

Original Article

***AvrStb6* diversity and isoform variation in *Zymoseptoria tritici*, the fungal wheat pathogen from Golestan Province, Iran**Vala Rezvani¹ , Parissa Taheri² , Mohammad Javan-Nikkhah¹ , Naser Mohammadi³ , Amir Mirzadi Gohari¹ ✉¹Department of Plant Protection, Faculty of Agriculture, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran.²Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran³Dryland Agricultural Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Maragheh, Iran 10.22092/mi.2026.372598.1346**ABSTRACT**

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici*, is a major disease of wheat worldwide. The avirulence effector *AvrStb6* is central to the gene-for-gene interaction with the wheat resistance protein *Stb6*. In this study, the *AvrStb6* locus was analyzed in three isolates from Golestan Province, Iran. Sequencing identified two haplotypes that encoded two distinct protein isoforms of 82 amino acids. Structural modelling using SWISS-MODEL showed that many nonsynonymous substitutions were located in surface-exposed loops, potentially affecting effector–host interactions. Pathogenicity tests revealed a clear association between isoform sequence and virulence: an I01-like isoform produced very low pycnidial coverage on *Stb6*-containing wheat, whereas divergent isoforms produced high pycnidial coverage. Notably, the persistence of an I01-like isoform in Iran contrasts with global reports of its absence. Global phylogenetic analysis placed the Golestan haplotypes within Turkish–European lineages. These findings demonstrate rapid effector diversification in Golestan province and emphasize the importance of diversified resistance breeding for durable STB management.

KEYWORDSAvirulence, Effector isoforms, Phylogenetic analysis, Protein structure modelling, *Septoria tritici* blotch.**INTRODUCTION**

The co-evolutionary arms race between plants and pathogens has resulted in a complex relationship defined by millions of years of mutual selection. During infection, pathogens secrete effector proteins that alter host metabolism and decrease immune responses, enabling colonization (Djamei et al. 2011, Lo Presti et al. 2015). Plants, in turn, have evolved a complex immune system that includes surface and intracellular receptors, as well as resistance (R) genes capable of recognizing specific effectors and establishing effective defense responses (Jones and Dangl 2006, Cook et al. 2015, Kanyuka and Rudd 2019).

The recognition of a pathogen effector protein, termed an avirulence (Avr) factor, by a corresponding plant resistance (R) protein imposes strong directional selection on the pathogen. This pressure drives the pathogen to either acquire novel effectors or accumulate mutations within existing effector genes to evade host immunity. Pathogens can escape recognition through various mechanisms, including frameshift or nonsense mutations, transposon insertions, repeat-induced point mutations (RIPs), promoter modifications, epigenetic alterations, or point mutations that maintain protein functionality while avoiding recognition (Luderer et al. 2002, Rouxel et al. 2011, Zhang et al. 2015, Plissonneau et al. 2017, Wang et al. 2020). Effector genes are frequently situated in dynamic genomic areas abundant in transposable elements (TEs), facilitating their fast diversification (Dong et al. 2015).

Zymoseptoria tritici (*Dothideomycetes*) is a haploid, hemibiotrophic fungus with a genome of 13 core and 8 auxiliary chromosomes. It causes *Septoria tritici* blotch (STB), one of the world's most damaging wheat foliar diseases (Goodwin et al. 2011, Croll and McDonald 2012, McDonald et al. 2015). Under favourable conditions, STB can reduce

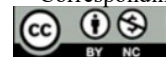
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photosynthesis, decrease grain filling, and induce premature leaf senescence, resulting in yield losses of 30-50% or more (Fones and Gurr 2015, Ghiasi Noei et al. 2022). Changes in agricultural practices (Bearchell et al. 2005, Dean et al. 2012, Wahdan et al. 2020), including the increased cultivation of semi-dwarf and rust-resistant wheat cultivars (Eyal et al. 1987, Karlsson et al. 2017), together with climate change, have increased the importance of this disease. Consequently, STB is now considered the third most important wheat disease worldwide (Yang et al. 2022), the most important wheat disease in Europe (Brennan et al. 2019), and the third most important in the United States (Ponomarenko et al. 2011). In Iran, this disease frequently occurs as regional epidemics during wet years in major wheat-producing provinces and leads to significant yield losses (Kia and Torabi 2008, Eslahi and Mojerlou 2018, Omrani et al. 2023). Khuzestan and Golestan are among the most important regions affected by the disease, although it has also been reported from Ardabil, Ilam, Lorestan, Fars, and Markazi (Kia et al. 2018). During infection, *Z. tritici* secretes a range of putative effectors, including the well-characterized avirulence effector AvrStb6. This small, cysteine-rich secreted protein is recognized by the wheat resistance protein Stb6, a wall-associated receptor-like kinase, consistent with a gene-for-gene interaction model (Brading et al. 2002, Zhong et al. 2017, Kema et al. 2018, Saintenac et al. 2018).

AvrStb6 was initially cloned from the reference isolates IPO323 (Goodwin et al. 2011) and 1E4 (Switzerland, 1999), while Stb6 was cloned from the wheat cultivar Cadenza and the landrace Chinese Spring (Kema et al. 2018, Saintenac et al. 2018). More than 50% of European commercial wheat cultivars carry functional Stb6 alleles, which have most likely existed in wheat populations for millennia (Chartrain et al. 2005, Saintenac et al. 2018). As a result, *Z. tritici* populations face substantial selective pressure to evolve *AvrStb6* variants that evade Stb6 recognition. High haplotype diversity at AvrStb6 has been found in global populations of *Z. tritici*, along with evidence of positive selection and rapid changes in haplotype frequencies (Brunner and McDonald 2018, Stephens et al. 2021). Although complete gene deletion has not been documented, the originally described avirulent isoform (I01) appears to have been extensively eliminated from contemporary populations. This suggests that AvrStb6 confers a significant, but yet unidentified, fitness advantage (Brunner and McDonald 2018).

