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# Allelic variation of the AvrSr50 effector increased virulence of the *Puccinia graminis* f. sp. *tritici* on *Sr50*-carrying wheat lines

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**Abstract**

Wheat stem rust, caused by the biotrophic pathogen *Puccinia graminis* f. sp. *tritici* (Pgt), is an important disease worldwide. To overcome host immunity, Pgt secretes several effectors, including AvrSr35 and AvrSr50. These effectors are recognized by resistant cultivars carrying corresponding receptors, resulting in incompatible reactions. In this study, we assessed the resistance of *Sr35*- and *Sr50*-carrying wheat monogenic lines to 30 Pgt isolates through inoculation with urediniospores of pure isolates. Virulence analyses were evaluated for resistant IF 0-2 and susceptible 3-4 reactions and revealed that Pgt20-6 and Pgt20-12 isolates showed increased virulence on the *Sr50*-carrying line. The presence of *AvrSr35* and *AvrSr50* genes was assessed in the genome of all isolates through PCR. Visualization of amplified fragments on agarose gel confirmed the presence of *AvrSr35* and *AvrSr50* genes in the genome of all 30 isolates. Despite the presence of Avr genes in all isolates, the lack of active recognition for two isolates on the *Sr50*-carrying line suggested point mutations in their sequence. Sequencing analysis of *AvrSr50* confirmed an A178T mutation unique to these two virulent isolates. Homology modelling based on the solved crystal structure of AvrSr50 showed a nonsynonymous mutation at the protein surface that may be involved in the interaction with Sr50 to escape from recognition. This study showed the constant genetic change of fungus and recalled the necessity of continuously monitoring the fungal population to take appropriate strategies in wheat breeding programs.

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## Introduction

Wheat (*Triticum aestivum*) is one of the main grains due to its nutritional value for humans. Wheat production is about 750 million tons worldwide, and the estimated for wheat requirement by 2050 is 56% increase of current production (Guarin et al., 2022). Wheat production dealed with various biotic and abiotic stresses that cause considerable yield losses, annually (Olayinka Bolaji et al., 2021). Among them, wheat stem rust disease caused by *Puccinia graminis* f. sp. *tritici* (Pgt) is widespread that severely damages the host plant in favorable condition. This fungus is an obligate biotrophic pathogen and is dependent on its host for reproduction. To successfully overcome host immunity, Pgt secretes a large repertoire of virulence factors (effectors) (Hofer, 2018) that are secreted from the specialized haustoria during host colonization (Garnica, et al., 2014), *AvrSr35* and *AvrSr50* effector genes have been discovered through comparative genomics of wild type and mutants of Pgt isolates (Andres Salcedo, 2017; Jiapeng Chen, 2017). In 2021, *AvrSr27* also was discovered by genomic analysis of mutants and their natural parental isolate (Upadhyaya et al., 2021). More recently, *AvrSr13* and *AvrSr22* were discovered based on the transient wheat protoplast transformation (Arndell et al., 2024; Lubega et al., 2024); however, the function of all these effectors is still unknown. Consistent with the gene for gene relationship, wheat cultivars carrying resistance genes recognize their corresponding effectors leading to incompatible interaction. Due to sexual reproduction and genetic mutations, this pathogen is

able to frequently generate new races to escape from host recognition (Patpour et al., 2022). Effective mutations at the interface of both proteins ruin the host recognition leading to gain of virulence in individuals of fungus (Jiapeng Chen, 2017).

In this study, various isolates that were virulent on monogenic lines carrying other Sr genes were evaluated for the presence of genes encoding *AvrSr35* and *AvrSr50* as well as their virulence on monogenic lines carrying *Sr35* and *Sr50* genes. These R genes encode cytoplasmic receptors that activate host defense upon recognition of their cognate fungal effectors AvrSr35 and AvrSr50, respectively (Andres Salcedo, 2017; Jiapeng Chen, 2017). These R genes are an important source as they confer resistance to a wide range of Pgt isolates, including the Ug99 race group (Luo et al., 2021; Saintenac et al., 2013). Therefore, it is crucial to monitor the genetic change of fungus to manage farming programs to prevent the breakdown of resistance to the devastating Pgt isolates in the field. AvrSr50 is shown to escape from host recognition through DNA insertion, stop codon loss or by amino-acid substitution at its interacting site with Sr50 (Ortiz et al., 2022). AvrSr35 also directly interacts with Sr35 (Zhao et al., 2022) and similarly, removing or inactivating these effectors through mutations leaves the plant defenseless and susceptible to Pgt (Andres Salcedo, et al., 2017). In this study, we assessed the virulence of 30 Pgt isolates on lines carrying *Sr35* and *Sr50* genes. Despite the presence of effector genes in all isolates, two isolates showed increased virulence on *Sr50*-carrying lines, suggesting that AvrSr50 was not (properly) recognized by the corresponding receptor.

