Saturation Mapping of a Major Effect QTL for Stripe Rust Resistance on Wheat Chromosome 2B in Cultivar Napo 63 Using SNP Genotyping Arrays

Jianhui Wu1, Qilin Wang1, Shengjie Liu2, Shuo Huang2, Jingmei Mu2, Qingdong Zeng1, Lili Huang1, Dejun Han2* and Zhensheng Kang1*

1 State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling, China, 2 State Key Laboratory of Crop Stress Biology for Arid Areas, College of Agronomy, Northwest A&F University, Yangling, China

Stripe rust or yellow rust (YR), caused by Puccinia striiformis f. sp. tritici (Pst), is one of the most important diseases of wheat (Triticum aestivum L.). Widespread deployment of resistant cultivars is the best means of achieving durable disease control. The red grain, spring wheat cultivar Napo 63 produced by CIMMYT in the 1960s shows a high level of adult-plant resistance to stripe rust in the field. To elucidate the genetic basis of resistance in this cultivar we evaluated 224 F2:3 lines and 175 F2:6 recombinant inbred lines (RILs) derived from a cross between Napo 63 and the Pst-susceptible line Avocet S. The maximum disease severity (MDS) data of F2:3 lines and the relative area under the disease progress curve (rAUDPC) data of F2:6 RILs were collected during the 2014–2015 and 2015–2016 wheat growing seasons, respectively. Combined bulked segregant analysis and 90K single nucleotide polymorphism (SNP) arrays placed 275 of 511 polymorphic SNPs on chromosome 2B. Sixty four KASP markers selected from the 275 SNPs and 76 SSR markers on 2B were used to identify a chromosome region associated with rust response. A major effect QTL, named Qyrnap.nwafu-2BS, was identified by inclusive composite interval mapping and was preliminarily mapped to a 5.46 cM interval flanked by KASP markers 90K-AN34 and 90K-AN36 in chromosome 2BS. Fourteen KASP markers more closely linked to the locus were developed following a 660K SNP array analysis. The QTL region was finally narrowed to a 0.9 cM interval flanked by KASP markers 660K-AN21 and 660K-AN57 in bin region 2BS-1-0.53. The resistance of Napo 63 was stable across all environments, and as a QTL, explained an average 66.1% of the phenotypic variance in MDS of F2:3 lines and 55.7% of the phenotypic variance in rAUDPC of F2:6 RILs. The short genetic interval and flanking KASP markers developed in the study will facilitate marker-assisted selection, gene pyramiding, and eventual positional cloning of Qyrnap.nwafu-2BS.

Keywords: adult-plant resistance, bulked segregant analysis, molecular markers, Puccinia striiformis, Triticum aestivum
INTRODUCTION

Global wheat production is affected by many diseases, among which the rusts are the most important (McIntosh et al., 1995; Hovmøller et al., 2010). Stripe or yellow rust (YR) caused by *Puccinia striiformis f. sp. tritici* (*Pst*) is a constant threat and as one of the most destructive diseases, it is capable of causing 5–25% yield losses, or sometimes more, in almost all wheat-growing regions (Chen, 2005; Wellings, 2011). Major stripe rust epidemics have occurred in China, with losses in some instances amounting to several million metric tons (Li and Zeng, 2002; Wan et al., 2007; Chen et al., 2009; Kang et al., 2010; Zhao et al., 2016). The most sustainable control strategy is resistant commercial cultivars (McIntosh et al., 1995; Li and Zeng, 2002; Wiesnerhanks and Nelson, 2016).

Stripe rust resistance is usually categorized as all-stage resistance (ASR) and adult-plant resistance (APR) (Chen, 2005, 2013). All-stage resistance is expressed during all plant growth stages and can be detected in seedlings. ASR is often preferred by breeders because the individual genes confer high levels of resistance and are easily selected in breeding. However this type of resistance is more vulnerable to pathogen evolution when deployed singly in cultivars (Chen, 2005; Chen et al., 2010). In contrast, APR, which is characterized by variable levels of response determined by actual growth stage and temperature, usually provides protection against a broader range of races and tends to be more durable (Chen, 2013; Brown, 2015; Niks et al., 2015). Acceptable levels of APR are often due to the interactive effects of two or more genes. The best known *Pst*-APR genes *Yr18*, *Yr36*, and *Yr46* that confer a degree of resistance to multiple races have been cloned, thus permitting development of ‘perfect’ markers for marker-assisted breeding (Fu et al., 2009; Krattiger et al., 2009, 2013; Forrest et al., 2014; Moore et al., 2015), and most importantly these genes have shown durability. Therefore, combining APR genes with race specific ASR genes is a preferred strategy for wheat breeding as it may prolong the life of the ASR genes and will significantly reduce losses if virulent races do develop (Chen, 2013; Ellis et al., 2014). This highlights the significance of identifying new resistance genes, especially those effective against a broad spectrum of pathogen races.