Zymoseptoria tritici first emerged in the Middle East alongside wheat domestication, subsequently spreading to North Africa, Europe, the Americas, and Oceania (Stukenbrock et al. 2007, Feurtey et al. 2023). A population genomic analysis of over 1,000 genomes revealed eleven well-supported genetic clusters, which largely correspond to continental boundaries, with two unique clusters found in the Middle East (Feurtey et al. 2023). Effector diversification in *Z. tritici* is significantly influenced by transposable elements, which comprise 16.5–24% of the genome and are particularly enriched in subtelomeric regions (Sánchez-Vallet et al. 2018, Badet et al. 2020).

The genetic diversity of *AvrStb6* has recently been examined in Iran. A study of 78 isolates collected from East Azerbaijan and Ardabil provinces revealed high sequence diversification, multiple nonsynonymous mutations, and associations with virulence traits, placing Iranian populations in a distinct global context (Rad et al. 2023). However, nothing is currently known about the genetic diversity and evolutionary patterns of AvrStb6 in other major wheat-growing regions of Iran, such as Golestan Province.

This study presents a focused examination of AvrStb6 diversity, the structural implications of amino acid-altering mutations, and the phylogenetic placement of identified variants in *Z. tritici* isolates collected from Golestan Province, a major wheat-growing region in northern Iran. Using Sanger sequencing, we characterized three Iranian isolates, determined their *AvrStb6* haplotypes and corresponding protein isoforms, modelled the three-dimensional effects of non-synonymous substitutions, and integrated the local haplotypes into a global phylogenetic context. A clear association was identified between AvrStb6 isoform sequence and reproductive success on Stb6-containing wheat. Our findings provide new effector-level insights into *Z. tritici* populations in northern Iran and contribute to a better understanding of the adaptive dynamics shaping pathogenicity in this important wheat pathogen, particularly in regions of the Middle East that remain under-sampled.

MATERIALS AND METHODS

Fungal isolates

Three *Z. tritici* isolates (NM9, NR6, and NM10a) collected from naturally infected wheat fields in Golestan Province, Iran, were used in this study. These isolates were previously obtained and characterized by Esfehni et al. (2026). The reference isolate IPO323 (avirulent on Stb6-containing wheat) was included as a control. All isolates were recovered from single spores and cultured on potato dextrose agar (PDA) at 18 °C in the dark for 7–10 days. For long-term storage, blastospore suspensions were maintained in 30% (v/v) glycerol at –80 °C.

AvrStb6 amplification, sequencing, and sequence analysis

The complete *AvrStb6* locus, including the coding region and flanking untranslated regions (UTRs), was amplified using a pair of primers (AvrStb6-F and AvrStb6-R; Supplementary Table 1). The forward and reverse primers were located approximately 40–50 bp upstream and downstream of the *AvrStb6* coding region, respectively. PCR reactions were performed using Taq DNA Polymerase (New England Biolabs) in a total volume of 25 µL containing 1 µL of template DNA, 12.5 µL of Master Mix, 1 µL of each primer, and 9.5 µL of sterile distilled water (Daroodi et al., 2022). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 minutes; 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 45 seconds, and extension at 72 °C for 1 minute; and a final extension at 72 °C for 10 minutes. PCR products were purified and Sanger-sequenced in both directions using the same primers at

Pishgam Biotech Co., Tehran, Iran. Sequence chromatograms were manually checked and assembled using Geneious Prime (Biomatters Ltd.).

Sequence analysis, isoform determination, and protein structure modelling

Retrieved *AvrStb6* sequences from the three Golestan isolates (NM9, NR6, and NM10a) were edited and aligned using the MAFFT algorithm (Katoh et al., 2019) in Geneious Prime with default settings, with the IPO323 sequence as reference. The nucleotide sequences were translated into amino acid sequences to identify *AvrStb6* isoforms. Two distinct isoforms were detected among the three isolates. Nonsynonymous substitutions were identified by comparing the translated sequences to the reference I01 isoform. The impact of nonsynonymous substitutions on protein structure was predicted by generating three-dimensional models using SWISS-MODEL (Yang et al. 2023). Structural differences between isoforms were visualized and compared using PyMOL v2.5 (Schrödinger, Inc., USA) to assess potential effects on folding, surface properties, and disulfide bonds.

Pathogenicity assays

Pathogenicity of the three Golestan isolates and the reference isolate IPO323 was evaluated on wheat cultivars Shafir (carrying *Stb6*) and Tajan (susceptible check). Plants were grown under controlled greenhouse conditions (18/16 °C day/night, 70% relative humidity (RH) until the first leaf was fully expanded. Inoculum was prepared on yeast malt dextrose agar (YMDA) at a concentration of 1×10^6 spores mL⁻¹ in 0.15% Tween 20. Inoculation was performed by spraying the spore suspension until runoff, followed by 48 h at 100% RH and subsequent incubation at 18-25 °C with >90% RH, and a 16-hour photoperiod. Disease severity was assessed 21 days post-inoculation (dpi) by quantifying the percentage of leaf area covered by lesions (PLACL) and the percentage of leaf area covered by pycnidia (PLACP) using ImageJ software (Schneider et al. 2012). The experiment included three biological replicates. Data are presented as mean $\pm$ standard deviation (SD).