## Material and Methods

### Collection of wheat stem rust isolates

During June-July 2020, stem rust samples were collected from wheat fields in the north, west and northwest of Iran, including Mazandaran, Golestan, Ardabil, Hamedan, Lorestan and East Azerbaijan provinces. The geographical data including latitude, longitude and altitude as well as the date were recorded for each sample (Table 1). Wheat stems containing developed uredia were used for purification and propagation of isolates.

**Table 1.** Date and geographical location of collected Pgt isolates. Samples were collected at 2020. Geographical location of samples was indicated in three separate columns.

| Isolate | Year | location | Latitude | Longitude |
|---|---|---|---|---|
| Pgt20-1 | 2020 | Mazandaran | 36.61961787 | 53.24757337 |
| Pgt20-2 | 2020 | Mazandaran | 36.69047455 | 53.45640099 |
| Pgt20-3 | 2020 | Mazandaran | 36.71806725 | 53.71491677 |
| Pgt20-4 | 2020 | Golestan | 36.7747739 | 54.02812366 |
| Pgt20-5 | 2020 | Golestan | 36.81113697 | 54.16549373 |
| Pgt20-6 | 2020 | Golestan | 36.81123598 | 54.26649373 |
| Pgt20-7 | 2020 | Golestan | 36.82573499 | 54.26306802 |
| Pgt20-8 | 2020 | Golestan | 36.85204065 | 54.52057592 |
| Pgt20-9 | 2020 | Lorestan | 33.43210552 | 49.45595012 |
| Pgt20-10 | 2020 | Hamedan | 34.87909749 | 48.53954011 |
| Pgt20-11 | 2020 | Hamedan | 34.94205744 | 48.55843577 |
| Pgt20-12 | 2020 | Hamedan | 34.84106733 | 48.56744568 |
| Pgt20-13 | 2020 | Hamedan | 35.02636871 | 48.53498161 |
| Pgt20-14 | 2020 | Hamedan | 35.07787146 | 48.63222511 |
| Pgt20-15 | 2020 | Hamedan | 35.07667791 | 48.67855379 |
| Pgt20-16 | 2020 | Hamedan | 37.26606827 | 47.23983118 |
| Pgt20-17 | 2020 | Hamedan | 37.95542517 | 47.05119621 |
| Pgt20-18 | 2020 | Hamedan | 37.94872449 | 47.10035873 |
| Pgt20-19 | 2020 | East Azarbayjan | 38.27170775 | 48.31434639 |
| Pgt20-20 | 2020 | East Azarbayjan | 38.2768584 | 48.31909921 |
| Pgt20-21 | 2020 | Ardebil | 38.28763139 | 48.33046234 |

| | | | | |
|---|---|---|---|---|
| Pgt20-22 | 2020 | Ardebil | 38.33218891 | 48.54746261 |

**Table 1.** (*continued*)

| Isolate | Year | location | Latitude | Longitude |
|---|---|---|---|---|
| Pgt20-23 | 2020 | Ardebil | 38.31984629 | 48.55272856 |
| Pgt20-24 | 2020 | Ardebil | 38.24736766 | 48.39154022 |
| Pgt20-25 | 2020 | Ardebil | 38.25937017 | 48.47955935 |
| Pgt20-26 | 2020 | Ardebil | 38.21556932 | 48.3287213 |
| Pgt20-27 | 2020 | Golestan | 37.08610204 | 55.21903664 |
| Pgt20-28 | 2020 | Golestan | 37.08132025 | 55.25344233 |
| Pgt20-29 | 2020 | Golestan | 37.08132025 | 55.25344223 |
| Pgt20-30 | 2020 | Golestan | 37.17232025 | 55.35343853 |