Quantitative trait loci (QTL) mapping is the currently preferred method of dissecting the genetic components of disease resistance (Niks et al., 2015; Wiesnerhanks and Nelson, 2016). However, the biggest bottleneck for conventional QTL mapping in bread wheat is lack of high-density polymorphic markers; consequently target QTL are often located across a large region and markers may not be sufficiently accurate for map-based cloning of candidate genes or for marker-assisted selection (MAS) in crop breeding (St Clair, 2010; Yang et al., 2015). Recent progress in genome sequencing and high throughput single nucleotide polymorphism (SNP)-based genotyping technologies in wheat has facilitated faster development of trait-linked markers (Yang et al., 2015). Compared with earlier markers, SNP have a distinct advantage in abundance and polymorphism. Recent applications of next-generation sequencing (NGS) greatly improved the efficiency and throughput of SNP discovery that contributed to the use of assay platforms (Wang et al., 2015). Current SNP assay platforms, including Illumina Bead Array™, Affymetrix GeneChip™ and Kompetitive allele-specific PCR (KASP™), have been rapidly adopted in mapping and MAS studies (Barabaschi et al., 2016; Rasheed et al., 2016). Moreover, the recently developed high-density 9, 35, 90, 660, and 820K arrays for wheat are efficient for genetic mapping and exceed precious resources in fine mapping (Allen et al., 2013, 2016; Cavanagh et al., 2013; Wang et al., 2014; Jia and Zhao, 2016; Winfield et al., 2016). A lot of research on genome-wide association (GWAS) and QTL mapping of resistance to stripe rust by wheat SNP arrays has already been carried out (Zegeye et al., 2014; Hou et al., 2015; Liu et al., 2015, 2016; Maccasferri et al., 2015b).

Bulked segregant analysis (BSA) (Michelmore et al., 1991), involving selected and pooled DNA samples from contrasting phenotypic groups, provides a simple and rapid approach to search for markers linked to specific genomic regions associated with a trait of interest. Combining the BSA strategy with a high-throughput NGS technology is a common practice for gene identification and QTL mapping. Many studies have outlined methodologies and applications of high-throughput sequencing in BSA for qualitative and quantitative traits (Abe et al., 2012; Takagi et al., 2013; Win et al., 2016).

In a previous study we screened a diverse panel of over 1,000 wheat germplasms for resistance to stripe rust in field nurseries at Yangling in Shaanxi province, and Tianshui in Gansu province. We identified a number of common wheat genotypes with resistance to prevalent Chinese *Pst* races (Han et al., 2012). Napo 63 with the pedigree Frocor//Frontana/Yaqui 48/3/Narino 59 is a red grained spring wheat cultivar produced by the International Maize and Wheat Improvement Center (CIMMYT) in the 1960s. Here, we identify Napo 63 as possessing typical APR to stripe rust. The objectives of the study were to (1) fine map the major QTL for stripe rust resistance using SNP arrays following BSA, (2) identify the candidate resistance genes, and (3) develop and verify the applicability of KASP markers to enable MAS in wheat breeding programs.

MATERIALS AND METHODS

Plant Materials

The mapping population comprised 175 *F*$_{5:6}$ recombinant inbred lines (RILs) developed by single-seed descent from the cross Avocet S × Napo 63. A second population of 222 *F*$_{2:3}$ lines from the same cross was derived from a single *F*$_{1}$ plant. Avocet S is a highly susceptible spring wheat selection from Australia. The wheat cultivars (or lines) Avocet S, Mingxian 169 (MX169) and Xiaoyan 22 (XY22) were used as susceptible controls throughout the study. Two hundred and fifteen wheat entries were used to evaluate polymorphisms of molecular markers flanking the

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1[^1]: [http://www.Lgcgenomics.com](http://www.Lgcgenomics.com)

2[^2]: [http://wgb.cimmyt.org/gringlobal/search.aspx](http://wgb.cimmyt.org/gringlobal/search.aspx)
resistance lines from the major winter wheat growing areas in China (Zeng et al., 2014), 28 landraces and 85 foreign germplasms with resistance to stripe rust (Han et al., 2012).

### Greenhouse Trials

Seedling and adult-plant tests were conducted under controlled greenhouse conditions to characterize the APR of Napo 63. Five races (CYR31, CYR32, CYR33, V26/CH42, and V26/Gui22-9) were used. Their virulence/avirulence characteristics were previously reported by Wu et al. (2016). For seedling tests, 9–15 plants of Avocet S and Napo 63 were grown in previously reported by Wu et al. (2016). For seedling tests 9 cm × 9 cm × 9 cm pots, and for adult-plant tests three plants were grown in larger 20 cm × 20 cm × 15 cm pots. Seedlings at the two-leaf stage (14 days after planting) and adult-plants at the booting stage were separately inoculated withurediniospores of each race mixed with talc (approximately 1:20). Inoculated plants were incubated at 10°C in a dew chamber in darkness for 24 h, and then transferred to a greenhouse at 17 ± 2°C with 14 h of light (22,000 lx) daily. Infection types (ITs) were recorded 18–21 days after inoculation using a 0–9 scale (Line and Qayoum, 1992). Plants with ITs 0–6 were considered resistant, and plants with ITs 7–9 were considered susceptible. In order to confirm and clarify ITs of the entries, the tests were repeated at three different times.

