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Research Article

Integrated Molecular and Phenotypic Screening of Pakistani Wheat Landraces for Resistance to Stem Rust (*Puccinia graminis* f. sp. *tritici*)

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ABSTRACT

Stem rust caused by *Puccinia graminis* f. sp. *tritici* remains a major threat to global wheat production, necessitating the identification and deployment of durable resistance genes. Among these, the adult plant resistance gene Sr2 in *Triticum aestivum* is widely recognized for conferring partial but durable resistance. This study aimed to determine the presence of Sr2 in 86 Pakistani wheat landraces using the linked molecular marker stm598tcac and to evaluate their phenotypic response to stem rust under field conditions at multiple disease assessments. PCR amplification with the Sr2-linked marker failed to produce the expected 56 bp diagnostic fragment in any accession, indicating the apparent absence of Sr2 in the evaluated germplasm. Field evaluations revealed limited resistance across observations. At the first assessment, only a few landraces exhibited resistant (R) reactions, while most were moderately susceptible (MS) or susceptible (S). Disease severity increased at the second observation, where only seven accessions remained resistant and the majority showed high susceptibility (≥80S). By the third assessment, nine accessions were classified as resistant (R/10MR), eleven as moderately resistant, four as moderately susceptible, and the remainder as susceptible (≥60-100S). Notably, accessions 210904, 210898-210903, and 220072 consistently expressed resistance across stages. In conclusion, the absence of the Sr2-linked marker and the predominance of susceptible reactions indicate that Sr2-mediated resistance is largely lacking in these Pakistani wheat landraces. However, a small subset of accessions demonstrated stable phenotypic resistance, suggesting the possible presence of alternative resistance sources valuable for future stem rust breeding programs.

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Introduction

Wheat stem rust, caused by *Puccinia graminis* f. sp. *tritici*, remains one of the most destructive diseases of bread wheat worldwide. Historically, stem rust epidemics have resulted in catastrophic yield losses, and the disease continues to threaten global food security due to the pathogen's high evolutionary potential and capacity for long-distance dispersal through windborne urediniospores. Climate change, characterized by rising temperatures, altered precipitation patterns, and increased frequency of extreme weather events, has further enhanced pathogen survival, mutation rates, and transboundary migration, thereby complicating disease forecasting and management strategies (Singh et al., 2020; Hodson et al., 2022).

The emergence and spread of highly virulent races, particularly the Ug99 lineage (race TTKSK and its derivatives), has renewed global concern. First detected in East Africa in 1999, Ug99 overcame the widely deployed stem rust resistance gene Sr31 and subsequently acquired additional virulence against other major resistance genes. Its spread across eastern and southern Africa and into parts of the Middle East highlights the vulnerability of global wheat production systems that rely heavily on race-specific resistance genes (Singh et al., 2020; Hodson et al., 2022). Although significant progress has been made in resistance breeding, cultivars carrying single, race-specific (major) genes often lose effectiveness rapidly due to directional selection pressure imposed on pathogen populations.

In contrast, adult plant resistance (APR) genes provide partial, quantitative, and generally non-race-specific resistance. APR is typically expressed at later growth stages and is associated with reduced disease severity, slower epidemic development, and enhanced durability across diverse agro-ecological environments (Juliana et al., 2020; Bhavani et al., 2021). Among these, Sr2 is the most extensively characterized APR gene and has served as the cornerstone of durable stem rust resistance for nearly a century. Originally derived from emmer wheat (*Triticum turgidum* ssp. *dicoccum*), Sr2 confers a "slow-rusting" phenotype by reducing pustule size, sporulation rate, and disease progression rather than completely preventing infection. When combined with minor-effect quantitative trait loci (QTLs) or other APR genes (e.g., Sr55/Lr67, Sr57/Lr34), Sr2 forms the basis of durable, multi-genic resistance.

However, phenotypic identification of Sr2 is challenging because its expression is influenced by environmental conditions and genetic background. The associated pseudo-black chaff (PBC) phenotype, traditionally used as a morphological indicator, is not always consistently expressed. Therefore, molecular marker-assisted selection (MAS) has become a reliable approach for detecting Sr2 in breeding germplasm. PCR-based markers tightly linked to Sr2 enable efficient introgression and pyramiding of resistance loci, accelerating the development of durable cultivars. Recent studies report variable frequencies of Sr2 in modern wheat cultivars depending on breeding history and geographic origin (Li et al., 2021; Yu et al., 2023).

