°C and 8 h dark at 15°C. After 42 days, with a fully emerged flag leaf (Zadoks stage 39), total genomic DNA was extracted and purified in a BioSprint 96 – DNA Plant kit (QIAGEN). DNA was quantified using a Quant-ITTM PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) in Synergy 2 Multi-Mode Reader (BioTek) and normalized to 20 ng · μl⁻¹ using QIAgility (QIAGEN).

Molecular markers

The RIL population was genotyped with single nucleotide polymorphism (SNP) markers using the Illumina Infinium assay with Wheat SNP 9K and 90K iSelect BeadChips (Cavanagh *et al.* 2013; Wang *et al.* 2014). SNP genotyping was performed on BeadStation and iScan instruments at the USDA-ARS Biosciences Research Laboratory, Fargo, ND, USA. Additionally, over 1,000 SSR markers were tested for polymorphism between Louise and Penawawa.

Microsatellite sequences and their chromosomal locations were obtained from Graingenes (http://wheat.pw.usda.gov/). SSR markers were amplified by PCR with M13-tailed (5'-CACGACGTTGTAAAAC-GAC-3') forward primers and M13 dye labeled primers (Schuelke 2000).

Three more sets of expressed sequence tags (EST) based SSR markers were tested. Thirty ESTs from differential expression profiling using the NGS transcriptomics analysis of Louise × Penawawa population, 30 based on rice synteny analysis, and 24 based on ESTs from chromosome 2B, using chromosome-based draft sequence of the hexaploid wheat (GenomeZipper) generated by the International Wheat Genome Sequencing Consortium (IWGSC) (Marcussen *et al.* 2014). Primers were designed using Primer-BLAST tool (https://www.ncbi.nlm.nih.gov). PCR reaction performed in 25-μl reactions containing 50 to 100 ng of genomic DNA, 5 μl GoTaq buffer (Promega), 1.25 unit GoTaq polymerase, 10 mM dNTPs, and 10 μM of forward and reverse primers. Amplification conditions were set as follows: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 59 to 61°C (specific to primers) for 30 s, extension at 72°C for 1 min. The final extension step was at 72°C for 5 min. Amplified reactions were split into two groups. The first group was analyzed using 2% agarose gel electrophoresis with EtBr staining directly after amplification. The second group was split and digested with a set of restriction endonucleases (*Mnl*I, *Hae*III, *Alu*I, *Msp*I, *Nla*III, *Cvi*AII, *Hha*I, *Fnu*4HI, *Mbo*I, *Cvi*KI-1), selected on the basis of NEBcutter V2.0 restriction site analysis, using the manufacturer's protocol for each enzyme (New England Biolabs). Digested reactions were also analyzed using 2% agarose gel electrophoresis with EtBr staining.

Quantitative trait loci mapping

IT and DS data from RILs and parental lines were used to calculate the area under the disease progress curve (AUDPC). Relative AUDPC (rAUDPC) was calculated for each line as a percentage of the most susceptible AUDPC value in each of the experiments. The statistical analysis of rAUDPC was performed within each environment using the statistical package SAS (SAS Institute, Raleigh, NC, USA). Genotype and replication by genotype effects were tested using the PROC GLM (regression, ANOVA). Broad-sense heritability (h^2) was calculated using the formula: $h^2 = Var(G)/Var(P)$. The mean rAUDPC values for each line within each environment were used in QTL mapping.

A combined set of SNP, SSR, and CAPS EST-based markers was used to construct linkage groups with JoinMap version 4.0 (van Ooijen and Voorrips 2001). Linkage groups were calculated with a logarithm of odd (LOD) threshold of 3. Genetic distances (cM) were calculated using the Kosambi mapping function. The linkage map was drawn using MapChart v2.3 software (Voorrips 2002).

QTL analysis was performed with Windows QTL Cartographer V2.5_011 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). The linkage map was constructed in JoinMap and consisted of 170 markers on chromosome 2B. DS and IT mean values were used as an input. Composite interval mapping (CIM) algorithm

(Zeng 1994) was applied to estimate the association of each marker to the traits, including the additive effect and the percentage of phenotypic variation explained by each QTL. The LOD score (significance threshold = 3) for QTL was calculated with 1000 permutations ($p = 0.05$) and a walk speed of 0.5 cM.