### Field Trials

Field trials were conducted in five environments at three locations (Yangling in Shaanxi, Tianshui in Gansu, and Jiangyou in Sichuan) in two winter wheat seasons (2014–2015 and 2015–2016), with each year location being considered a single environment. The 175 Avocet S × Napo 63 lines were tested at all three locations during 2015–2016 and the 222 F2,3 lines were tested at Yangling and Tianshui during 2014–2015. All trials were arranged in randomized complete blocks with three replicates. An individual plot consisted of a single 1 m row with 30 cm between adjacent rows. Each plot was sown with approximately 30 seeds of each RIL or parent, whereas each F2,3 line and parents were grown from 25 seeds. The parents and susceptible variety XY22 were planted after every 20 rows throughout the field. Another susceptible control MX169 was planted in 5-row blocks after every 50 rows and around the field area. XY22 and MX169 served as inoculum spreaders to ensure uniform stripe rust development throughout the field. Since, there is reliable natural occurrence of stripe rust at Tianshui and Jiangyou the experiments were conducted under naturally infected conditions. The Yangling site was inoculated withurediniospores of race CYR32 suspended in liquid paraffin sprayed onto MX169 at flag leaf emergence. Disease severities (DS) were recorded three times for each line, when MX169 had 60–100% and XY22 had 30 to >90% severity during the period April 1 to 15 at Jiangyou, May 1 to 20 at Yangling, and May 25 to June 15 at Tianshui. DS were assessed visually using percentage diseased leaf area based on the modified Cobb scale (Peterson et al., 1948). DS for the F6 RIL population were converted to area under the disease progress curve (AUDPC) values following Chen and Line (1995). Relative AUDPC (rAUDPC) values were calculated for each RIL and parent as a percentage of the mean AUDPC value of the susceptible parent, Avocet S (Lin and Chen, 2007). Maximum disease severities (MDS) of F2,3 lines were scored when YR levels on Mingxian 169 and Avocet S reached their maxima around 15–20 May at Yangling and 10–15 June at Tianshui. Non-segregating lines were scored as a single value; segregating lines were scored as two or more values that were later converted to an average value for analysis.

### Statistical Analyses

Mean MDS of each F2,3 line and rAUDPC of each F6 RIL were used in analyses of variance (ANOVA). Analyses of variance and Pearson’s correlation coefficients were performed with AOV functionality in the QTL IciMapping V4.1 software package (Wang, 2009). Broad-sense heritability (h2) of stripe rust resistance was calculated by $h^2 = \frac{\sigma_g^2 + \sigma_{ge}^2 + \sigma_{re}^2}{\sigma^2}$ (Allard, 1960), where $\sigma_g^2$ is $(MS_{g} - MS_{e})/re$, $\sigma_{ge}^2$ is $(MS_{g} - MS_{e})/r$, and $\sigma_{re}^2$ is $MS_r$; in this formula, $\sigma^2 = \sigma_g^2 + \sigma_{ge}^2 + \sigma_{re}^2 = \sigma^2 = \text{genotypic} \times \text{environment interaction variance}$, $\sigma^2 = \sigma^2 = \text{error variance}$, $MS_g = \text{mean square of genotypes}$, $MS_e = \text{mean square of genotype} \times \text{environment interaction}$, $MS_r = \text{mean square of error}$, $r = \text{number of replications}$, and $e = \text{number of environments}$.

### Combined Bulk Segregant Analysis with the 90 and 660K SNP Arrays

DNA from parents, F2 plants and F5-derived lines were extracted from young leaves of greenhouse-grown plants following Song et al. (1994). BSA was performed to identify markers polymorphic between the resistant parent Napo 63 and susceptible parent Avocet S, and between the resistant DNA (R-bulk) and susceptible parental DNA (S-bulk) bulks. DNA of 10 F2,3 lines and 10 F5:6 RIL homozygous resistant (IT 1, DS ≤ 5) and homozygous susceptible (IT 9, DS ≥ 90) in all environments were separately pooled to contrast advertising bulks that along with the parents were genotyped with the 90 and 660K SNP arrays from CapitalBio Corporation (Beijing, China) (Figures 1A–C). SNP genotype calling and clustering was performed with Genome Studio Polyploid Clustering v1.0 software (Illumina). SNP markers that were monomorphic, poor quality showing more than 20% missing values, ambiguous in calling, or with minor allele frequencies below 5% were not used. Polymorphic SNPs associated with resistance in BSA were localized to chromosomes based on the high-density 90K (Wang et al., 2014) or 660K genetic maps (Jizeng Jia, personal communications). The sequences of all SNP were used to blast version 0.4 of the assembled Chinese Spring survey sequence [The International Wheat Genome Consortium (IWGSC)4] to determine their physical positions.

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1. http://www.isbreeding.net/software/default.aspx?type=detail&type2=detail2&id=18
2. http://www.capitalbio.com
3. http://www.illumina.com
4. http://www.wheatgenome.org/
FIGURE 1 | Overview of analyses. F$_{2:3}$ lines and F$_{5:6}$ recombinant inbred lines (RILs) were derived from the cross AvS × Napo 63. (A) Phenotypes of AvS, Napo 63 and their progenies across all environments and data collected at heading-flowering stage. (B) Frequency distribution of maximum disease severity (MDS) for 221 F$_{2:3}$ lines grown at Yangling and Tianshui in 2015. (C) Frequency distribution of relative area under the disease progress curve (rAUDPC) for 175 F$_{6}$ RILs grown at Yangling, Jiangyou and Tianshui in 2016. Black arrows indicate the parental line means. Distributions of the polymorphic SNPs in each chromosome by 90K (D) and 660K (F) SNP arrays and positions of SNPs in chromosome 2B (E,G). Selected SNPs (in the red dotted boxes) were analyzed in KASP assays.
Molecular Marker Analysis and Genotyping