Global genetic diversity, haplotype network, and phylogenetic analysis

To contextualize the phylogenetic relationships of the three examined Iranian isolates with a global panel, 40 publicly available *AvrStb6* sequences were retrieved from NCBI GenBank, primarily from Stephens et al. (2021) and Curran et al. (2023). These sequences, together with the three Iranian isolates and reference isolate IPO323, formed a final dataset of 44 sequences. Multiple sequence alignment was performed using MAFFT in Geneious Prime (Biomatters Ltd.) with default settings. Genetic diversity parameters for the global dataset (n= 44) were calculated using DnaSP v5.10 (Librado and Rozas 2009), including the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and total number of mutations (Eta). Haplotype relationships were inferred using the TCS haplotype network algorithm in PopART v1.7 (Leigh and Bryant 2015). In the resulting network, each circle represents a unique haplotype, with circle size proportional to haplotype frequency, and hatch marks on branches indicate the number of mutational steps. A maximum likelihood phylogenetic tree was constructed in Geneious Prime 1.3 using the Tamura-Nei model with gamma-distributed rate variation among sites. Branch support was assessed with 1,000 bootstrap replicates, and the tree was rooted with the *AvrStb6* paralog *Mycgr3G82331* located on chromosome 10.

RESULTS

Sequence variation of the *AvrStb6* gene

The complete *AvrStb6* coding region (365 bp) was Sanger-sequenced from three *Z. tritici* isolates (NM9, NR6, and NM10a) collected from Golestan Province, Iran (Esfehani et al. 2026). An alignment of these sequences against the reference avirulent isolate IPO323 (I01) revealed two distinct haplotypes (H_IR1 and H_IR2) among the Iranian isolates (Fig. 1, Table 1). Isolate NM9 shared a nearly identical *AvrStb6* sequence with IPO323. In contrast, isolates NR6 and NM10a carried an identical haplotype that differed from IPO323 at 42 nucleotide positions. The 42 nucleotide differences were primarily located in exons 2 and 3, regions previously identified as hotspots for *AvrStb6* polymorphism (Stephens et al. 2021).

AvrStb6 isoforms and structural consequences of mutations

Translation of the nucleotide sequences yielded two protein isoforms, each 82 amino acids in length. A protein sequence alignment was performed using the reference avirulent isoform IPO323 (I01) (Fig. 2A). The protein sequence alignment of different *AvrStb6* isolates revealed significant differences between the Iranian isolates and the reference isolate IPO323. Isolate NM9 exhibited a high level of amino acid identity with IPO323, with only six amino acid substitutions. In contrast, isolates NM10a and NR6, despite showing complete sequence similarity to each other, displayed the highest number of variations with twenty amino acid substitutions compared to IPO323, including: I4V, L8F, R20K, V21A, S22K, G25S, I26V, K31A, A32R, D34Q, G41E, T42Y, Y50F, H59N, F60W, Q61K, N72T, V75T, I76V, and L77W. Among these variations, the substitution at position 41 (Glycine to Glutamic acid: G41E) in both isolates NR6 and NM10a is of particular significance due to the known importance of this site in the interaction of the *AvrStb6* protein with *Stb6* (Stephens et al. 2021). However, mutations at position 34 and 60, which are present in both isoforms, are considered important for evading recognition of the fungus by wheat cultivars carrying the *Stb6* resistance

gene (Brunner and McDonald, 2018), where NM9 carries D34A (Aspartic acid to Alanine) and F60Y (Phenylalanine to Tyrosine) substitutions, while NM10a and NR6 carry D34Q (Aspartic acid to Glutamine) and F60W (Phenylalanine to Tryptophan) substitutions. Furthermore, the presence of several consecutive substitutions in isolate NR6 within a region spanning positions V21A to D34Q, and the clustered mutations in isolate NM9 in the C-terminal region (positions 60 to 77), suggest these areas are susceptible to evolutionary changes and may potentially impact protein function. Position 43 remained unchanged in all investigated isolates. Three-dimensional models of the two Iranian isoforms were generated using SWISS-MODEL, with IPO323 (I01) as the reference. The models were superimposed in PyMOL. Structural comparison revealed that most nonsynonymous mutations are located in surface-exposed loops rather than in the helical core. Several substitutions, including 34, 41, 60, 61, and 75, appear to alter local surface charge and loop flexibility. Although the overall helical backbone remains relatively conserved, these surface changes may influence the effector's interaction with the host receptor Stb6 or its stability in the plant apoplast (Fig. 2B).

Pathogenicity assays

To assess virulence, the three Golestan isolates (NM9, NR6, and NM10a) were evaluated alongside the reference isolate IPO323 on the wheat cultivars Shafir (carrying Stb6) and Tajan (susceptible check). Disease assessments were carried out 21 days post-inoculation (dpi) by measuring the percentage of leaf area covered by lesions (PLACL) and the percentage of leaf area covered by pycnidia (PLACP). The experiment was performed with three biological replicates. On the susceptible cultivar Tajan, all isolates caused high disease severity. IPO323 produced a mean PLACL of $95.3\% \pm 1.5$ and PLACP of $90.3\% \pm 1.1$. The Golestan isolates also showed high aggressiveness on Tajan, with NM9 causing a mean PLACL of $95.8\% \pm 12.4$ and PLACP of $11.1\% \pm 0.9$, NR6 causing a mean PLACL of $96.8\% \pm 9.3$ and PLACP of $10.0\% \pm 0.8$, and NM10a exhibiting the highest pycnidial production with a mean PLACL of $98.9\% \pm 2.2$ and PLACP of $35.5\% \pm 0.7$. On the Stb6-containing cultivar Shafir, IPO323 was avirulent, causing only minimal necrosis (PLACL = $5.1\% \pm 0.4$) with no pycnidia formation. All three Golestan isolates caused extensive necrosis on Shafir, indicating that they can overcome Stb6-mediated resistance to initiate infection. However, they differed markedly in their ability to reproduce on this resistant cultivar. NM9 caused a PLACL of $83.4\% \pm 12.4$ with very low pycnidial production (PLACP = $0.33\% \pm 0.06$). In contrast, NR6 and NM10a produced both high necrosis ($90.7\% \pm 9.3$ and $98.9\% \pm 0.4$, respectively) and moderate to high pycnidial coverage (PLACP = $8.8\% \pm 0.5$ and $7.8\% \pm 0.1$), respectively (Fig. 3).