Results

The IT of the susceptible parent, Penawawa, ranged from 6 to 8, while the IT of Louise, the resistant parent and donor of HTAP, ranged from 2 to 3, based on field observations. The DS for Louise ranged from 1 to 15% and for Penawawa from 20 to 70%. The DS values for the whole RIL population across multiple environments ranged from 25 to 97% with an average of 36% for resistant lines and 72% for susceptible lines (Fig. 1). Tested with race PST-100, the most predominant race in the United States since 2005 (Chen *et al.* 2010; Wan and Chen 2012, 2014), both parents exhibited susceptibility at the seedling stage, suggesting the absence of ASR genes effective against this race.
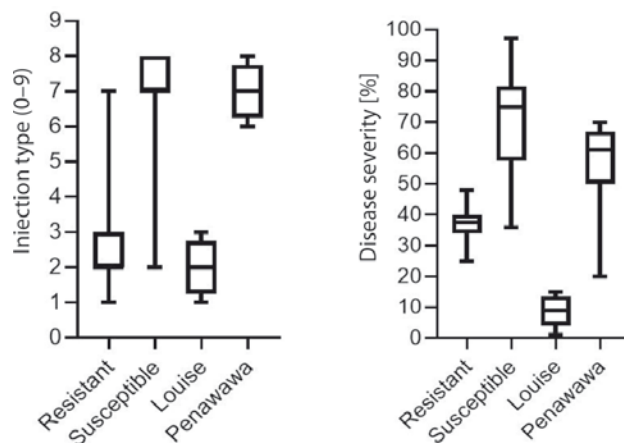

Fig. 1. Infection type (IT) and disease severity (DS) distribution. IT and DS values for all 188 RILs and parental lines plotted as boxes with upper and lower quartiles defining the edges. Medians are shown as horizontal lines inside the boxes and extreme values are represented as whiskers

A working set of 142 SNP and 28 SSR markers mapped to chromosome 2B and was based on the data from Graingenes, 9K and 90K SNP assays, the previous Louise HTAP study (Carter *et al.* 2009), and those generated from
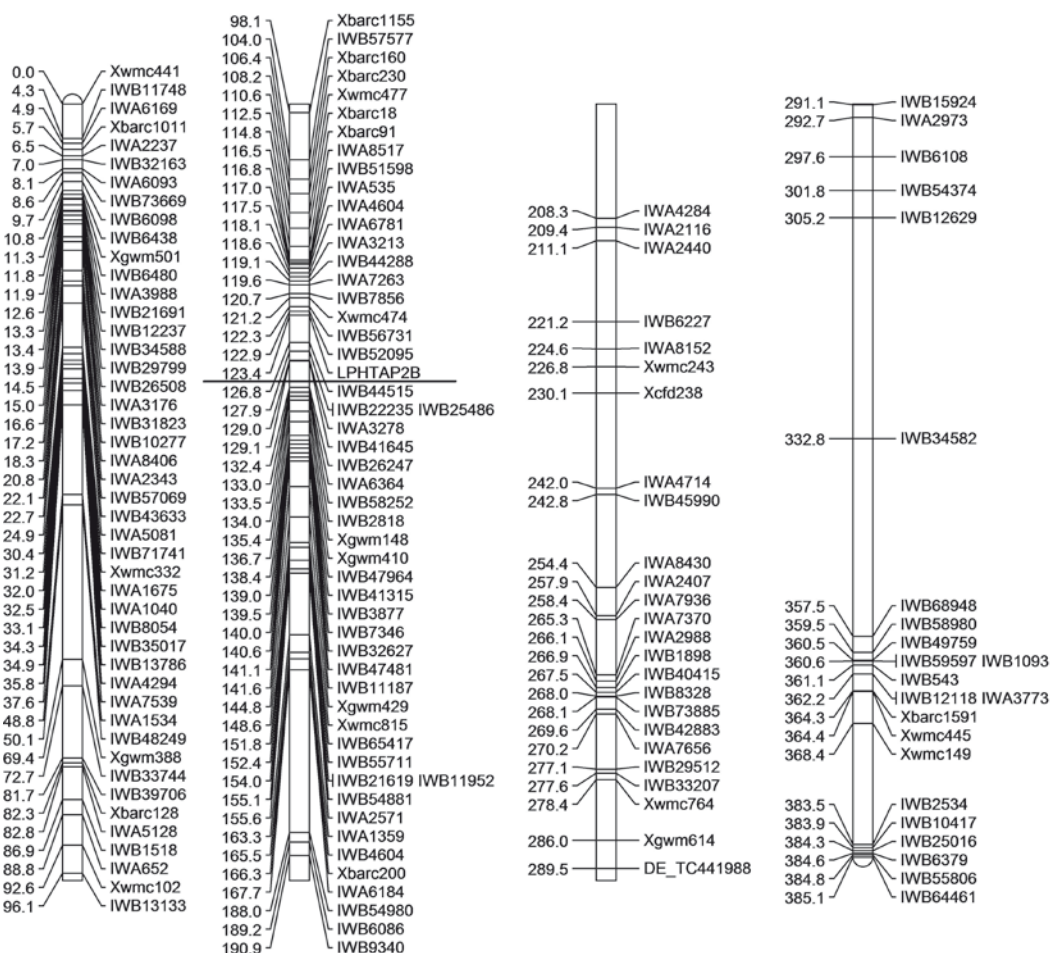