Following chromosome location based on polymorphic SNPs, the parents and contrasting DNA bulks were used to screen published wheat SSR markers on that chromosome (Somers et al., 2004). The SSR assays were performed in a S1000 Thermal Cycler (BIO-RAD) with reaction mixtures (15 µL) containing 50–100 ng of template DNA, 1.0 U of Taq DNA polymerase, 1.5 µL of 10 × buffer (50 mmol KCl, 10 mmol of Tris-HCl, pH 8.3), 2.0 mmol of MgCl₂, 200 mmol of dNTPs, and 1.0 mmol of the forward and reverse primers. The PCR program was: denaturation at 94°C for 4 min, followed by 15 cycles of 94°C for 30 s, touchdown starting at 65°C for 45 s (decreasing 1.0°C per cycle), 72°C for 1 min, 20 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 1 min, and a final extension for 10 min at 72°C. PCR products were separated in 6% denaturing polyacrylamide gels, visualized using silver staining for polyacrylamide gels, and photographed.

Based on the integrated genetic map (Maccarferri et al., 2015a,b) and wheat genome assembly, KASP PCR markers were developed for polymorphic SNPs using a similar approach to that described in Ramirez-Gonzalez et al. (2015). KASP assays were performed following the protocol of LGC Genomics using a KASP mix containing universal FRET (fluorescence resonance energy transfer) cassettes (FAM and HEX), ROX™ passive reference dye, Taq polymerase, free nucleotide, and MgCl₂ in an optimized buffer. End-point fluorescence data were visualized with a microplate reader (FLUOstar Omega, BMG LABTECH, Germany) and analyzed by Klustering Caller software. Reaction mixtures consisted of final volumes of 5 µL containing 2.5 µL of 2× KASP V4.0 Mastermix (LGC Genomics), 0.056 µL of assay primer mix (12 mM of each allele-specific primer and 30 mM of common primer) and 50–100 ng of genomic DNA. An S1000 Thermal Cycler (Bio-Rad) was used with cycling conditions: 94°C for 15 min, nine cycles of 94°C for 20 s, touchdown starting at 65°C for 60 s (decreasing 0.8°C per cycle), 32 cycles of 94°C for 20 s, and 57°C for 60 s.

Genetic Map Construction and QTL Analysis

Genotypic data from our populations were used to construct a genetic map with the regression function in JoinMap version 4.0 (Van Ooijen, 2006). Recombination fractions were converted to centiMorgans (cM) using the Kosambi function (Kosambi, 1943) and a LOD score of 3.0 was used as a threshold. The linkage map was graphically visualized with Mapchart V2.3 (Voorrips, 2002). The resistance locus was assigned to a chromosomal bin by location of tightly flanking markers based on the deletion maps of Sourdille et al. (2004). QTL mapping was conducted with QTL IciMapping V4.1 (Wang, 2009) using an inclusive composite interval mapping (ICIM) analysis. A logarithm of odds (LOD) threshold of 2.5 was set to declare a significant QTL. The MDS score for each F₂,₃ line and rAUDPC score for each F₆ RIL at each field site were used in the respective QTL analyses. The phenotypic variances explained (PVE) by individual QTL were also obtained using ICIM.

Identification of Candidate Genes and Comparative Genomics Analysis

The polymorphic SNP markers located in the target region were blasted on the Ensemble Plants website to find Gene Identifiers (IDs) that were used for gene annotation from the Human-Readable Description data for gene function. Each corresponding wheat gene sequence was also used to perform a BLAST search against the genome sequence databases of Brachypodium and rice to identify orthologous gene sets.

RESULTS

Phenotypic Evaluation

In greenhouse experiments with Pst races CYR31, CYR32, CYR33, V26/CH42 and V26/Gui22-9, Napo 63 was susceptible (IT 8–9) in seedling tests but highly resistant (IT 1) in adult-plant stage tests. The susceptible parent, AvS was susceptible (IT 8–9) at both growth stages. In field tests, stripe rust developed well across environments. F₁ plants displayed IT 1-2 and DS 10–35 (necrotic flecks, without sporulation) at Yangling (Figure 1A) during 2014–2016 indicating that APR in Napo 63 was partially dominant. Napo 63 was rated with a mean MDS of ≤5% or a mean rAUDPC value of <5%, whereas AvS had a mean MDS ≥ 95% or a mean rAUDPC value of 100% in all environments. The frequency distributions of mean MDS for the 221 F₂,₃ lines in two environments and mean rAUDPC for 175 F₅,₆ RILs in three environments revealed continuous variation (Figures 1B,C), indicating quantitative inheritance. MDS data of F₂,₃ lines were significantly correlated between the two environments, with correlation coefficients of 0.90 (P < 0.0001) (Table 1), and heritability was 0.80 (Table 2). The rAUDPC data for the F₅,₆ RILs were highly correlated across three environments (r = 0.77–0.92, P < 0.01) (Table 1), and heritability was 0.87 (Table 2). ANOVA revealed significant differences (P < 0.001) in MDS and rAUDPC between lines, environments, replicates within environments and line × environment interactions (Table 2). The results suggested that the expression of APR was consistent across the different environments.