Despite extensive deployment of Sr2 in improved germplasm, its prevalence and distribution in South Asian wheat landraces remain insufficiently documented. Traditional landraces represent valuable reservoirs of genetic diversity shaped by long-term adaptation to local agro-climatic conditions. They often harbor novel alleles and complex polygenic resistance mechanisms involving minor-effect loci and gene networks that contribute to quantitative disease resistance (Kumar et al., 2019; Singh et al., 2023). Systematic molecular screening of landraces can therefore uncover untapped resistance sources and facilitate their integration into contemporary breeding programs.

Given the strategic importance of durable resistance under changing climatic scenarios, the present study aimed to molecularly characterize previously unexamined Pakistani wheat landraces for the presence of Sr2 using a PCR-based marker system. The findings will provide baseline information on the distribution of Sr2 in indigenous germplasm and support future resistance breeding and gene discovery efforts aimed at strengthening stem rust resilience in wheat production systems.

Materials and Methods

Plant material

A total of eighty-six wheat (*Triticum aestivum* L.) landraces originating from Pakistan were obtained from the USDA National Small Grains Collection (NSGC), Aberdeen, Idaho, USA (Table 1). The accessions represented diverse agro-ecological regions, including Punjab (27 accessions), Sindh (52 accessions), Azad Jammu and Kashmir (2 accessions), and the North West Frontier region (5 accessions). These landraces were selected to capture broad genetic diversity for the assessment of stem rust resistance-associated markers.

Table 1. Summary of Pakistani wheat landraces used in the study.

Region	Number of Accessions	Plant ID
Punjab	27	40951,210896
Sindh	52	182079,182120
Azad Kashmir	2	250411,250412
North West Frontier	5	219737,219748

DNA extraction

Genomic DNA was extracted from young, healthy seedling leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol following Doyle and Doyle (1990). Leaf tissues were ground in liquid nitrogen to ensure efficient cell disruption. DNA quality was assessed by electrophoresis on 1.5% agarose gels, and purity was evaluated based on band integrity and the absence of degradation. DNA concentrations were standardized to uniform working concentrations suitable for polymerase chain reaction (PCR) amplification.

PCR marker for *Sr2* detection

The presence of the stem rust resistance gene *Sr2* was assessed using the closely linked simple sequence repeat (SSR) marker stm598tcac (Hayden et al., 2004). Amplification was performed using forward primer stm598tcac-F (5′-GTTGCTTTAGGGGAAAAGCC-3′) and reverse primer stm598tcac-R (5′-TCTCTCTCTCTCTCACACACAC-3′), which are reported to produce a diagnostic fragment of approximately 56 bp associated with the *Sr2* locus.

PCR amplification

PCR reactions were carried out in 25 µl volumes containing 50-100 ng genomic DNA, 0.25 µM of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 U Taq DNA polymerase. Amplification was performed in a thermal cycler under the following conditions: an initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min (optimized temperature), and extension at 72°C for 2 min; followed by a final extension at 72°C for 7 min.

All PCR assays were conducted at least twice to confirm reproducibility and consistency of amplification patterns. Due to the unavailability of authenticated *Sr2*-positive control genotypes, band identification was based on the expected fragment size reported in previous studies. Therefore, marker detection should be considered indicative rather than definitive.

Electrophoresis and data analysis

Amplified PCR products were resolved on 6% high-resolution agarose gels prepared in 1× TBE buffer and stained with ethidium bromide. Bands were visualized under ultraviolet (UV) illumination, and fragment sizes were estimated using a 50 bp DNA ladder as a molecular size standard.

The presence or absence of the expected 56 bp fragment was scored for each accession. It is acknowledged that fragments of approximately 56 bp approach the lower resolution limit of conventional agarose gel systems. Consequently, faint or closely migrating bands may not have been clearly distinguishable, potentially leading to underestimation (false-negative scoring) of marker presence. Marker frequency was calculated as the proportion of accessions exhibiting the diagnostic band relative to the total number of genotypes analyzed.

Results

PCR amplification using the Sr2-linked marker stm598tcac failed to produce the expected 56 bp diagnostic fragment in any of the 86 wheat landraces analyzed (Figure 1). Due to the unavailability of a locally maintained Sr2-positive control, band interpretation was based on the validated diagnostic fragment size (56 bp) previously reported by Hayden et al. (2004). None of the tested accessions exhibited the characteristic 56 bp amplicon associated with the Sr2-linked allele, indicating the apparent absence of the Sr2 resistance gene in the evaluated germplasm under the conditions of this assay.