Fig. 2. Linkage map – chromosome 2B. Wheat chromosome 2B separated into 4 sections. The left side in each section represents the position in cM and the right side, the marker's name. The co-segregating marker is underscored with a black line

GenomeZipper ESTs. These markers were polymorphic in the Louise × Penawawa mapping population and clustered together in one linkage group with LOD scores >4, 148 of them had LOD >10. The final linkage map for chromosome 2B spanned 385 cM (Fig. 2).

Among the EST based markers from all tested sets, five showed polymorphism between the parents. During the linkage map construction, three markers from rice synteny analysis and one from the transcriptomics study were clustered in different regions from chromosome 2B. One marker from the transcriptomics study and one from wheat GenomeZipper clustered in the 2B linkage group with LOD > 10. Furthermore, marker LPHTAP2B co-segregated with the resistant phenotype. The marker was based on a 729-nucleotide EST, WHE3591_A09_A17ZS, reported in GenomeZipper from the IWGSC wheat draft sequence. In order to seek polymorphism between the parents and in the mapping population, this EST region was amplified using AGGAGCTTGAGGGATTTGGC – forward and TCCTCCCTGATCCGCAAAAC – reverse primers, followed by digestion with *Mbo*I. The amplicon of the resistant parent Louise was digested into four
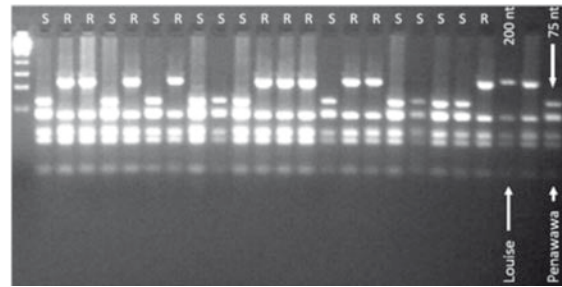

Fig. 3. LPHTAP2B restriction profile. Parental bands marked with arrows, all RILs have clear bimodal digestion. Polymorphic fragment sizes are pointed to with arrows on the top

