Heterologous Expression and Characterization of *ToxA1* Haplotype from India and its Interaction with *Tsn1* for Spot Blotch Susceptibility in Spring Wheat

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Abstract

Background: *ToxA*, a necrotrophic effector protein, is present in the genome of fungal species like *Parastagnospora nodorum*, *Pyrenophora tritici-repentis* and *Bipolaris sorokiniana. Tsn1* is the sensitivity gene in the host whose presence indicates more susceptibility to *ToxA* carrying pathogen, and *ToxA-Tsn1* interaction follows an inverse gene-for-gene relationship.

Methods and Results: The present study involved cloning and expressing the *ToxA1* haplotype from *B. sorokiniana*. It was found that the amplicon exhibited an expected product size of 471 bp. Sequence analysis of the *ToxA1* nucleotide sequence revealed the highest identity, 99.79%, with *P. tritici-repentis*. The protein expression analysis showed peak expression at 16.5kDa. Phylogenetic analysis of the *ToxA1* sequence from all the *Bipolaris* isolates formed an independent clade along with *P. tritici-repentis* and diverged from *P. nodorum*. *ToxA-Tsn1* interaction was studied in 18 wheat genotypes (11 *Tsn1* and 7 *tsn1*) at both seedling and adult stages, validating the inverse gene-for-gene relationship, as the toxin activity was highest in the K68 genotype (*Tsn1*) and lowest in WAMI280 (*tsn1*).

Conclusion: The study indicates that the haplotype *ToxA1* is prevailing in the Indian population of *B. sorokiniana*. It would be desirable for wheat breeders to select genotypes with *tsn1* locus for making wheat resistant to spot blotch.

Keywords: *Bipolaris sorokiniana*, effector-triggered susceptibility, inverse gene-for-gene relationship, sensitivity genes, *ToxA1*, *Triticum aestivum*

Introduction

Host selective toxins (HSTs) are the effector molecule(s) released during the invasion of necrotrophic pathogens and act as major determinants of pathogenicity (1). These necrotrophic effectors are specific biomolecules that are structurally complex, chemically diverse, and responsible for causing some key diseases in important crops. HSTs are very important in host-pathogen interaction but are produced by limited genera of fungi involving multiple toxins (2). Spot blotch of wheat is a damaging disease caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker, syn. *Cochliobolus sativus* (S. Ito & Kurib.) a hemi-biotrophic pathogen. This disease is mainly prevalent in South Asia's warm, humid region, especially in the eastern Indo-Gangetic plains of India. Wheat productivity can decline by 40% due to spot blotch alone (3). Spot blotch has been reported to cause up to 17.5% yield loss in the Indian sub-continent, and about 25mha of wheat is affected by spot blotch worldwide (4). Plant pathogens have their genetic makeup for causing host infection, which can decided by the presence of a single gene that confers host susceptibility or resistance (5, 6). The proteinaceous necrotrophic effector ToxA a major HST produced by *B. sorokininana*. Additionally, ToxA is known to be produced from two necrotrophic pathogens, *Parastagonospora nodorum* (Berk.) Quaedvl., Verkley & Crous, syn. *Phaeosphaeria nodorum* (E. Müll.) Hedjar. and *Pyrenophora tritici-repentis* (Died.) Drechsler and cause Septoria nodorum blotch and tan spot, respectively (7, 8). ToxA protein was reported initially in a culture filtrate of the wheat pathogen *P. tritici-repentis* (8). The ToxA protein was first discovered by Tomás and Bockus (9) from the culture filtrate of *P. tritici-repentis*. The *ToxA* manifested high nucleotide diversity in *P. nodorum,* whereas it showed a lack of variation in *P. tritici-repentis*. This pattern of nucleotide diversity and the recent emergence of tan spot suggests the recent introduction of *ToxA* into *P. tritici- repentis* genome (6). The *ToxA* sequences from *P. nodorum* and *P. tritici-repentis* depicted differences at four fixed nucleotide sites resulting in two predicted amino acid changes consistent with the