Mapping QTL for Stripe Rust Resistance with F₂,₃ Lines

A total of 509 SNPs showed polymorphisms between the DNA bulks after genotyping by the 90K SNP array; 275 of these SNPs were blasted on the Ensemble Plants website to find Gene Identifiers (IDs) that were used for gene annotation from the Human-Readable Description data for gene function. Each corresponding wheat gene sequence was also used to perform a BLAST search against the genome sequence databases of Brachypodium and rice to identify orthologous gene sets.
TABLE 1 | The correlation analysis of the mean maximum disease severity (MDS) for F₂₃ lines across two environment and relative area under the disease progress curve (rAUDPC) for F₆ recombinant inbred line population across three environment.

| Environment   | Yangling 2015 | Tianshui 2015 | Yangling 2016 | Tianshui 2016 | Jiangyou 2016 |
|---------------|---------------|---------------|---------------|---------------|---------------|
| Yangling 2015 | 1             |               |               |               |               |
| Tianshui 2015 | 0.896***      | 1             |               |               |               |
| Yangling 2016 |               |               | 1             |               |               |
| Tianshui 2016 |               | 0.767***      |               | 1             |               |
| Jiangyou 2016 |               |               |               | 0.922***      | 1             |

***Significant at P < 0.001.

TABLE 2 | Variance components of MDS scores for F₂₃ lines and rAUDPC for F₆ recombinant inbred line population derived from AvS/Napo 63.

| Source of variation | F₂₃ lines | F₆ RILs |
|---------------------|-----------|---------|
|                      | Df | Mean square | Df | Mean square |
| Lines               | 221| 1633.78***  | 174| 0.669***    |
| Replicates (environments) | 4  | 265.59***  | 6  | 0.117***    |
| Environments        | 1  | 2661.93***  | 2  | 0.422***    |
| Lines × Environments | 220| 149.86***  | 343| 0.042***    |
| Error               | 445| 63.00       | 1020| 0.013      |
| $\sigma^2_g$        | 285.82 |            | 0.055 |           |
| $h^2$               | 0.80  |            | 0.87  |            |

***Significant at P < 0.001.

FIGURE 2 | Comparative genetic linkage maps of stripe rust resistance gene Qyrnap.nwafu-2BS. The QTL region for Qyrnap.nwafu-2BS was identified by QTL mapping using phenotypic and marker data from AvS × Napo 63. (A) Genetic linkage maps of Qyrmap.nwafu-2BS on chromosome 2BS produced from results of by using F₂₃ lines (A) and F₆ RILs (B). (C,D) Orthologous genomic regions of Qyrmap.nwafu-2BS on Brachypodium chromosome 1 and rice chromosome 5.

were located on chromosome 2B and the others were distributed across other chromosomes (Figure 1D). Most of the SNPs on 2B were within an interval of 50–250 Mb (Figure 1E). Sixty four chromosome-specific SNPs in the region were selected for conversion to KASP markers and then screened on the parents and bulks to confirm polymorphisms before being genotyped on the entire population; 49 failed to distinguish the contrasting parents and bulks. Four of 76 published wheat SSR markers on...
chromosome 2B were also polymorphic between parents and bulks.

A genetic map was constructed using the 4 SSR and 15 KASP markers genotyped on the 224 F2 individuals, resulting in a linkage group spanning 16.1 cM. A major effect QTL Qyrnap.nwafu-2BS was placed in this map by ICIM using the mean MDS data across environments. The QTL was preliminarily located between the KASP markers 90K-AN34 (Kukri_c36026_68) and 90K-AN36 (w SNP_Ex_c62844_62315607) in an interval of 5.46 cM (Figures 2A, 3A).

Validation of the QTL Using F5:6 RILs and Fine Mapping of Qyrnap.nwafu-2BS

A second genetic map was constructed for the RIL population using the same markers. (Figures 2A, B). The major QTL for stripe rust resistance was also detected by ICIM using mean rAUDPC data across environments (Figure 2B). Approximately, 7,000 SNPs showed polymorphisms in BSA with the 660K SNP array; 5,263 were located on all 21 chromosomes based on the w7984/Opata85 map (Figure 1F) (Jizeng Jia, personal communications). More than 130 of 2,689 SNPs in this map occurred in the chromosome 2B interval 90K-AN34—90K-AN36 according to the wheat genome assembly (Figure 1G). To fine map Qyrnap.nwafu-2BS and develop improved markers for routine breeding applications 14 more closely linked chromosome-specific SNP markers were converted to KASP assays and eight polymorphic KASP markers were used to map the F2:3 and F5:6 populations. All the sequences of KASP markers are listed in Supplementary Table S1.