fragments with a distinct fragment of 200 nucleotides, whereas the amplicon from the susceptible parent Penawawa showed a distinct band of 75 nucleotides (Fig. 3). Two remaining fragments were <50 nucleotides and monomorphic between the parents. Genotyping with this marker showed perfect correspondence with the resistant and susceptible RIL phenotypes, considering IT 1–4 as resistant and IT 6–9 as susceptible. Furthermore, QTL analysis with the DS data estimated LPHTAP2B as being responsible for 62% of phenotypic
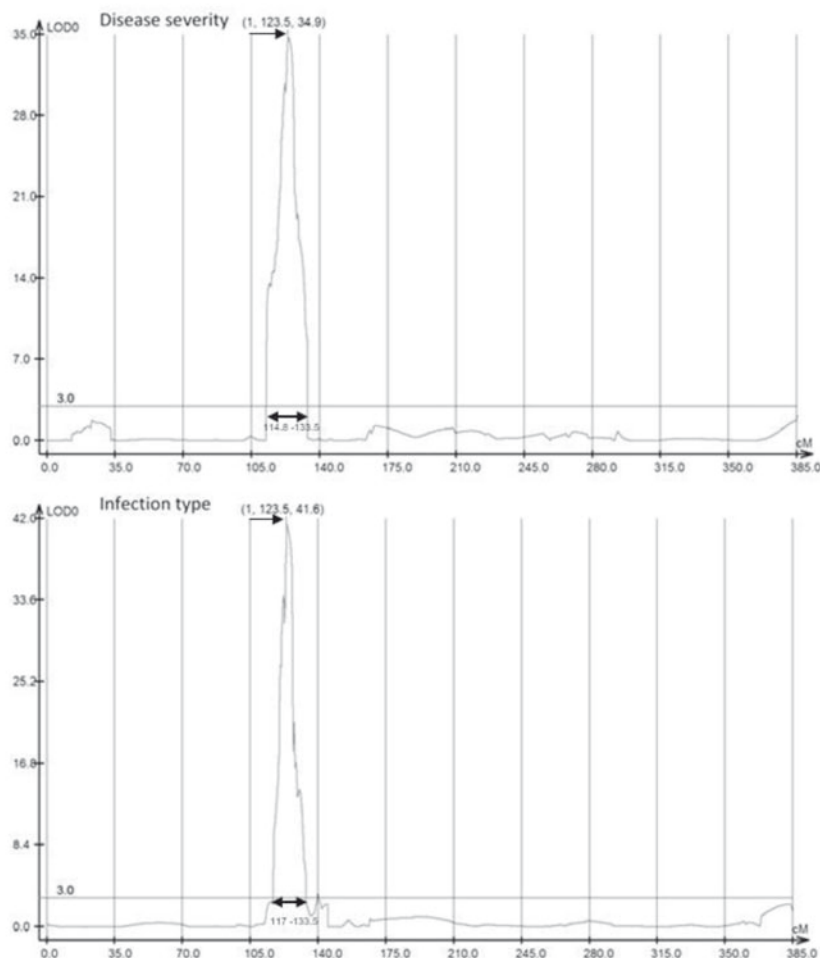

Fig. 4. Quantitative Trait Loci (QTL) analysis using disease severity (DS) and infection type (IT) data. The X axes represent logarithm of odd (LOD) scores and the Y axes, the location in centimorgans. LPHTAP2B marker maps directly to the peaks for both DS and IT

variance (R^2) with –17.6 additive effect attributed to Louise, and LOD = 34.8 (Fig. 4). QTL analysis with IT data showed 58% of the phenotypic variance explained by LPHTAP2B with –2.5 additive effect and LOD = 41.6 (Fig. 4).

Discussion

Several genes that confer resistance to stripe rust have been located on wheat chromosome 2B, including *Yr5, Yr7, Yr27, Yr31, Yr41, Yr43, Yr44, YrC51, Yr53*, and *YrSP* (McIntosh *et al.* 2017). Colocalization of these ASR genes with LPHTAP2B can be ruled out since the Louise × Penawawa mapping population showed susceptibility in the seedling stage. A few studies have also reported HTAP resistant QTL on chromosome 2B. QTL *QYrid.ui-2B.2* was derived from IDO444 and mapped to a similar region between markers *gwm429* and *barc91* in the Rio Blanco × IDO444 mapping population (Chen *et al.* 2012). Although this QTL spans the Louise HTAP region, the magnitude of effect is significantly lower. It explained 20% of the phenotypic variation versus 60% in Louise, and the peak with the highest LOD was 15 cM distal. Pedigree analysis for up to seven generations did not show shared ancestry with Louise, suggesting a different source of the resistance. The source of Louise HTAP is derived from the Pacific Northwest cultivar Wakanz. Marker analysis performed for both *QYrlo.wpg-2BS* and LPHTAP2B showed no polymorphism between Louise and Wakanz (Fig. 5).

The HTAP QTL *QYr.caas-2BS*, identified in Chinese landrace Pingyuan 50, was also mapped in a similar region, but only reached the LOD 3 threshold under one experimental condition and was below LOD 2 in four other experiments. Furthermore, its peak was >20 cM proximal to LPHTAP2B (Lan *et al.* 2010). The cultivar Luke carried HTAP QTL *QYrlu.cau-2BS2*, which explained up to 40% of the phenotypic variation, but was shifted more than 30 cM in centromere proximal (Guo *et al.* 2008). Two QTLs located on chromosome 2B and associated with stripe rust resistance were observed in the French bread wheat cultivar Camp Remy (Mallard *et al.* 2005). *QYr.inra-2BL* was localized with Louise HTAP QTL in the *mc245 – gwm148* region, but was associated with all-stage resistance, whereas *QYr.inra-2BS* was associated with HTAP resistance but mapped >40 cM proximal to LPHTAP2B. Furthermore, pedigree analysis of six parental cross generations showed no shared ancestry of Louise and Camp Remy, suggesting a different source of the resistance.