## Purification and propagation of uredospores of Pgt

Based on the universal protocols (Olivera Firpo et al., 2017), to purify and amplify Pgt isolates, stem rust pustules obtained from collected samples were inoculated onto a newly established susceptible wheat cultivar known as Morocco. Seeds of this cultivar were subsequently planted in beds composed of peat moss. The seedlings were cultivated within a phytotron environment maintained at a temperature of 25 °C and a relative humidity range of 80-90%. At the single-leaf developmental stage, the seedlings were inoculated with Pgt pustules derived from the collected samples and were then incubated in a dark environment at 18 °C with 100% humidity for a duration of 20 hours, followed by an incubation period at 23±2 °C with 80-90% humidity under a photoperiodic lighting regime of 16 hours of light and 8 hours of darkness for two weeks. At 14 days post-inoculation (dpi), a single pustule was selected from each isolate, and new seedlings were subsequently inoculated. To ensure the integrity and purity of the isolates, this inoculation process was repeated three to four times. Urediniospores from the pure isolates were amplified under the same conditions outlined previously, and the harvested urediniospores were desiccated in a desiccator containing silica gel at a temperature of 4°C for a period of 48 hours. The collected urediniospores were then stored in individual microtubes at -80°C for future analytical procedures.

## Phenotyping of Pgt isolates on monogenic wheat lines carrying *Sr35* and *Sr50*

To determine the virulence of isolates, lines carrying one of stem rust resistance genes *Sr35* and *Sr50* were used. Seedlings at the first leaf stage were inoculated with spores of each isolate. Conditions for plant growth, inoculation and incubation of differential lines was comparable to those of purification section as stated above. Infection types were assessed at 14 dpi based on the 0 to 4 scale (Stakman et al., 1944). Infection types; 0 to 2 were regarded as incompatible reaction, i.e., type 0 (immune or fleck), 1 (small uredia with necrosis) and 2 (small to medium uredia with chlorosis or necrosis) were resistant. Compatible reactions include infection types; 3 (medium-size uredia with/without chlorosis) and 4 (large uredia without chlorosis or necrosis) were regarded as susceptible (Fig. 1). The variations were refined by modifying characters as follows: -, uredia somewhat smaller than normal for the infection type; +, uredia somewhat larger than normal for the infection type. To assess the virulence of the isolates, lines carrying either the stem rust resistance genes *Sr35* or *Sr50* were employed. Seedlings at the initial leaf developmental stage were subjected to inoculation with spores derived from each isolate. The conditions established for plant growth, inoculation, and the incubation of differential lines were consistent with those delineated in the purification section. The categorization of infection types was conducted at 14 days post-inoculation (dpi) utilizing a scale ranging from 0 to 4, as previously described (Stakman et al., 1962).

## DNA extraction

Seedlings of susceptible cultivar Morrocco containing stem rust pustules were harvested and ground in Chinese mortars using liquid nitrogen. Using a modified method described for DNA extraction (Gupta, 2019), amount of 1 ml extraction buffer was added to 200 mg of powdered tissue in 2 ml microtubes and homogenized mixture was incubated in a water bath at 65 °C for 1 hour. After incubation, samples were centrifuged at 25 °C at 12,000 rpm for 12 min. The supernatant was transferred into a clean tube and was mixed and homogenized with at equal volume of chloroform:

isoamyl alcohol (24:1). Microtubes were centrifuged for 10 minutes at 10,000 rpm and the supernatant was transferred into a clean tube and treated with 3 µl of RNase (10 mg/ml) and was incubated in 45°C for 30 minutes. An equal volume of isopropanol was added to microtube and centrifuged for 10 minutes at 10,000 rpm. Then, pellet was washed twice with 100% and 70% ethanol and slowly dried for 30 minutes. An amount of 50 µl of deionized water was added to the extracted DNA and concentration was measured by nanodrop.