Qyrnap.nwafu-2BS was confirmed by analyzing recombinants between KASP markers 90K-AN34 and 90K-AN36. Eighteen and 23 recombinants from F5:6 RILs and F2:3 lines were...
TABLE 3 | Summary of stripe rust resistance QTL detected by ICIM in the AvS × Napo 63 F₂:₃ population across two environments and F₅:₆ population across three environments.

| Location and year | Marker interval       | LODᵃ | Addᵇ | PVEᶜ |
|-------------------|-----------------------|------|------|------|
| MDS               |                       |      |      |      |
| Yangling 2015     | 660K-AN21—660K-AN57    | 48.6 | −27.3| 62.9 |
| Tianshui 2015     | 660K-AN21—660K-AN57    | 56.1 | −26.1| 69.2 |
| Average           |                       |      |      | 66.1 |
| rAUDPC            |                       |      |      |      |
| Yangling 2016     | 660K-AN21—660K-AN57    | 22.7 | −0.18| 45.7 |
| Tianshui 2016     | 660K-AN21—660K-AN57    | 36.0 | −0.26| 62.4 |
| Jiangyou 2016     | 660K-AN21—660K-AN57    | 34.6 | −0.24| 59.1 |
| Average           |                       |      |      | 55.7 |

ᵃLOD, logarithm of odds score.
ᵇAdd, additive effect of resistance allele.
ᶜPVE, percentages of the phenotypic variance explained by individual QTL.

TABLE 4 | Phenotype and alleles of KASP markers flanking Qyrnap.nwafu-2BS in Napo 63, Avocet S (AvS), susceptible checks and 19 wheat cultivars and landraces.

| Wheat line          | KASP markers | Severity (%) and reaction at adult-plant stageᵇ |
|---------------------|--------------|-----------------------------------------------|
|                     | 660K-AN3     | 660K-AN21 | 660K-AN57 | 660K-AN65 | Yangling 2014 | 2015 | 2016 | Tianshui 2014 | 2015 | 2016 |
| Napo 63             | AA           | GG       | TT       | TT        | 5R           | 1R   | 5R   | 5R           | 5R   | 5R   |
| Avocet S            | CC           | AA       | CC       | CC        | 100S         | 100S | 100S | 100S         | 100S | 100S |
| XY22 (CK)           | CC           | AA       | CC       | CC        | 100S         | 95S  | 100S | 100S         | 100S | 100S |
| MX169 (CK)          | CC           | AA       | CC       | CC        | 100S         | 100S | 100S | 100S         | 100S | 100S |
| IDO444              | CC           | AA       | TT       | CC        | −             | −    | −    | −             | −    | −    |
| Kariega             | AA           | GG       | TT       | TT        | −             | −    | −    | −             | −    | −    |
| Kenya Kudu          | AA           | GG       | TT       | TT        | −             | −    | −    | −             | −    | −    |
| Luke                | AA           | GG       | TT       | TT        | −             | −    | −    | −             | −    | −    |
| Louise              | AA           | GG       | TT       | TT        | −             | −    | −    | −             | −    | −    |
| Opata 85            | AA           | GG       | TT       | TT        | −             | −    | −    | −             | −    | −    |
| Buc/Bijy            | AA           | GG       | TT       | TT        | 1R            | 1R   | 5R   | 5R           | 10R  | 5R   |
| Bluejay“S”          | AA           | GG       | TT       | TT        | 1R            | 1R   | 1R   | 1R           | 5R   | 10R  |
| Mos“S”-Imu          | AA           | GG       | TT       | TT        | 1R            | 1R   | 5R   | 10R          | 5R   | 5R   |
| Taa 72              | AA           | GG       | TT       | TT        | 10R           | 10R  | 5R   | 15R          | 10R  | 5R   |
| Taa 73              | AA           | GG       | TT       | TT        | 15MR          | 10R  | 10R  | 10R          | 5R   | 5R   |
| Yang 11–59          | CC           | GG       | TT       | TT        | 70MS          | 90S  | 100S | 90S          | 80MS | 100S |
| Zhouyuan 9369       | AA           | GG       | TT       | CC        | 40MR          | 50MS | 40MR | 30MR         | 40MS | 50MR |
| Jimai 41            | CC           | AA       | TT       | TT        | 80S           | 90S  | 80S  | 100S         | 90S  | 90S  |
| Jimai 44            | CC           | AA       | TT       | TT        | 70S           | 90S  | 80S  | 90S          | 100S | 100S |
| Jimai 45            | CC           | AA       | TT       | TT        | 80S           | 100S | 90S  | 100S         | 90S  | 100S |
| Luo 8112            | CC           | AA       | TT       | TT        | 70S           | 90S  | 80S  | 100S         | 100S | 100S |
| Shengnong 1         | CC           | AA       | TT       | TT        | 60MS          | 80MS | 90S  | 70MS         | 80S  | 80S  |
| Suzhou 1310         | CC           | AA       | TT       | TT        | 70MS          | 90MS | 70MS | 80S          | 90S  | 100S |
| Suzhou 1313         | CC           | AA       | TT       | TT        | 60MS          | 80S  | 80S  | 90S          | 100S | 90S  |
| Suzhou 22           | CC           | AA       | TT       | TT        | 80MS          | 90S  | 100S | 80S          | 100S | 100S |
| Wanximai 2013–20    | CC           | AA       | CC       | TT        | 70S           | 100S | 90S  | 90S          | 90S  | 100S |
| Wanximai 2013–9     | CC           | AA       | CC       | TT        | 80S           | 100S | 70S  | 80S          | 90S  | 90S  |
| Zhongmai 170        | CC           | AA       | TT       | TT        | 80S           | 90MS | 80S  | 90S          | 70S  | 80S  |