Recently reported APR QTL – *Qyrnap.nwafu-2BS* in cultivar Napo 63 was mapped close to LPHTAP2B on the short arm of chromosome 2B, with similarly high R^2 – 55 to 66% and LOD scores 22 to 56 (Han *et al.* 2017). Although additional allelism studies are required to analyze the difference between these loci, the authors report 660K-AN21 and 660K-AN57 polymorphism in the region of putative gene *Traes_2BS_2B483208E*. This gene encodes glycosyltransferase activity, whereas LPHTAP2B marker is expressed and located in the GTP-binding protein with P-loop NTPase domain. Interestingly, the source of the resistant allele of *Qyrnap.nwafu-2BS* is Napo 63, which is present in the fourth generation of parental pedigree for Louise but belongs to the Wawawai ancestry which is shown to be susceptible and does not contain polymorphism for LPHTAP2B marker. Finally, a few additional markers reported from the genome-wide association study (GWAS) of stripe rust associated QTL in a worldwide collection of spring wheat, were located on chromosome 2B (Maccaferri *et al.* 2015). Of those, *IWA586, IWA226, IWA3206, IWA7312*, and *IWA5177* were located far outside of the Louise HTAP region, while *IWA905* and *IWA4606* were within 7 and 21 cM from LPHTAP2B, respectively. Although neither of those markers showed polymorphism in the Louise × Penawawa population, further allelism studies will be necessary to determine if these loci represent different resistance genes.

Since chromosome 2B carries multiple QTLs and genes associated with different types of stripe rust resistance, further analysis of their complex interactions and localizations is needed, especially for breeding programs aided by marker-assisted selection (MAS). MAS includes a set of tools that utilize DNA markers with tight linkage to the loci of interest. It aids or completely substitutes phenotypic selection, directly identifying a trait of interest, based on linked markers
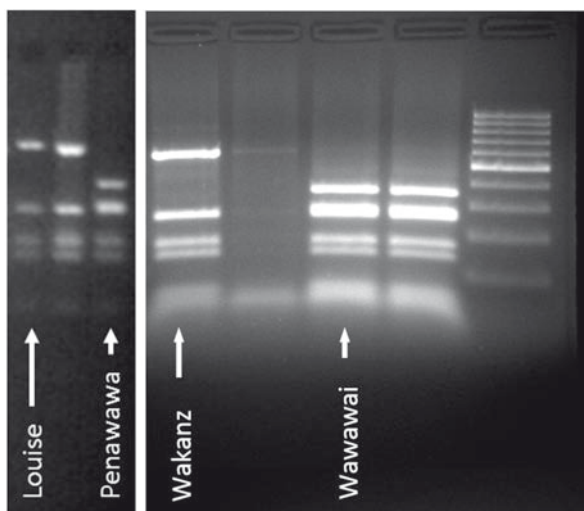

Fig. 5. LPHTAP2B donor polymorphism. Restriction fragments for Louise, Penawawa and Louise parents – Wakanz × Wawawai are shown after amplification with LPHTAP2B primers and MboI digestion. Louise and Wakanz restriction patterns are identical

(Collard *et al.* 2005). It is especially effective in selection for recessive and costly for phenotyping traits, and for pyramiding resistance genes. It has served as an effective tool in many wheat disease resistance programs (Zheng *et al.* 2014; Han *et al.* 2017). Lack of closely linked trait markers is one of the limiting factors of MAS. The function of marker efficiency is usually the degree of linkage or a proximity to a gene of interest. LPHTAP2B co-segregates with HTAP resistant phenotype and is based on expressed sequence. We hypothesize that it is a functional marker and it can be immediately incorporated in MAS breeding programs, adding a source of durable resistance, although further physical mapping of this region is needed to precisely locate and understand the relation between marker and HTAP gene.

The exact function of the putative gene that co-segregates with LPHTAP2B is unknown, although it aligns to a family of GTP-binding proteins. Proteins from this class are involved in plant defense signaling (Sano *et al.* 1995), and as regulators of cell death in rice (Kawasaki *et al.* 1999). LPHTAP2B EST also contains the region of the P-loop NTPase domain superfamily, which includes ATPase Binding Cassette (ABC) genes, previously reported to confer durable resistance to multiple fungal pathogens in wheat (Krattinger *et al.* 2009). Despite these associations, further physical mapping and gene cloning are needed in order to understand the Louise mechanism of HTAP resistance.

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