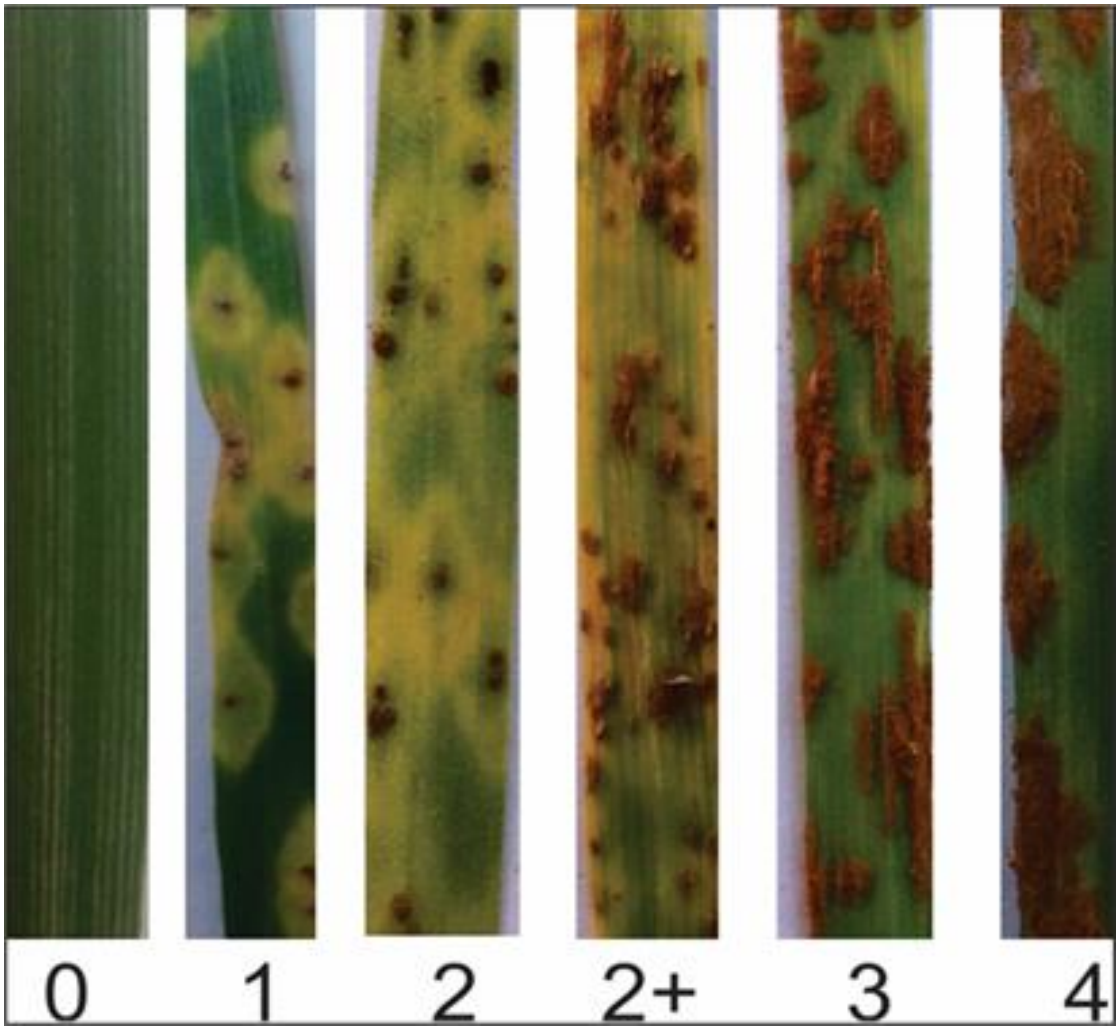


**Fig. 1.** Infection types of Pgt and host response used for this study (Provided by authors based on standard scale used by Stackman et al., 1962).

## PCR conditions

To assess the presence of *AvrSr35* and *AvrSr50* genes in the genome of isolates, specific primer pairs belonging to these avirulence genes were synthesized and used for PCR studies (Table 2). To ensure the quality of isolated DNA, specific primers of Pgt isolates at ITS region were used (Barnes & Szabo, 2007). PCR was carried out using 20 µL volume reaction involving 10 µl Master mix, 7 µl deionized water, 1 µL forward primer, 1 µL reverse primer, and 1 µL DNA. PCR reactions were performed as indicated in Table 3. PCR products were run on 1% agarose gel and visualized using UVITEC Imaging System.

**Table 2**. Primer pairs used in this study

| Allele | Primer (5□>3□) |
|---|---|
| *AvrSr35* | F GCCATGAGGAACTTTGCTGC |
| | R CAGGGGAAATTTTGGTGTTG |
| *Avrsr50* | F CTACCTGTGTTGGCGCCTT |
| | R GGTTCCCTTCCCGTCRTTC |

| | | |
|---|---|---|
| ITS | F | TGAACCTGCAGAAGGATCATTA |
| | R | TGAGAGCCTAGAGATCCATTGTTA |

**Table 3**. Program used to perform PCR reactions

| | ***Avrsr50*** | | | ***Avrsr35*, ITS** | | |
|---|---|---|---|---|---|---|
| Steps of PCR reaction | Temperature | Time | Cycles | Temperature | Time | Cycles |
| Initial denaturing | 95 °C | 3 minutes | 1 | 95 °C | 2 minutes | 1 |
| Denaturing | 95 °C | 30 second | | 95 °C | 30 second | |
| Annealing | 58 °C | 30 second | 35 | 60 °C | 30 second | 35 |
| Extension | 72 °C | 25 second | | 72 °C | 25 second | |
| Extension Final | 72 °C | 5 minutes | 1 | 72 °C | 5 minutes | 1 |
| Cooling | 4 °C | □ | | 4 °C | □ | |