interspecific transfer of this gene from *S. nodorum* into *P. tritici-repentis* (6), and this *ToxA* gene jumped horizontally from *P. tritici-repentis* to another pathogen *B. sorokiniana,* (10). *ToxA* and its surrounding 14 kb suggest that this gene was horizontally transferred, facilitated by a type II DNA transposon (11). *ToxA* is a single-copy gene, and it was found to be embedded within a 12-kb genomic element nearly identical to the corresponding regions in *P. nodorum* and *P. tritici-repentis* (10). The mature ToxA protein is 13.2 kDa, and it encodes a signal peptide of 23 amino acids and a pro-domain of 4.3 kDa (required for protein folding), and both are cleaved before the secretion of mature ToxA protein (1, 12, 13). Studies revealed that the host sensitivity of ToxA was mapped on wheat chromosome arm 5BL, and the sensitivity gene was named *Tsn1* (14). Further, it was confirmed that this gene was significant for both tan spot and SNB, and in both cases, the pathogen had a sensitivity gene such as *ToxA* (6, 15). For the wheat-*B. sorokiniana* pathosystem, ToxA confers virulence, and it is the only known virulence gene whose corresponding sensitivity gene (*Tsn1*) in the host has been identified. Further, it was also demonstrated that *Tsn1*-carrying wheat cultivars are more sensitive to *B. sorokiniana* isolates that harboured the *ToxA* gene. Cloning of the *Tsn1* gene unfolded characteristics similar to classical disease resistance nucleotide-binding leucine-rich repeat (NLR) gene that included serine/threonine kinase, leucine-rich repeats, nucleotide binding and protein kinase domains (14). The ToxA gene is transcribed as a necrotrophic effector protein responsible for infection in the host, with *Tsn1* being a resistance gene (16). A resistance-like gene *Tsn1* in wheat confers susceptibility to *ToxA* in *P. tritici-repentis* and *P. nodorum* isolates (6). This *ToxA–Tsn1* interaction system has been proved as an example of the inverse gene-for-gene relationship. It was realizing the occurrence of spot blotch in South Asia and the emergence of *ToxA1* as a key virulence gene driven us towards studying this host-pathogen relation. Keeping this in mind, we cloned the *ToxA1* gene from *B. sorokiniana* and conducted expression analysis

99 for the ToxA1 protein. We also validated the pathogenicity of *ToxA1* on the allelic status of
100 *Tsn1* by using 18 *Tsn1/tsn1* genotypes.

101 **Materials and Methods**

102 ***Primer designing:***

103 *ToxA* is a 534 bp-long gene sequence, and primers were designed using the web-based tool
104 Primer-BLAST (Basic Local Alignment Search Tool) from NCBI. The *ToxA* sequence from *P.*
105 *tritici-repentis* (U79662.1) was obtained in FASTA format from NCBI (National Centre for
106 Biotechnology Information) GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The
107 primers were utilized to amplify *ToxA* in *B. sorokiniana* isolate MCC 1533 (Pusa2). The isolate
108 MCC1533 (Pusa2) has been identified and deposited at National Center for Microbial
109 Resources (NCMR), Pune, India. The primer sequence used is given as Forward Primer (FP) -
110 CGCGGATCCGATCCCGGTTACGAAATCG and Reverse Primer (RP) - CGC
111 CTCGAGCTAATTTTCTAGCTGCATTCTC. Revised nomenclature of ToxA haplotypes
112 corresponds the

113 ***RNA extraction and cDNA synthesis***

114 *The B. sorokiniana* isolate MCC 1533 (Pusa2) was cultured for 10 days in a Potato Dextrose
115 Broth. Mycelium was harvested, and the total RNA was isolated using Invitrogen PureLink^{TM}
116 RNA Mini kit following the manufacturer's protocol. The integrity and size distribution of total
117 RNA purified with RNeasy Kit was checked by 1.2% agarose gel electrophoresis and ethidium
118 bromide staining, visualizing two sharp bands of 28S rRNA to 18S RNA. RNA was quantified
119 using Nanodrop2000 instrument (Thermo Scientific) and 1µg RNA reverse transcribed to
120 cDNA was synthesized using a Thermo cDNA synthesis kit following the manufacturer's
121 protocol.

122 An additional treatment of RNase was included during cDNA synthesis as supplied and
123 instructed with the kit and manufacturer's protocol to prevent chances of contamination of
124 genomic DNA during isolation.