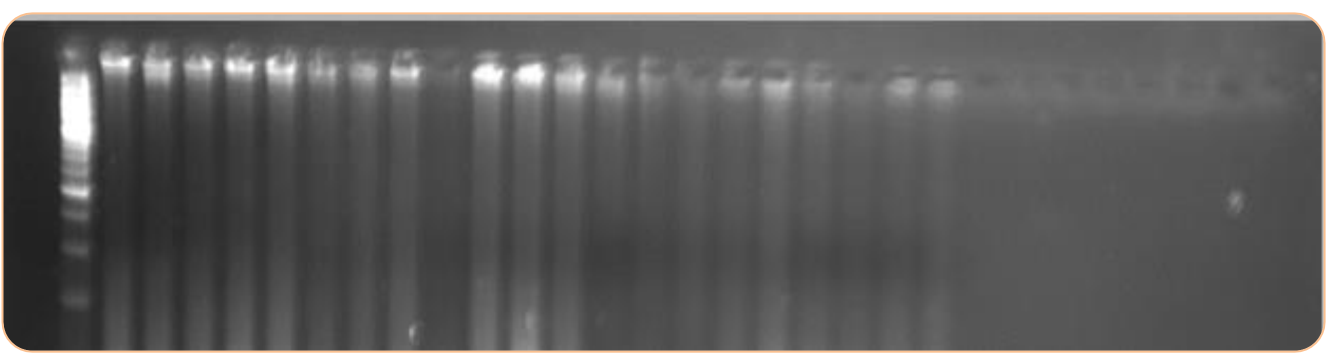

Figure 1. PCR amplification profile of the Sr2-linked marker stm598tcac in Pakistani wheat landraces. The expected 56 bp diagnostic fragment associated with the Sr2 gene was not detected in any accession. These results indicate that the Sr2-linked marker was absent under the present assay conditions, suggesting that resistance observed in certain accessions may be governed by alternative resistance loci.

Stem rust response at first observation

At first observation, few landraces exhibited resistant (R) reactions, while a moderate number were classified as moderately resistant (MR). The majority of accessions fell into the moderately susceptible (MS) group, indicating noticeable disease development. A smaller proportion showed susceptible (S) reactions (≥60% severity). Overall, resistant sources were limited, whereas most landraces displayed moderate to high susceptibility (Table 2).

Disease reaction of wheat landraces at second observation

At the second observation, only seven landraces (210904, 210898–210903, 220072) were resistant. Thirteen accessions were moderately resistant, and 29 were moderately susceptible. The majority of the remaining landraces exhibited high susceptibility (≥80S). Overall, resistant sources were limited, while most accessions showed moderate to high disease severity (Table 3).

Disease reaction at third observation

At the third observation, nine accessions (PI 210904, 210896, 210898-210903, and 220072) were classified as Resistant (R/10MR). Eleven accessions showed Moderate Resistance (20-30MR/MRMS), four were Moderately Susceptible (40MS), while the remaining accessions were Susceptible (≥60-100S) as shown in Table 4.

Table 2. Disease reaction of Pakistani wheat landraces to stem rust at first assessment

Group	Disease reaction	PI numbers (landraces)
Resistant (R)	R, 10R	182089, 182103, 182105, 210898-210904, 210900-210903, 220072, 220073-220075
Moderately Resistant (MR)	10-20MR, MRMS	182086–182088, 182090, 182106-182111, 189753, 193385, 210896, 210905-210906, 210908, 210913-210914, 250412-250413
Moderately Susceptible (MS)	30-50MS	40951, 40953, 181087, 182079, 182084, 182091-182098, 189743-189758, 193383, 193388-193389, 217544-217545, 219737-219749, 219752, 220071, 250236-250237, 250414, 250585-250586
Susceptible (S)	≥60S	182102, 182115-182124, 182126, 189739, 189744, 210907, 210909, 217546-217547, 218119, 219744-219748, 220076-220077, 250411, 250584

Table 3. Disease response classification of Pakistani wheat landraces at second assessment.

Group	Disease reaction	PI numbers (landraces)
Resistant (R)	R, 10MR	210904, 210898-210903, 220072
Moderately Resistant (MR)	20-30MR/MRMS	182089-182090, 182106, 193385, 210896, 210905, 210908, 210913-210914, 250412-250413
Moderately Susceptible (MS)	40-60MS	182086-182088, 182109-182111, 189753, 193383, 193388-193389, 210906-210907, 210915-210916, 217544-217545, 219737-219752, 220071, 250236-250237, 250414
Susceptible (S)	≥80S	Majority of remaining accessions including 40951, 40953, 182079, 182084, 182091-182098, 182102, 182115-182124, 182126, 189739, 189743-189744, 189757-189758, 217546-217547, 218119, 219744-219748, 220076-220077, 250411, 250584-250586

Table 4. Disease classification of Pakistani wheat landraces at the third assessment.