## Allele mining in the virulent and avirulent isolates of AvrSr50 and homology modelling

To study the allelic variation of *AvrSr50* in four rust isolates including two virulent isolates (Pgt99-6 and Pgt99-12) and two selected avirulent isolates (Pgt99-1 and Pgt99-28), DNA was isolated from Pgt uredospore as stated above. Full length gene fragments of *AvrSr50* were amplified using Phusion PCR mix and clean fragments were evaluated trough Sanger sequencing. Using SeqMan software, the AvrSr50 genomic sequences of these four isolates were aligned with that of wild avirulent isolate (Chen et al., 2017). Only a nucleotide change (A>G) at position 178 was observed in the whole sequence. Using the SWISS-MODEL homology-modelling server, the 3D model for amino acid sequence of AvrSr50 was developed based on the solved crystal structure of AvrSr50 (7mqq.1- QCMJC). Variation of residues at surface of protein was visualized in PyMOL**.**

## Results

## Phenotyping revealed increased virulence on wheat lines carrying *Sr50*

To determine whether the collected isolates are virulent on the monogenic lines carrying one of *Sr35* and *Sr50* resistance genes, Infection types (IF) of seedlings were scored after two weeks post-inoculation. Based on the standard scale, IF 0-2 and IF 3-4 were evaluated for incompatible and compatible reactions, respectively. In this study Morocco was used as susceptible line to all Pgt isolates. Results revealed that both monogenic lines carrying *Sr35* and *Sr50* were resistant to the majority of Pgt isolates, where two isolates (Pgt99-6 and Pgt99-12) showed increased virulence on *Sr50*-carrying line (Fig. 2). These data show that wheat lines carrying their corresponding resistance genes recognize these effectors leading to incompatible interaction except two isolates that escape recognition probably due to mutations or deletion of their corresponding effector genes.

## Genotyping revealed the presence of *AvrSr35* and *AvrSr50* in all Pgt isolates

To study whether gene deletion of *AvrSr35* and *AvrSr50* has occurred in the genome of virulent isolates, genes encoding AvrSr35 and AvrSr50 effectors were amplified on the DNA of fungal pustules. Primer pairs specific to ITS of Pgt (Barnes & Szabo, 2007) were used for the qualification of isolated DNA. These primers do not amplify fragments from wheat genome (Fig. 3). Mock-inoculated seedlings were used as a negative control. PCR fragments of 315 and 348 bp using primer pairs specific to *AvrSr35* and *AvrSr50* genes of Pgt were amplified and visualized on 1% agarose gel (Fig. 3). Results showed that *AvrSr35* and *AvrSr50* genes were present in the genome of all isolates. Increased virulence despite the presence of *AvrSr50* gene in the genome of Pgt99-6 and Pgt99-12 suggests that point mutations or frame shifts may have occurred in the genome of virulent isolates and could be depicted through sequencing.

## Variation in a single surface‑exposed residue of AvrSr50 may affects its recognition by Sr50

The allelic variation of *AvrSr50* in four rust isolates including two virulent isolates (Pgt99-6 and Pgt99-12) and two avirulent isolates (Pgt99-1 and Pgt99-28) was evaluated through Sanger sequencing. The *AvrSr50* genomic sequences of these four isolates were aligned

with the reported sequence of wild avirulent DNA (Chen et al., 2017). Data revealed a nucleotide change A178G that resulted in an amino acid change of Alanine to Threonine (A60T) (Fig. 4A). Homology modeling based on the solved crystal structure of AvrSr50 (7mqq.1- QCMJC) (Ortiz et al., 2022) in PDB database showed the presence of polymorphic amino acid at the surface of the protein. The polymorphic residue was unique to the virulent isolates and mapped to the surface of the protein (Fig. 4B) suggesting its involvement in the interaction of AvrSr50 with Sr50. This is a nonsynonymous mutation change from a nonpolar amino acid to a polar one at the surface of protein.