Global genetic diversity, haplotype network, and phylogenetic analysis

To place the Golestan isolates in a global context, 40 publicly available *AvrStb6* sequences were retrieved from NCBI GenBank and analyzed together with the reference isolate IPO323 (I01). The combined dataset of 44 sequences contained 67 polymorphic sites, with a haplotype diversity (Hd) of 0.914, a nucleotide diversity (Pi) of 0.05822, and a total number of mutations (Eta) of 82. Haplotype analysis revealed 23 distinct haplotypes worldwide. The two Golestan haplotypes clustered with sequences originating from Turkey and the United Kingdom. A maximum-likelihood phylogenetic tree was constructed using RAxML with 1,000 bootstrap replicates, rooted with the *AvrStb6* paralog *Mycgr3G82331* on chromosome 10 (Fig. 4). The tree showed that the Golestan haplotypes were nested within Turkish-European lineages. This topology is consistent with previously described genetic relationships among *Z. tritici* populations from the Middle East and Europe. A TCS haplotype network was also constructed and was largely congruent with the phylogenetic tree (Fig. 5).

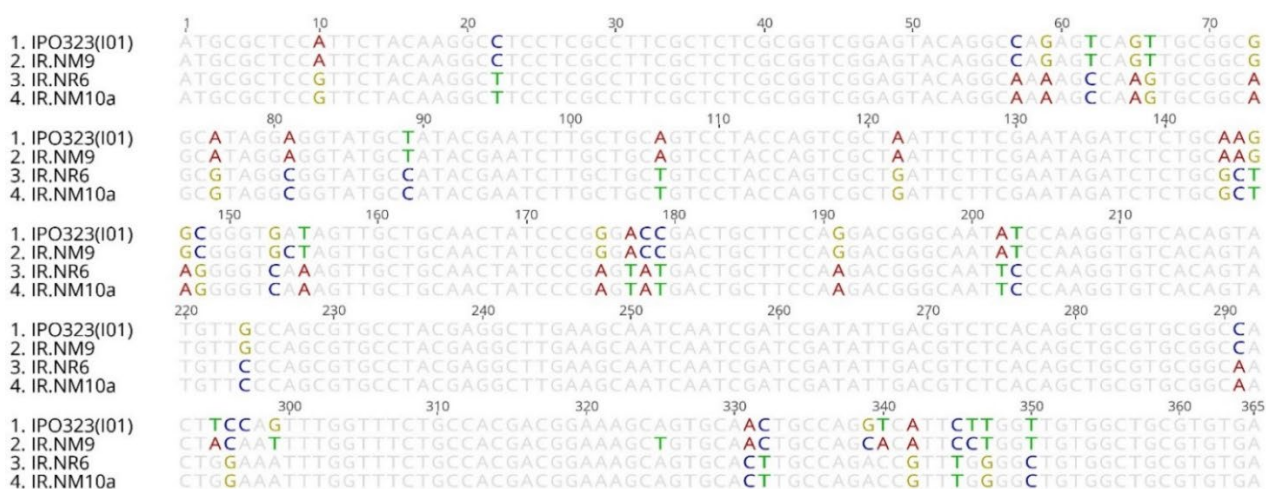
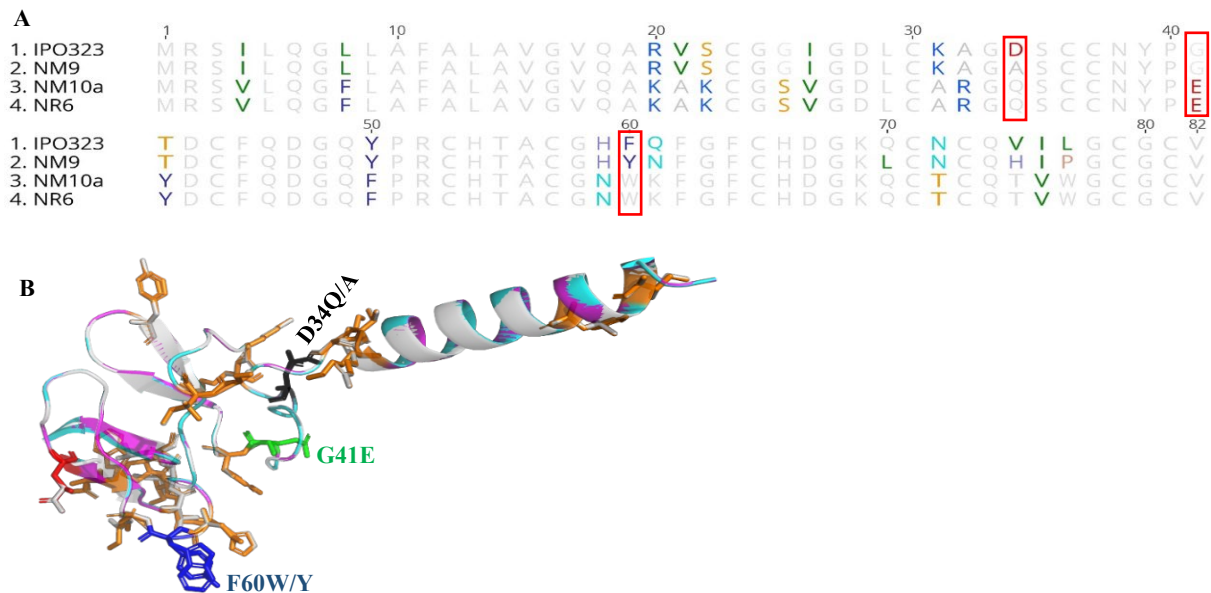


Fig. 1. Alignment of the *AvrStb6* coding region (365 bp). The reference avirulent isolate IPO323 (I01) is shown at the top, followed by Iranian isolates NM9, NR6, and NM10a. Identical nucleotides are indicated in gray. The alignment was generated using Geneious Prime software.