ᵃAA means the SNP locus.
ᵇA field response was a combination of disease severity recorded as a single value for each accession at each site from 0 to 100% of the foliage infected using Peterson et al. (1949) scale, and R, resistant; MR, moderately resistant; M, moderately resistant to moderately susceptible; MS, moderately susceptible; and S, susceptible.
grouped based on their haplotypes across the marker interval (Figures 3B,D). The corresponding *Pst* phenotypes of each group were displayed using the mean rAUDPC or MDS data (Figures 3C,E). The mean value and standard deviation of each group were calculated on basis of the individual rAUDPC or MDS of each line (Supplementary Tables S4, S5). With the help of these more closely linked markers, this QTL region was narrowed down to a 0.9 cM interval flanked by KASP markers 660K-AN21 (AX-10930296) and 660K-AN57 (AX-110457023) (Figures 2A,B, 3A). This explained 62.9 and 69.2% of the phenotypic variances in average MDS among F2,3 lines grown in two environments. For the F2,5 RILs the corresponding variances in rAUDPC explained ranged from 45.7 to 62.4% in three environments (Table 3). According to the position of the SSR markers in the 2B deletion bin map, the QTL was located in the 2BS-1-0.53 region (Figure 3A). Figures 4A,B showed genotypic data of F2,3 and F2,5 lines from the KASP assays.

**Evaluation of Linked Markers in Various Wheat Genotypes**

A set of 215 wheat genotypes was used to evaluate the robustness of KASP markers derived from 660K-AN3, 660K-AN21, 660K-AN57, and 660K-AN65 KASP in predicting the presence of *Qyrnap.nwafu-2BS* in the mapping population. The 660K-AN57 KASP marker did not detect the presence/absence of the resistance allele accurately and resulted in a high level of misclassification (Supplementary Table S2). Markers 660K-AN3, 660K-AN21, and 660K-AN65 amplified 1, 2, and 12 false positives among susceptible cultivars, respectively (Figures 4C,D), but no genotype had all SNP markers (Table 4), indicating that they should not be used alone to detect *Qyrnap.nwafu-2BS*. Ten cultivars with APR contained the same 660K-AN3, 660K-AN21, and 660K-AN65 alleles, as Napo 63. They were CIMMYT lines Buc/-Bjy, Kariega, Kenya Kudu, Louise, Bluejay“S”, Luke, Mos“S”-Imu, Opata 85, Taa 72 and Taa 73 indicating that they may have the resistance gene. Nonetheless, further genetic studies need to be performed to confirm allelism or otherwise. APR gene or QTL on chromosome 2BS have been identified in Kariega, Kenya Kudu, Louise, Luke and Opata 85.

**Comparative Genomic Analysis and Candidate Genes in the Target Genomic Region**

To assess collinearity of the target genomic region in wheat with *Brachypodium* and rice, the relevant wheat gene sequences were selected to identify orthologous genes in comparative genomic regions in *Brachypodium* and rice. For simplification only 18 polymorphic KASP-SNP markers located between 90K-AN30 and 90K-AN12 are listed (Figures 2C,D). Comparative analyses revealed that 11 KASP markers in wheat showed collinearity with *Brachypodium* and rice. Ten predicted *Brachypodium* genes, except Bradi1g19627.1, in this region have orthologous genes in rice, but arranged in a reverse order indicating an inversion in rice compared to *Brachypodium* and wheat.

The target region spanned a 4.3 Mb interval from 154.9 to 159.2 Mb in the wheat genome assembly, which has synteny with a 102.2 kb genomic region *Brachypodium* chromosome 1S (from Bradi1g20410.1 to Bradi1g20560.2) and a 188.4 kb region in rice chromosome 5S (from LOC_Os07g43530.1 to LOC_Os07g43870.1). A total of 67 SNP markers were blasted in the target region of wheat and 14 wheat genes were identified; several SNPs were mapped in the same gene (Supplementary Table S3). The target region contained a glycosyltransferase gene defined by marker AX-110644789 (gene *Traes_2BS_2B483208E*) that is possibly involved in plant disease resistance (Bolouri Moghaddam and Van den Ende, 2012) and therefore can be regarded as a potential candidate gene for *Qyrnap.nwafu-2BS*.