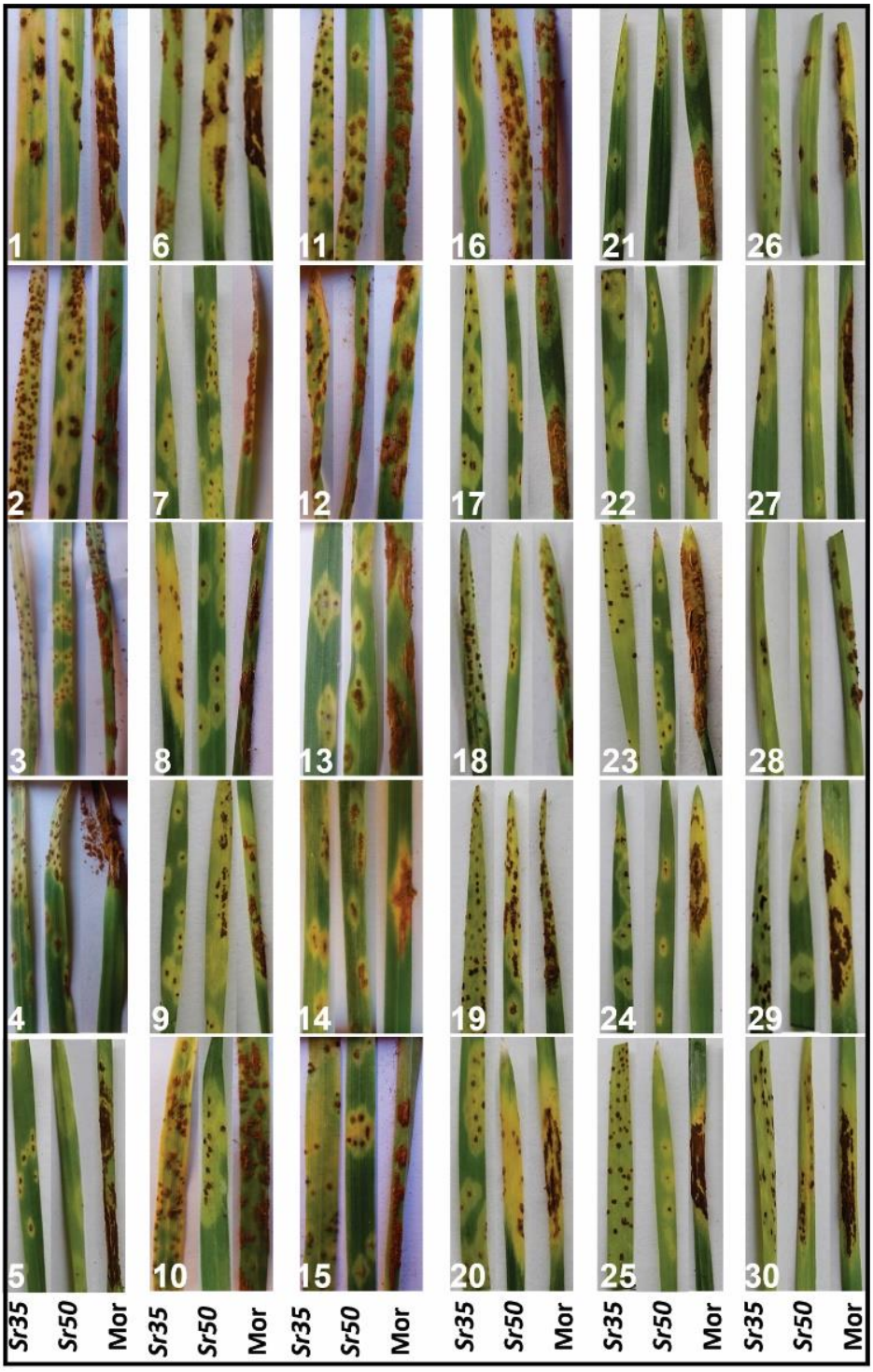


**Fig. 2.** Infection type of *Puccinia graminis* f. sp. *tritici* on *Sr24* and *Sr31*-carrying lines. The number of 30 isolates were inoculated on *Sr24* and *Sr31*-carrying lines as well as on susceptible cultivar Morocco. Low infection types 1 and 2 were

considered as incompatible and high infection types 3 and 4 as compatible reactions. Isolates Pgt99-6 and Pgt99-12 were virulent on *Sr50*-carrying monogenic lines showing developed pustules under leaf epidermis.