Table 1. *AvrStb6* haplotypes and GenBank accession numbers for three *Zymoseptoria tritici* isolates from Golestan Province, Iran. Isolate NM9 represents Haplotype 1 (H_IR1). Isolates NR6 and NM10a share Haplotype 2 (H_IR2).

Isolate	Haplotype	GenBank accession
NM9	H_IR1	PZ302446
NR6	H_IR2	PZ302447
NM10a	H_IR2	PZ302448

**Fig. 2.** (A) Protein Sequence Alignment of AvrStb6 Isolates. Amino acid sequences of Iranian isolates NM9, NR6, and NM10a were aligned with the reference avirulent isolate IPO323 (I01). Isolate NM9 exhibited a high level of amino acid identity with IPO323, with only six amino acid substitutions. Isolates NM10a and NR6 display twenty substitutions compared to IPO323. Key residues at positions 34, 41, and 60, critical for Stb6 recognition, are highlighted. (B) Three-dimensional superimposition of the AvrStb6 isoforms identified in *Z. tritici* isolates from Golestan Province together with the reference isoform I01 from IPO323. IPO323 is shown in gray, NM10a in yellow, NM9 in magenta, and NR6 in cyan. Key nonsynonymous mutation sites are highlighted as sticks (mutations in NM9 are shown in red, while those in NR6 and NM10a are shown in orange). The functionally important G41E substitution in NR6 and NM10a is specifically indicated in green, whereas the F60W/Y and D34Q/A substitutions are shown in black and blue, respectively, across NR6, NM10a, and NM9. Models were generated using SWISS-MODEL and visualized in PyMOL. Most substitutions are located in surface-exposed loop regions rather than in the helical core, suggesting possible effects on local surface charge, loop flexibility, and interaction with the host receptor Stb6.

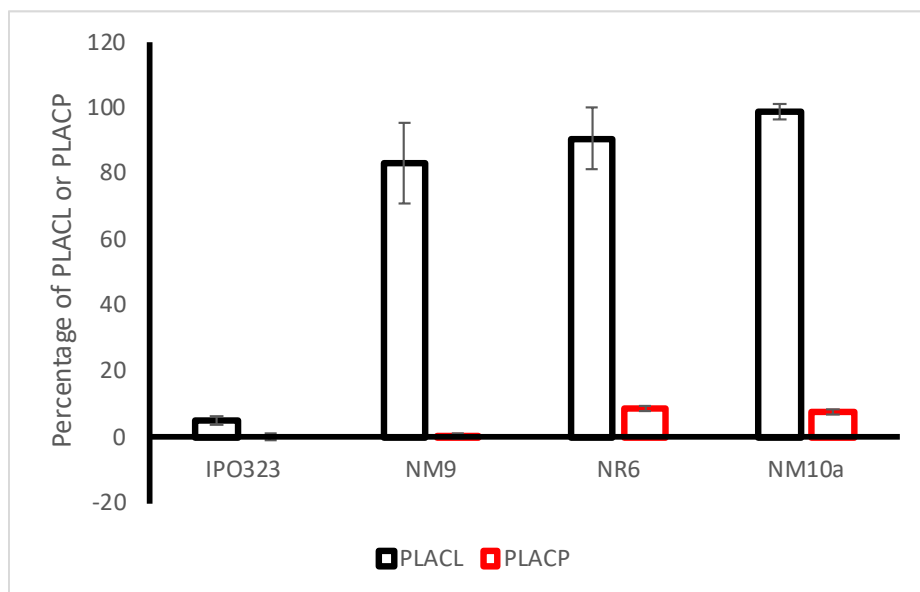
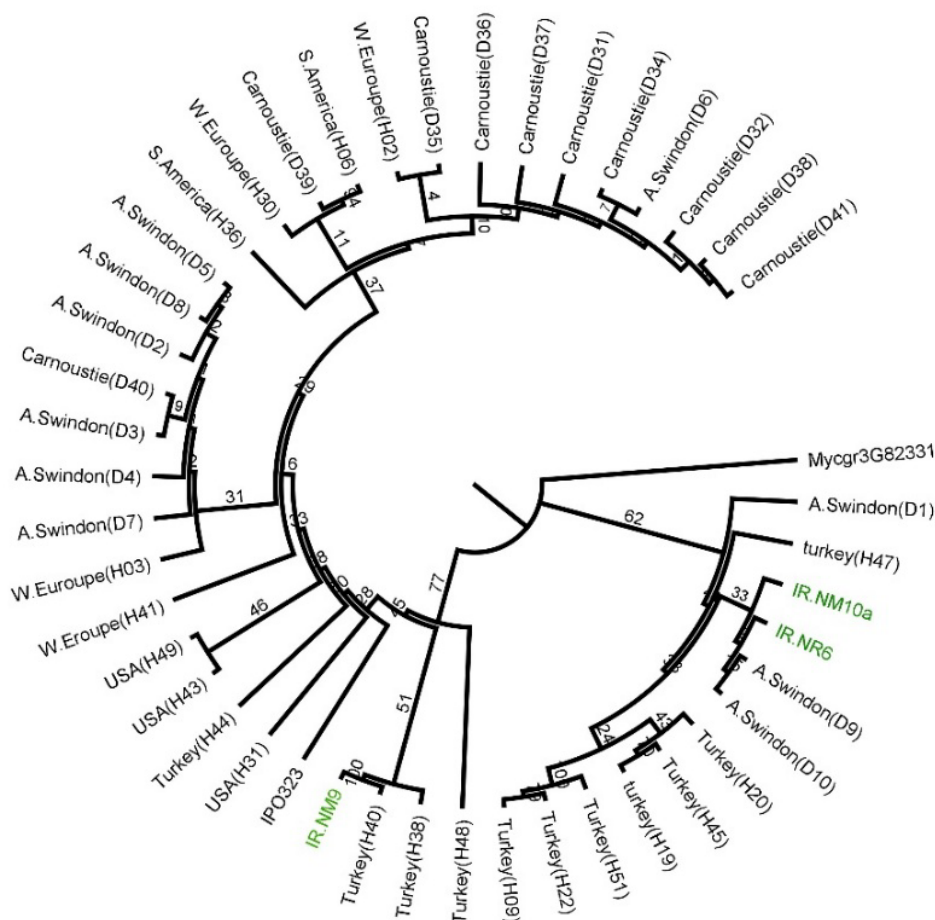