125 ***ToxA gene cloning***

126 The expression construct was produced in the bacterial expression vector pET28a that directs
127 the expression of HIS-tagged fusion proteins in *E. coli*. BamH1 and XhoI restriction sites were
128 introduced in forward and reverse primer for cloning in pET vector. Primers were designed
129 such that the PCR amplified portion of the *ToxA1* gene was generated with a 5' BamHI site and
130 a 3' XhoI site. The PCR product was digested with BamHI and XhoI, gel-purified with the
131 QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into the BamHI and XhoI
132 sites of pET28a. Primers FP-CGCGGATCCGATCCCGGTTACGAAATCG and RP- CGC
133 CTCGAGCTAATTTTCTAGCTGCATTCTC were used for the PCR amplification. PCR
134 amplification was performed using cDNA as a template in an Eppendorf Mastercycler using
135 the following cycles; initial denaturation of 95°C for 7 min followed by 35 cycles of 94°C (30
136 s), 55°C (45 s) and 72°C (1 min) and a final extension of 10 min at 72°C. The PCR product
137 was gel-purified, digested with BamHI and XhoI, and ligated into the respective restriction
138 sites of pET28a. The resulting construct contained the predicted effector protein gene fused to
139 a 6X histidine tag at the N-terminus. The ligated product was used to transform *E. coli*
140 DH5alpha and BL21 (DE3) strains (17) for cloning and protein expression, respectively.

141 ***Sequencing and characterization***

142 Cloned fragment of *ToxA* from *B. sorokiniana* isolate MCC1533 (Pusa2) was sequenced by
143 Sanger sequencing. The sequences obtained in two frames were merged, and Bioedit Sequence
144 Alignment Editor (v 7.2) was used to identify overlapping sequences in both orientations (18).
145 Integrated sequences were then prepared in a single frame. The sequence has been deposited
146 to the National Centre for Biotechnology Information (NCBI) database with Gene Bank

147 accession number (OP289649). The processed *ToxA* sequence was used as a query for BLASTn
148 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for finding homologous sequences with reference to
149 the NCBI nucleotide database (19). The processed *ToxA1* nucleotide sequence was then
150 translated to the amino acid sequence in BioEdit 7.2. Sequences of *P. tritici-repentis, P.*
151 *nodorum* and different isolates of *B. sorokiniana* were retrieved from the NCBI protein
152 database. The amino acid sequences retrieved using BLASTp searches were then used to create
153 multiple sequence alignments of 27 ToxA sequences. MEGA X (Molecular Evolution Genetics
154 Analysis) software (19) was then used to generate a phylogeny of *ToxA* amino acid sequences
155 of 49 isolates of *P. tritici-repentis, P. nodorum* and *B. sorokiniana* using the CLUSTAL W
156 alignment algorithm (20). All alignment parameters were left at their default values; 178 and
157 114 positions with and without gaps were obtained, respectively. Sequences were analyzed
158 using substitutions selected with complete deletion of gaps or absent data. The phylogenetic
159 tree was inferred using the JTT model and the maximum likelihood method (21). Using the
160 default setting and the Neighbor Joining method, the initial tree was inferred, and Nearest-
161 Neighbor Interchange was used as the ML heuristic search method. Phylogenetic tree
162 reconstructions were performed using the Maximum Likelihood Method. For the Poisson
163 correction model, 1000 bootstrap replicates were used to assess node support (22).

164 ***Protein modelling and topographical properties***

165 The predicted protein sequence of cloned Pusa2 *ToxA* was subjected to the SWISS-MODEL
166 homology modelling online tool (23). Proteins were modelled utilizing Protein Data Bank
167 (PDB) entry 1zld.1.A (*P. tritici-repentis*) as reference. The quality of modelled protein was
168 judged based on GMQE (Global Model Quality Estimation) and QMEAN (Qualitative Model
169 Energy Analysis) Z-score parameters (24, 25). The best model was selected based on the
170 GMQE score, which ranges from 0 to 1, with a higher value indicating better reliability. On a
171 global scale, the QMEAN Z-score estimates the "degree of nativeness" of the structural features

172 observed in the model. QMEAN Z-scores around zero indicate good agreement between the
173 model structure and experimental structures. Scores of -4.0 or below are an indication of
174 models with low quality. The quality of the resulting models was also monitored with
175 PROCHECK and ProsSA-WEB (26). Geometrical and topographical properties of the
176 modelled proteins were identified using the Computed Atlas of Surface Topography of Proteins
177 (CASTp) 3.0 online server (27). The UCSF Chimera software was used to create images of
178 molecular graphics (28). The alpha helix and the beta-sheet percentage were analyzed using
179 PDBMD2CD.