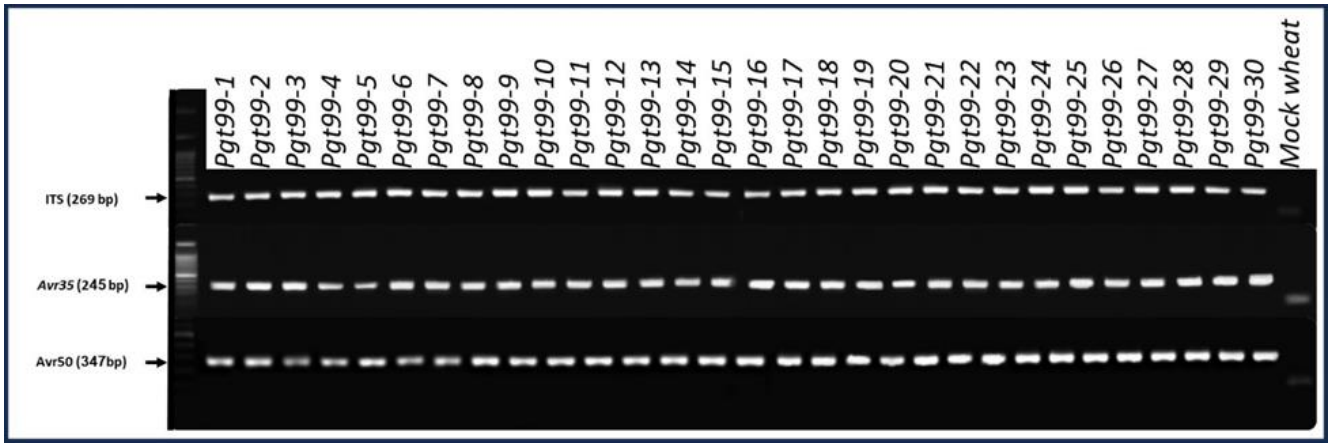


**Fig. 3.** Amplification of fragments from ITS, Avr*Sr35* and Avr*Sr50* genes. For each isolate DNA was extracted from pustules of infected leaves. Using ITS primers the quality of DNA was confirmed. Specific primers were used to assess the presence of Avr*Sr35* and Avr*Sr50* genes in the genome of 30 Pgt isolates and amplified fragments were loaded on 1% agarose gel. The size of fragments and name of genes were indicated in the figure. On top of pictures the name of isolates was indicated. The last lane of each gel, DNA from mock-inoculated wheat leaves were used for PCR as negative control

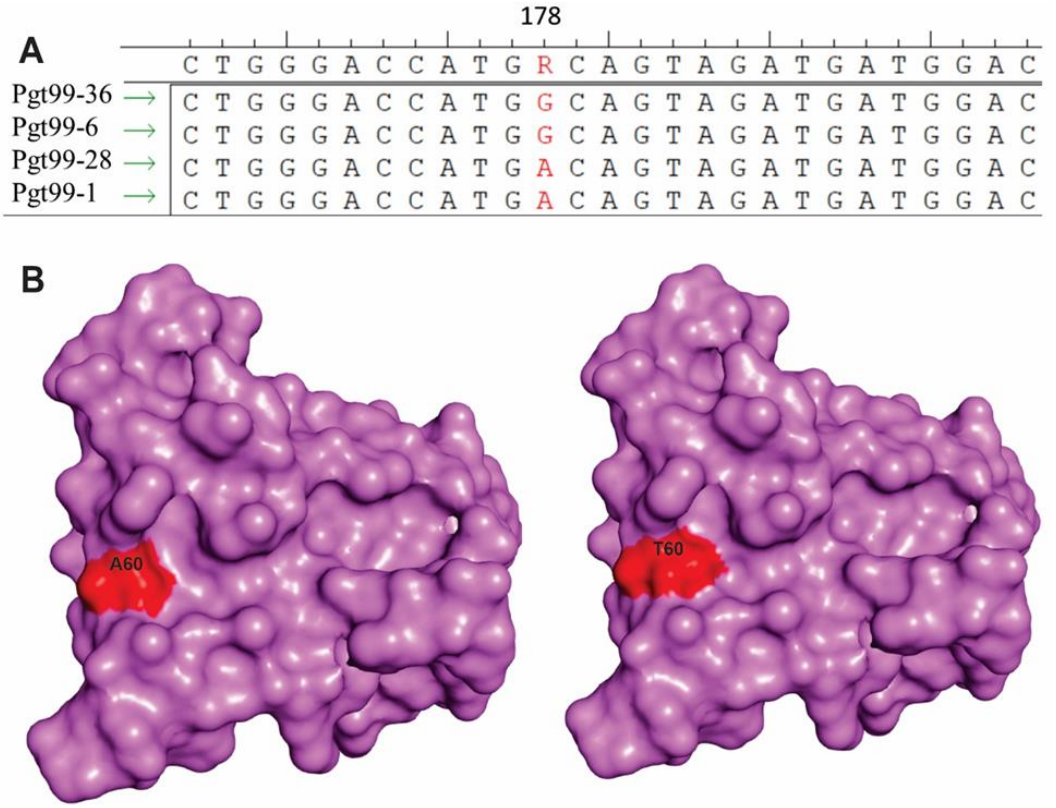


**Fig. 4.** Sequencing analysis and homology structural model for native and mutants of AvrSr50. A. Alignment shows the variation of AvrSr50 sequence for avirulent (Pgt99-1 and Pgt99-28) and virulent (Pgt99-6 and Pgt99-12) isolates at the polymorphic sites (A>G). B. To generate the 3-dimensional model, the full amino acid sequence of AvrSr50 was submitted to SWISS-MODEL online server. The best model was built based on the solved crystal structure of AvrSr50 (7mqq.1-QCMJC) (Ortiz et al., 2022) and illustrated by PyMol. Alanine and Threonine at position 60 were shown in native (left) and mutant (right) models of AvrSr50 (in red).