Fig. 3. Mean percentage of leaf area covered by lesions (PLACL) and percentage leaf area covered in pycnidia (PLACP) caused by the reference isolate IPO323 and three *Zymoseptoria tritici* isolates from Golestan Province, Iran (NM9, NR6, and NM10a), on wheat cultivar Shafir (carrying the *Stb6* resistance gene). Error bars represent the standard deviation from three biological replicates. All three Golestan isolates caused extensive necrosis, but isolate NM9 produced markedly lower pycnidia compared to NR6 and NM10a.



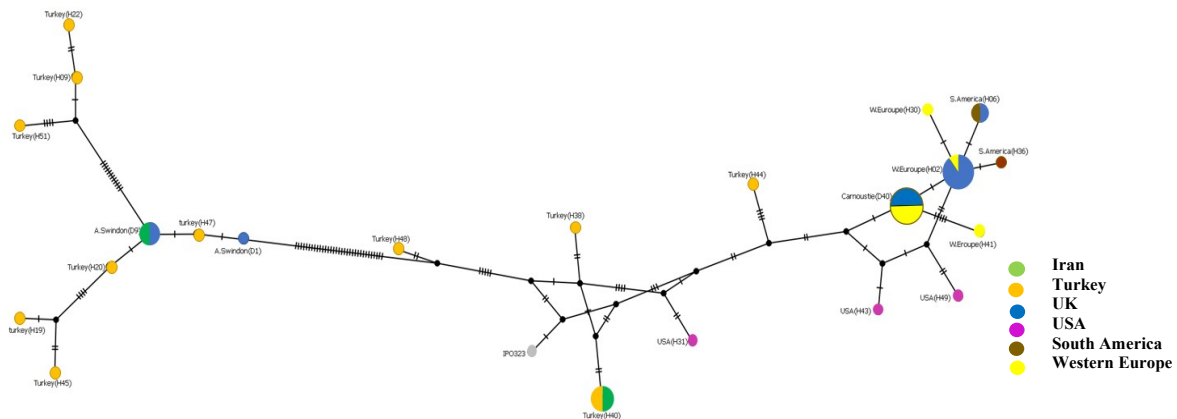


Fig. 5. TCS (statistical parsimony) haplotype network of the *AvrStb6* gene constructed from 44 sequences, including three Iranian isolates from Golestan Province, Iran, reference isolate IPO323, and 40 global sequences retrieved from NCBI GenBank. The nodes are painted in a variety of colors based on the geographic region(s) from which the isolates of the same node were first identified. Each circle represents a unique haplotype, with circle size proportional to the number of isolates. Hatch marks on branches indicate the number of mutational steps. The two Golestan haplotypes (H2, represented by NM9; H15, represented by NR6 and NM10a) are highlighted in green. The network shows clustering of Golestan haplotypes with Turkish and UK sequences.

DISCUSSION

The present study provides a focused yet in-depth characterization of AvrStb6 effector diversity in *Z. tritici* isolates from Golestan Province, northern Iran. Although based on only three isolates, the results uncover substantial effector variation: two haplotypes that translate into two distinct protein isoforms of 82 amino acids, differing by 21–22 non-synonymous substitutions. Structural modelling using SWISS-MODEL, followed by PyMOL visualization, revealed that the majority of these substitutions are located in surface-exposed loops rather than the conserved helical core.

Several amino acid substitutions identified in the Iranian isolates (NM9 and NR6), including NR6-specific changes (I4V, L8F, R20K, V21A, S22K, G25S, I26V, K31A, A32R, D34Q, G41E, T42Y, Y50F, H59N, F60W, Q61K, N72T, V75T, I76V, and L77W) and NM9-specific mutations (D34A, F60Y, Q61N, Q70L, V75H, L77P), are predominantly located in surface-exposed regions of the protein. Structural modeling suggests that these substitutions may collectively influence local surface charge distribution and loop flexibility and potentially modulate the effector–host interaction interface. These structural alterations suggest that even limited sequence variation can generate functionally relevant isoform diversity in local populations.