**DISCUSSION**

The resistance gene-rich chromosome 2BS region is known to possess many genes for both ASR and APR, including *Yr27, Yr31, Yr41, YrC51, YrF, YrH9014, YrKK, YrP81, YrSp*, and *YrTp1* (Maccaferri et al., 2015b). Resistances conferred by *Yr27, Yr31, Yr41, YrP81*, and *YrSp* have been overcome by Chinese *Pst* races (Zheng et al., 2014; Zeng et al., 2015). All confer all-stage or race-specific resistance. However, *YrKK,*
characterized in the CIMMYT-derived common wheat cultivar Kenia Kudu (Kenya 131/Kenya 184P), confers immunity in adult-plants and had a small effect on seedling response (Li et al., 2013). Pedigree analyses revealed that Napo 63 has a distant relationship with Kenia Kudu through the common parent Florence (Supplementary Figure S1). Several stripe rust QTLs with major APR effects for on chromosome 2BS have been reported (Rosewarne et al., 2013). For example, QYrlu.cau-2BS1 and QYrlu.cau-2BS2 in Luke, QYr.id.ui-2B.1 and QYr.id.ui-2B.2 in IDO444, QYr.sgi-2B.1 in Kariage, QYrl.wpg-2BS in Louise, QYr-2B in Opata 85 and QYr.intra-2B.1 in Camp Remy conferring large effects were located in a similar region (Boukhatem et al., 2002; Mallard et al., 2005; Guo et al., 2008; Carter et al., 2009; Prins et al., 2011; Chen et al., 2012). Based on the integrative genetic map (Maccasferri et al., 2015b) all of the above major QTLs were within the interval 19.9–50.2 cM, within which we placed Qyrap.nwfu-2BS between 40.02 and 43.44 cM. Pedigree analyses indicated that all cultivars except IDO444 were derived from CIMMYT germplasm14. IDO444 (PI 578278) is a winter wheat developed by the University of Idaho wheat breeding program in 1994 without CIMMYT germplasm in its pedigree (XM Chen, personal communication15). Moreover, in our study, the same alleles of KASP markers 660K-AN3, 660K-AN21, 660K-AN57, and 660K-AN65 were detected in Kariage, Kenia Kudu, Louise, Luke and Opata 85. Based on molecular detection assays, origin, and chromosome location it appears likely that the same gene is present in at least some of these varieties. Although BSA has a wide range of applications in genetics and genomics, it can also be used for dissection of relatively simple traits controlled by major genes (Sun et al., 2010; Zou et al., 2016). For quantitative traits that are controlled by polygenes with different effects, BSA can be improved through increasing population size and marker density, using multiple bulked samples, and precision phenotyping. In this study, F2.3 lines and S5.6 RILs were chosen for genotyping by 90 and 660K SNP arrays using segregates with extreme differences in phenotype across all environments (Figure 1). A large number of SNPs associated with the resistance were then selected. Based on SNP location a major QTL region was identified and chromosome-specific SNPs were selected and converted to KASP markers for further screening by BSA. Fifteen KASP markers developed from 64 SNPs from the 90K SNP assay were employed to generate a genetic map and the resistance locus was located in an interval of 5.46 cM spanned by 90K-AN34 and 90K-AN36. The same procedure was used to fine map the QTL with the 660K SNP array and Qyrap.nwfu-2BS was narrowed to a 0.9 cM interval between SNP markers 660K-AN21 and 660K-AN57. Thus, validation of SNP arrays with KASP-SNP assays improves the accuracy of fine mapping in QTL regions. Comparative genomics showed that the target genomic region has synteny with Brachypodium and rice, and except that the region was inverted in rice, there were no rearrangements to complicate future map-based cloning.

To date, three adult-plant stripe rust resistance genes have been cloned. Yr18 encodes an ATP-binding cassette (ABC) transporter (Krattiger et al., 2009), Yr36 encodes a protein containing both kinase and START domains (Fu et al., 2009), and Yr46 encodes a hexaspore transporter that inhibits hexose uptake from the apoplast by host cells (Moore et al., 2015). Based on the functions of the identified proteins, vesicle trafficking and protein/metabolite transportation are probably common physiological processes involved in APR (Niks et al., 2015). In this study, bioinformatics analysis of the mapped SNPs in the target region for stripe rust resistance showed that wheat gene Traes_2BS_2B483208E encoded a glycosyltransferase. Sugars are involved in many metabolic and signaling pathways in plants. Sugar signaling may also contribute to immune responses against pathogens when changing concentrations or ratios of sugars in plant tissue can induce plant defense genes, influence plant hormone pathways, and induce resistance to various diseases (Bolouri Moghaddam and Van den Ende, 2012; Dodds and Lagudah, 2016). Traes_2BS_2B483208E function in energy metabolism and transport is similar to that of Lr67/Yr46. Prediction of candidate genes sets a basis for the next step in map-based cloning. Nevertheless, further genetic studies and more detailed analyses are needed to confirm the roles of this and other candidate genes in stripe rust response.

Marker-assisted selection provides an efficient way to incorporate and pyramid genes in breeding programs. However, the markers must be reliable, specific, and easily used in an economic way. This is particularly true for disease resistance where remotely located disease nurseries may be needed for phenotyping to ensure reliable and repeatable results (Chen, 2013). KASP genotyping technology provides a high throughput platform at low cost. In this study, we identified 660K-AN3, 660K-AN21, and 660K-AN65 as flanking markers suitable for selection of Qyrap.nwfu-2BS but when tested on a set of diverse genotypes the markers were insufficiently robust to determine presence or absence of Qyrap.nwfu-2BS and therefore should be used together.

**AUTHOR CONTRIBUTIONS**

JW: conducted the experiments, analyzed the data, and wrote the manuscript. QW and DH: identified the resistant parental line, made the cross and participated in the field experiments. SL, SH, and JM: participated in field experiments and contributed to the genotyping experiment. QW and QZ: assisted in analyzing the data. LH: revised the manuscript. DH and ZK: conceived and made the cross and participated in the field experiments. SL, SH, and JM: participated in field experiments and contributed to the genotyping experiment. QW and QZ: assisted in analyzing the data. LH: revised the manuscript. DH and ZK: conceived and directed the project and revised the manuscript.

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14http://wgb.cimmyt.org/gringlobal/search.aspx
15https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1473249
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MAPPING STRIPE RUST RESISTANCE QTL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00653/full#supplementary-material

FIGURE S1 | Pedigrees of Napo 63 and Kenya Kudu.
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