180 ***Isolation of proteins from E. coli culture***

181 *E. coli* colony harboring the expression construct was inoculated in 5 ml of LB (Sambrook et
182 al. 2006) with 100µg of kanamycin per ml (LBA) and grown overnight at 37°C and shaking at
183 250 rpm (G24 Incubator Shaker, New Brunswick Scientific, Edison, NJ). The overnight culture
184 was used to inoculate 25 ml of fresh LBA, which was then incubated with shaking at 37°C for
185 one hour. The expression of fusion proteins was induced by the addition of isopropyl-beta-
186 Dthiogalactopyranoside (IPTG) to a final concentration of 1 mM, followed by an additional 4
187 hours of incubation under the same conditions. After 20 minutes of centrifugation at 4,000 g
188 and 4°C, cell particles were resuspended in a cold sonication buffer(SB; 50 mM Na2HPO4, 5
189 mM Tris-base, 300 mM NaCl, pH 8.0) with 0.5% Triton-x-100 and 10 mM DTT. Resuspended
190 pellets were sonicated three times, 30 s each, with 2 min on ice between periods. Insoluble
191 material was then pelleted by centrifugation at 10,000 × *g,* 4°C, for 10 min. Pellets were washed
192 thrice with SB with 0.5% Triton-× -100 and 10 mM DTT. The final washed pellets, containing
193 inclusion bodies, were either stored frozen at –80°C or resuspended in 1 ml of 8 M guanidine
194 HCl (Sigma) in refolding buffer (250 mM NaCl, 100 mM sodium phosphate buffer, 10 mM
195 Tris-base, 4% glycerol, 1 mM EDTA, and 0.005 % Tween 20, pH 8.0) with freshly prepared
196 DTT added to a final concentration of 10 mM. Solubilized proteins were sonicated briefly twice

197 (for 5 to 10 s) and then heated to 37°C for 10 min and 50°C for an additional 5 min to assist in
198 the solubilization of proteins. Insoluble material was removed from the samples by centrifuging
199 at 12,000 ×*g* for 10 min. Then, solubilized proteins were quantitated with Bradford assay
200 reagent (Bio-Rad, California, USA) with bovine serum albumin used as a standard. Fusion
201 proteins solubilized in guanidine HCl were used immediately.

202 ***Screening of ToxA1 –Tsn1 interaction on different wheat genotypes***

203 The screening of 18 wheat genotypes was conducted for *Tsn1/tsn1,* out of which 11 genotypes
204 harboring *Tsn1* and the rest 7 genotypes with *tsn1* (see Table 1) were sown in 3 replications at
205 polyhouse, Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University in
206 1m rows at a distance of 22.5cm between two rows. Infiltration of ToxA1 protein, as well as
207 control (Uninduced protein), was done at both the seedling stage (3-5 leaf stage) and adult stage
208 (Zadoks growth stage 55) (29). 250µg of ToxA protein was infiltrated by a sterilized syringe,
209 and symptoms were recorded 7 days after infiltration. The 5th emerged leaf was infiltrated for
210 seedlings, while the flag leaf was infiltrated with toxin ToxA1 for adult plants. The temperature
211 was maintained at 25±2°C. The light/dark period was maintained at 14hrs light and 10hrs of
212 darkness. Photographic data was captured using a Nikon camera. The lesion size was measured
213 in length, width, and area of lesions using ImageJ software (Version 1.53s) (30).

214 ***Statistical Analysis***

215 The statistical analysis was conducted for phenotypic traits, including length, width, and area
216 of necrotic lesions of wheat genotypes with the *Tsn/tsn1* gene at both seedling and adult stages.
217 The point biserial correction coefficients between disease components and the
218 presence/absence of *Tsn1* were calculated (32). The analysis in R software V 4.2.1 was
219 performed using packages' *phytools', 'stats', 'agricolae', 'ggplot2', 'ggtree'* (31).

220 **Results**

221 ***Cloning and expression study of ToxA1***

222 The *ToxA* gene from *B. sorokiniana* was amplified using primers designed from *PtrToxA*
223 through RT-PCR. The amplicon exhibited the expected product size of 471bp, which was then
224 cloned in the pET28a vector. The cloned gene was validated using colony PCR of the
225 transformed cell. The *ToxA1* gene, after validation, was subjected to sequencing, and the
226 sequence data after processing revealed successful cloning of the *ToxA* gene (now *ToxA1- B.*
227 *sorokininana*). This *ToxA1* nucleotide sequence was further used for BLASTn analysis,
228 revealing the highest identity, 99.79%, with *P. tritici-repentis* and E-value close to zero.
229 Expression of ToxA1 was done by inducing the transformed *E.coli* cell through IPTG of
230 different concentration, namely, at 0.4mM, 0.6mM, 0.8mM and 1.0mM. Maximum expression
231 of ToxA1 was obtained at 1.0mM concentration with an expected band size of 16.5kDa in
232 sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 1).