Consistent with previous studies, in addition to the key mutation at position 41 (Stephens et al. 2021), amino acid substitutions at the major sites 34 and 60 play important roles in the interaction between this fungus and wheat lines carrying the Stb6 resistance gene. These sites are considered to be under diversifying selection (Brunner and McDonald 2018). Furthermore, a study conducted in 2018 demonstrated that these positions represent primary targets initially selected for evasion of recognition by wheat cultivars carrying the Stb6 resistance gene. Subsequently, compensatory mutations at positions associated with these sites (including positions 43, 59, and 61) are suggested to mitigate potential fitness costs associated with reduced effector functionality (Brunner and McDonald 2018). It is important to note that no experimentally resolved crystal structure of AvrStb6 is currently available, limiting direct structural interpretation of residue function. Consequently, the functional relevance of specific amino acid positions has mainly been inferred from structural modeling, comparative sequence analyses, and evolutionary or mutational studies. Brunner and McDonald (2018) applied Bayesian Graphical Models (BGM) to identify co-evolving amino acid sites potentially associated with host-driven selective pressures, highlighting positions 34 and 60 as candidate functionally important residues. In addition, previous studies by Kema et al. (2018) and Stephens et al. (2021) identified substitutions at positions 41 and 43 as being associated with changes in host recognition and resistance breakdown. Therefore, the putative functional importance of the positions identified in the present study is supported by convergent evidence from independent computational and comparative analyses, although direct structural and biochemical validation is still lacking.

A key finding of this study is the association between *AvrStb6* sequence variation and virulence on Stb6-containing wheat. Isolate NM9, which carried an I01-like isoform closely related to the avirulent reference IPO323, produced very

low pycnidial densities on the resistant cultivar Shafir. In contrast, isolates NR6 and NM10a, which carried divergent isoforms with 21–22 nonsynonymous substitutions relative to I01, produced high pycnidial densities on the same resistant cultivar. This association suggests that the observed amino acid substitutions, particularly those in surface-exposed loops, may enable evasion of Stb6-mediated recognition while preserving effector function.

However, the observed associations between specific *AvrStb6* haplotypes or mutations and phenotypic variations (such as virulence and pycnidiation levels) in *Z. tritici* isolates should be interpreted as correlations rather than definitive causation. Pathogenicity and pycnidium formation are highly complex, polygenic traits governed by a vast genomic network of virulence factors in *Z. tritici*. While *AvrStb6* plays a pivotal role in triggering Stb6-mediated resistance, other effector genes, redundant cellular pathways, or the overall genetic background likely contribute to the final aggressive phenotype and reproductive fitness of these isolates. Therefore, future functional validation, such as gene knock-out or allele-swapping experiments, remains essential to definitively isolate and confirm the precise contribution of individual *AvrStb6* mutations to these quantitative pathogenic traits. Furthermore, although *AvrStb6* and *Stb6* represent a classic genetically defined gene-for-gene pair, it is important to note that a direct physical interaction between these two proteins has not been biochemically proven. Previous yeast two-hybrid (Y2H) assays failed to detect direct binding between *AvrStb6* and either the full-length *Stb6* protein or its isolated domains (Kema et al., 2018). Thus, the recognition of *AvrStb6* by *Stb6* might not occur through direct receptor-ligand binding, but rather via an indirect mechanism. This could involve the monitoring of host 'guardee' or 'decoy' proteins by *Stb6*, or the participation of additional, yet-unidentified host co-receptors or components in a multi-protein recognition complex. To definitively resolve this ambiguity and fully map the interactome, the application of targeted molecular techniques, such as co-immunoprecipitation (Co-IP) assays, will be essential in future investigations. Therefore, our references to this interaction throughout this study imply functional and genetic recognition rather than demonstrated physical binding. Notably, isolate NM9 carried an *AvrStb6* isoform closely related to the avirulent reference I01, which was reported to be absent from global populations sampled between 2013 and 2017 (Stephens et al. 2021). The persistence of an I01-like isoform in Golestan Province suggests that Iran may represent a refuge for avirulence alleles, possibly due to different wheat cultivation practices (e.g., use of landraces) or lower deployment of *Stb6*-containing cultivars. All three Golestan isolates caused necrotic lesions on *Stb6* wheat, but they differed markedly in pycnidial production. The absence of premature stop codons or complete gene deletion, even in the highly virulent isoforms, supports the hypothesis that *AvrStb6* retains an essential but still unknown fitness function beyond its avirulence role (Brunner and McDonald 2018, Sánchez-Vallet et al. 2018). Recent functional work has further shown that *AvrStb6* accumulates near stomata and triggers localized defense responses (Ghiyasi Noei et al. 2022), implying that the surface mutations observed here may fine-tune recognition while preserving core activity.

When placed in a global context, the two Golestan haplotypes clustered with Turkish and UK (A. Swindon) lineages in both the TCS haplotype network and the RAxML phylogenetic tree (rooted with the *AvrStb6* paralog Mycgr3G82331). This positioning is consistent with previously described genetic connections between Middle Eastern and European *Z. tritici* populations, supporting the known origin of the pathogen in the Middle East and its subsequent long-distance dispersal (Stukenbrock et al. 2007, Feurtey et al. 2023). Importantly, this study is among the first in Iran to move beyond basic haplotype detection and examine *AvrStb6* at the isoform and structural level in isolates from Golestan Province. Previous Iranian research has mainly focused on virulence profiling or general sequence diversity (Mirzadi Gohari et al. 2015, Rad et al. 2023, Esfehiani et al. 2026), whereas the present work links sequence variation directly to predicted protein structure and potential functional consequences.

From a practical breeding perspective, the observation that an I01-like isoform still exists in Iran raises the possibility that *Stb6* may still be effective against some local *Z. tritici* genotypes. However, the presence of highly virulent isolates (NR6, NM10a) in the same region indicates that *Stb6*-mediated resistance alone is unlikely to be durable. The structural changes observed in the virulent isoforms could allow the pathogen to maintain effector function while evading recognition, a scenario already documented in European populations (Brunner and McDonald 2018, Stephens et al. 2021). These results reinforce the need for gene pyramiding strategies that combine *Stb6* with other major *Stb* genes and quantitative resistance loci (Brown et al., 2015).