## Discussion

Phenotyping data revealed that monogenic lines carrying *Sr35* and *Sr50* genes were resistant to the majority of Pgt isolates, meaning that wheat lines carrying these resistance genes recognize their corresponding effectors leading to incompatible interaction. However, two isolates Pgt99-6 and Pgt99-12 escape recognition probably due to mutations or deletion of their corresponding effector genes. So far, no virulence of Pgt on lines carrying these two resistance genes was reported from Iran. Therefore, it was important to study it in further details. The *Sr50* gene is derived from rye and similar to its orthologous *Mla* (*mildew locus a*) in barley encode a protein that directly binds to AvrSr50 (Saur et al., 2019). Sr50 is a type of NB-LRR protein with a coiled- coil (CC) at the N-terminus of the protein (Jubic, Saile, Furzer, El Kasmi, & Dangl, 2019). Similarly, recognition of AvrSr35 by host NB-LRR receptor Sr35 induces host resistance at early stages of infection, even before haustorium formation (Andres Salcedo, et al., 2017; Saintenac et al., 2013). It seems *AvrSr35* truncations by miniature inverted transposable elements (MITE) are the origin of natural *Sr35*-virulent Pgt isolates. These elements are abundant in the genome of rust fungi compared to other fungi (Duplessis et al., 2011). Polymorphism studies of AvrSr50 in variants of Pgt showed that Q121K is a polymorphic residue that impacts on AvrSr50 recognition by Sr50 (Ortiz et al., 2022). In this study, isolates Pgt99-6 and Pgt99-12 showed bigger pustules on leaves of *Sr35*-carrying line, where inoculation of other isolates induced the host defense leading to small pustules surrounded with discolored halo on wheat seedlings (Fig. 1). Based on North American Stem Rust Nomenclature Code Sheet, isolates Pgt99-6 and Pgt99-12 belong to races PTTTF and TTTTF, respectively (Khanboluki, Nasrollahi, & Karimi-Jashni, 2024). These races belong to clade IV (Szabo, Olivera, Wanyera, Visser, & Jin, 2022) with no virulence on *Sr24* and *Sr31*-carrying lines.

Genotyping study showed that *AvrSr35* and *AvrSr50* genes are present in the genome of all isolates, however through Sanger sequencing, the allelic variation of *AvrSr50* in two virulent isolates (Pgt99-1 and Pgt99-28) was confirmed. This nucleotide change lead to an amino acid change of nonpolar amino acid Alanine to a polar amino acid Threonine at the surface of the protein. Previously, allelic variation studies showed a surface-exposed mutation (K>Q) led to loss of AvrSr50 recognition by Sr50 (Ortiz et al., 2022). Therefore, Feasible methods like transient co-expression of A60T variants of AvrSr50 with native Sr50 in *Nicotiana benthamiana* or in cell suspensions will further confirm the effect of A60T in effector-receptor interaction.

## Conclusion

Results of this study show the genetic change of fungus leading to increased virulence on resistant sources. It is very important to constantly monitor the genetic changes of fungus to avoid incidence of epidemies. The *Sr50* resistance gene has not been commonly deployed in cultivated varieties (Mago et al., 2015). Together with *Sr24*, combination of *Sr35+Sr50* are valuable sources for resistance to many Pgt races from clade I(Ug99) to most common races belong to clades IV. These could include other minor and major R genes for more durability (Luo et al., 2021). Information about population genetic of fungus and homozygosity of resistance loci in wheat lines help to take appropriate strategies in wheat breeding programs.

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## Conflict of interest

The authors declare that there are no conflicts of interest present.

## CRediT author statement

**M. Karimi-Jashni:** conceived the project, methodology, laboratory works, writing, reviewing & editing. **F. Khanboluki:** laboratory works, writing, reviewing. **M. Nasrollahi:** laboratory and experimental works. All authors have approved the manuscript and agree with submission to JAPP.

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