Group	Disease reaction	PI numbers (landraces)
Resistant (R)	R, 10MR	210904, 210896, 210898–210903, 220072
Moderately Resistant (MR)	20-30MR/MRMS	182089, 182090, 182106, 193385, 210902, 210905, 210908, 210913-210914, 250412-250413
Moderately Susceptible (MS)	40MS	210915, 210916, 217544, 250414
Susceptible (S)	≥60-100S	All remaining accessions

Discussion

This study integrated marker-assisted screening for the adult-plant resistance (APR) gene Sr2 with multi-stage field phenotyping to evaluate stem rust resistance in 86 Pakistani wheat landraces. The Sr2-linked marker (stm598tcac) was not detected in any accession, indicating that Sr2 is absent or extremely rare in this panel. This observation aligns with previous reports that Sr2 has primarily been incorporated into modern cultivars through targeted breeding rather than being widely conserved in traditional farmer-maintained germplasm (Bhavani et al., 2021; Yu et al., 2023). Because Sr2 confers partial, slow-rusting resistance rather than complete immunity, it is less likely to have been historically selected by farmers who favored visually apparent resistance and superior agronomic performance. Similar patterns have been documented in South Asian germplasm (Juliana et al., 2020; Singh et al., 2023).

Importantly, the absence of the Sr2 marker does not equate to a lack of stem rust resistance. Field evaluation at Jhuddo revealed considerable phenotypic variability, with a subset of landraces consistently expressing resistant to moderately resistant reactions across successive observations despite high disease pressure. This discrepancy between marker results and phenotypic performance suggests that resistance in these accessions is likely governed by alternative APR genes, minor-effect loci, or polygenic quantitative resistance rather than Sr2 alone. Comparable findings in diverse landrace panels indicate that slow-rusting phenotypes are frequently controlled by complex genetic architectures involving multiple loci with small to moderate effects (Kumar et al., 2019; Gao et al., 2024). The detection of stable partial resistance under natural infection conditions highlights the potential of these accessions as sources of non-race-specific resistance.

The predominance of susceptible reactions in later observations highlights the vulnerability of most landraces under intense epidemic pressure. Nevertheless, the few accessions maintaining low disease severity represent valuable genetic resources for breeding programs aimed at pyramiding quantitative resistance. Incorporating such germplasm could broaden the resistance base, reduce reliance on single major genes, and enhance durability against evolving stem rust races, including highly virulent lineages such as Ug99 (Singh et al., 2020; Hodson et al., 2022). These landraces may contribute novel alleles that complement established APR genes such as Sr2, Sr55/Lr67, and Sr57/Lr34 in future gene pyramids.

From a methodological perspective, marker-assisted detection is an efficient strategy for tracking Sr2; however, false negatives may arise due to recombination between the marker and gene or sequence polymorphisms at primer-binding sites (Li et al., 2021). The absence of authenticated Sr2-positive controls in this study constitutes a limitation and restricts definitive validation of marker performance. Future research should incorporate validated positive controls and multiple Sr2-linked markers to enhance diagnostic reliability. Integration of molecular screening with multi-environment phenotyping will provide a more robust framework for identifying durable resistance.

Overall, these findings emphasize the untapped potential of Pakistani wheat landraces as reservoirs of quantitative stem rust resistance, even in the apparent absence of Sr2. Comprehensive genomic approaches, including genome-wide association studies, QTL mapping, and functional validation, are warranted to elucidate novel APR loci underlying slow-rusting phenotypes. Strategic utilization of this germplasm in breeding pipelines will strengthen resilience against emerging stem rust races under changing climatic conditions and support long-term regional food security.

Authors' Contribution

MSS conceptualized and designed the study, performed the laboratory experiments, conducted data analysis, and prepared the original manuscript draft. ARJ and ARM supported the experimental design and assisted in laboratory experimentation. DKK and MHA contributed to data interpretation and provided critical revisions to the manuscript. LC and MJ assisted in data curation and manuscript editing. All authors reviewed and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 12: Responsible Consumption and Production
SDG 13: Climate Action

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