To understand the remarkable diversity of *AvrStb6* in Iran, we must examine the evolutionary histories of the host and the pathogen. Iran lies within the Fertile Crescent, the historic cradle of wheat domestication. Because wild and domesticated wheat relatives have co-existed with *Z. tritici* in this region for millennia, Iran represents a major center of origin and genetic diversity for both species. This long history of co-evolution has allowed the pathogen to continuously adapt to ancestral and modern host resistance genes, including *Stb6*. The selection pressure imposed by these diverse host backgrounds over thousands of years—coupled with the modern deployment of *Stb6*-containing cultivars—has likely driven the accumulation of the numerous haplotypes we observed. This deep evolutionary reservoir makes the Iranian *Z. tritici* population highly dynamic and capable of rapidly generating virulent variants. Consequently, tracing these effector dynamics in the Fertile Crescent is not only crucial for local agriculture but also provides essential clues about how virulence evolves globally.

While this investigation operates primarily as an initial case study rather than an exhaustive, population-wide screen due to the limited number of extensively characterized isolates, the integration of sequence analysis, isoform identification, protein structure modelling, pathogenicity testing, and global phylogenetic reconstruction offers a coherent preliminary picture of *AvrStb6* evolution in Golestan Province. Future studies should expand sampling across more

regions of Iran and include functional validation (e.g., agroinfiltration or yeast two-hybrid assays) to determine how the identified mutations affect effector activity and host interaction. Such information will be critical for designing durable resistance against an evolving pathogen in the face of changing climate and agricultural practices.

CONCLUSION

In conclusion, this study demonstrates that AvrStb6 in *Z. tritici* isolates from Golestan Province, Iran, exists as two distinct protein isoforms with multiple surface-exposed nonsynonymous substitutions. A key finding is the association between isoform sequence and virulence: the I01-like isoform (NM9) produced very low pycnidial densities on Stb6-containing wheat, whereas divergent isoforms (NR6 and NM10a) produced high pycnidial densities. Notably, the persistence of an I01-like isoform in Iran contrasts with global reports of its absence (Stephens et al. 2021), suggesting that Iran may represent a refuge for avirulence alleles. Phylogenetic clustering with Turkish and UK lineages is consistent with previously described genetic connections between Middle Eastern and European populations. These findings provide new insight into the molecular evolution of a key effector in an important wheat-growing region of Iran and underscore the urgent need for diversified resistance breeding strategies to sustain effective management of the STB disease.

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AUTHOR CONTRIBUTION

Vala Rezvani: Performed all *in Planta* Assays, Bioinformatic Analyses, and Data visualization, Analyzed the data, and wrote the original draft manuscript; Parissa Taheri: Provided scientific comments and revised the manuscript; Mohammad Javan-Nikkhah: Provided scientific comments and revised the manuscript; Naser Mohammadi: Provided scientific comments and revised the manuscript; Amir Mirzadi Gohari: Conceptualized and designed the study, coordinated the research, interpreted the results, and revised the manuscript. All authors reviewed the manuscript and agreed to the submission.

DATA AVAILABILITY

The *AvrStb6* nucleotide sequences generated in this study have been deposited in GenBank under accession numbers PZ302446, PZ302447, and PZ302448. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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DECLARATION

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

Not applicable.

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تنوع و تفاوت‌های ایزوفرمی AvrStb6 در قارچ بیماری‌زای گندم *Zymoseptoria tritici* از استان گلستان، ایران

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چکیده

بیماری لکه برگ‌گی سپتوریایی گندم (STB)، که توسط قارچ *Zymoseptoria tritici* ایجاد می‌شود، یکی از مهم‌ترین بیماری‌های گندم در سراسر جهان است. افکتور غیر بیماری‌زا AvrStb6 نقش مهمی در برهم‌کنش ژن-در برابر-ژن با پروتئین مقاومت Stb6 در گندم دارد. در این مطالعه، جایگاه AvrStb6 در سه جدایه از استان گلستان-ایران بررسی شد. توالی‌یابی این جدایه‌ها وجود دو هاپلوتایپ را نشان داد که به دو ایزوفورم پروتئینی متمایز، هر یک با طول ۸۲ اسید آمینه ترجمه می‌شوند. مدل‌سازی ساختاری با استفاده از SWISS-MODEL نشان داد که بسیاری از جایگزینی‌های غیرهم‌معنی در لوپ‌ها و در سطح قرار دارند و احتمالاً بر برهم‌کنش افکتور-میزبان تأثیر می‌گذارند. آزمون‌های بیماری‌زایی ارتباط روشنی بین توالی ایزوفورم و بیماری‌زایی نشان دادند؛ به‌طوری‌که یک ایزوفورم مشابه I01 پوشش پیکنید بسیار پایینی را روی گندم‌های دارای Stb6 ایجاد کرد، در حالی که ایزوفورم‌های واگرا، پوشش پیکنید بالایی را تولید کردند. نکته قابل توجه این است که پایداری یک ایزوفورم مشابه I01 در ایران با گزارش‌های جهانی مبنی بر عدم حضور آن در تضاد است. تحلیل فیلوژنتیکی جهانی نیز هاپلوتیپ‌های گلستان را درون دودمان‌های ترکی-اروپایی قرار داد. این یافته‌ها تنوع‌یابی سریع افکتورها در استان گلستان را نشان می‌دهد و اهمیت استفاده از راهبردهای متنوع اصلاحی برای دستیابی به مدیریت پایدار بیماری STB را برجسته می‌کند.

واژگان کلیدی: ایزوفورم‌های افکتور، آنالیز فیلوژنتیکی، لکه برگ‌گی سپتوریایی گندم، مدل‌سازی ساختار پروتئین، *Septoria tritici*.