233 ***ToxA1 characterization and Phylogenetic analysis***

234 The identification of *ToxA1* after showing sequence similarity with *P. tritici-repentis ToxA1*
235 using BLASTn analysis on NCBI was conducted. Further characterization of ToxA1 was
236 conducted by translating the nucleotide sequence to amino acid sequence using the web-based
237 tool Expasy (https://www.expasy.org/resources/translate). The amino acid sequence of ToxA1
238 was subjected to BLASTp analysis on NCBI, which depicted 100% similarity with *P. tritici-*
239 *repentis* showing E value $9e^{-109}$. Multiple sequence alignment of 25 ToxA protein sequences
240 and *ToxA1* MCC1533 (Pusa2) translated sequence in Bioedit revealed that amino acid
241 differences were found in most *P. nodorum* strains. However, the cloned Pusa2_*ToxA1*
242 sequence showed complete similarity to *P. tritici-repentis* ToxA sequence. *P. nodorum ToxA*
243 sequence depicted amino acid substitution for Glutamate, Aspartate, Leucine, Valine and
244 Asparagine (Fig. 2).

245 The phylogenetic analysis depicts the evolutionary relatedness of the *ToxA1* amino acid
246 sequence with other ToxA sequences. The tree indicates that *ToxA1* sequences originate from

247 a common ancestor but form different clades during evolution. The *ToxA1* sequence from all
248 *Bipolaris* isolates formed an independent clade along with *P. tritici-repentis* and diverged from
249 *P. nodorum*. The phylogeny indicates sequence similarity among *B. sorokiniana* and *P. tritici-*
250 *repentis,* whereas it differed from *P. nodorum* (Fig. 3).

251 ***Protein modelling and Topographical properties***

252 The structure of the ToxA1 protein of a cloned gene of *B. sorokiniana* isolate MCC1533
253 (Pusa2) was predicted using the SWISS-MODEL homology modelling online server. For
254 checking the reliability of models GMQE (ToxA1: 0.56), QMEAN Z scores are included
255 QMEAN -0.33, Cbeta –1.79, torsion 0.53 and these scores indicated that these models are
256 reliable and good quality. The quality of the resulting models was monitored with
257 PROCHECK. Ramachandran plot analysis revealed that 88.1% of the ToxA1 protein structure
258 residues fell within the most favoured regions, with a further 11.9% in the additionally allowed
259 region (Fig. 4). No residues were found in the generously allowed or disallowed regions. The
260 number of non-glycine and non-proline residues was 100%. The number of end residues
261 (excluding Gly and Pro) is 2, and the number of glycine and proline residues was 12 and 3,
262 respectively.

263 Predicted ToxA1 protein structures of *B. sorokiniana* MCC1533 (Pusa2) isolate based
264 on homology modelling are depicted in Fig. 4. The protein sequences of ToxA1 protein showed
265 sequence identity of 100% with template PDB entry 1zld.1.A (*P. tritici-repentis*, *PtrToxA*).
266 When generated structures were compared in UCSF Chimera, it was observed that the RGD
267 motif (toxic region) was present in the 3D structure of ToxA1 protein and found that arginine
268 is present in the alpha helix of the loop at 118 positions. At the same time, Glycine and Aspartic
269 acid is present in the β-sheet of the protein structure at 119 and 120 positions of ToxA1 protein
270 (Fig. 5). The percentage of the alpha helix was 3.39%. At the same time, antiparallel sheet 1

271 was 35.55%, antiparallel sheet 2 was 12.76%, the parallel sheet was 0.85%, the turn was 7.63%,
272 and the other was 39.83%.

273 ***Bioactivity of ToxA1 effector protein and its interaction with Tsn1 at both seedling and adult***
274 ***stage***

275 The bioactivity of the *ToxA1* effector was studied on necrotic lesions symptoms caused due to
276 *ToxA1* protein and was found to be more prominent at the adult stage. At the seedling stage,
277 the maximum symptomatic area (0.977 cm^2) was recorded in genotype K68(*Tsn1*) and the
278 minimum (0.265 cm^2) in WAMI280 (*tsn1*). At the same time, the length varied from 2.872 cm
279 (K68, *Tsn1*) to 0.860cm (PBW343, *tsn1*). However, the maximum width recorded was 0.432
280 cm for genotype WAMI288 (*Tsn1*), and the minimum in genotype harbouring *tsn1*(LOK1) was
281 0.307cm.

282 At the adult stage, the length of the symptom was maximum (4.613cm) in genotypes
283 harbouring*Tsn1*(K68) and minimum (1.305 cm) WAMI280 (*tsn1*), and the width also had the
284 same trend ranging from 0.633cm (C306, *Tsn1*) to 0.327cm (WAMI280, *tsn1*). The area was
285 measured for quantifying the necrotrophic symptom, and it found that the maximum area in
286 the genotype harboring *Tsn1* was 2.326 cm^2 (K68), and the minimum was 0.411
287 cm^2(WAMI280, *tsn1*). A significant difference was found between the seedling and adult
288 stages and for *Tsn1* and *tsn1* (Table 1, Fig. S2, S3, S4).

289 **Discussion**

290 Studies on necrotrophic effectors producing HSTs have provided a new dimension to host-
291 pathogen interaction and established an inverse gene-for-gene relationship (6, 34). The HSTs
292 are effectors proteins and induce a susceptible response in the host harbouring the dominant
293 susceptibility gene. ToxA is a necrotrophic effector protein, one of the key factors for causing
294 the most devastating disease, spot blotch in wheat, prevalent in warm, humid regions of South
295 Asia. The present study focuses on cloning of *ToxA1* gene, followed by its characterization and

296 purification of the resultant protein and its expression analysis. This study successfully cloned
297 and characterized the *ToxA1* gene from *B. sorokiniana* isolate MCC1533 (Pusa2). This gene
298 was found initially in *P. nodorum* and horizontally transferred to *P. tritici-repentis* and, more
299 recently, to *B. sorokiniana* (6, 35). The cloned gene and its expression was studied in the
300 present study, and the purified protein showed peak expression at a concentration of 1.0 mM
301 IPTG with band migration at 16.5kDa in size. Manning et al. (36) also reported similar findings
302 where lysates with His-ToxA1 expressed a phosphorylated band that migrated at 16 kDa
303 compared to lysates without His-ToxA1 (control). However, mass spectral analyses have
304 indicated a 13.2 kDa mass of purified mature ToxA1 protein from *P. tritici-repentis* (1, 8). The
305 importance of a protein purification study for ToxA1, which has selective host toxicity for
306 genetic analysis of pathogenicity in the *Pyrenophora*-wheat interaction, was also suggested by
307 Touri et al. (8).
308 The *ToxA1* gene was truncated in the present study to remove the signalling sequences
309 comprising 22 amino acids, as the mature ToxA1 gene is released after the proteolytic cleavage
310 of this signal sequence. Previous studies have also described these signalling peptides in ToxA
311 protein that involve the first 22 amino acids, which are proteolytically cleaved after import into
312 the endomembrane system (1). The release of mature toxin involves additional proteolytic
313 processing, and as the ToxA1 protein is blocked at the N terminus, such modifications would
314 incur a mass increase (1). The ToxA1 cloned sequence obtained in our study has been compared
315 with the already reported ToxA1 sequences from *P. nodorum*, *P. tritici-repentis* and *B.*
316 *sorokiniana*. Multiple sequence alignment was performed on the amino acid sequences of 26
317 isolates. The results indicate that the ToxA regions of *B. sorokiniana* and *P. tritici-repentis* are
318 more closely related to one another than they are to *P. nodorum*. This conclusion is supported
319 by the identical ToxA1 sequence shared by *B. sorokiniana* and *P. tritici-repentis* and the six-
320 position difference between *P. nodorum* haplotypes. (Fig. 2). The sequence identity between

321 the three fungal species accounts for the common evolutionary origin of *ToxA* and the potential
322 of exchanging DNA between these species (10). The significance of phylogenetic studies in
323 establishing evolutionary relationships among the different pathogen races or deriving the
324 specific gene evolution between different genera is well known. The sequencing data for *ToxA1*
325 showed a high similarity between *B. sorokiniana* and *P. tritici-repentis* (10, 37, 38). Previous
326 studies have also suggested that the ToxA1 sequence did not differ for *B. sorokiniana* and *P.*
327 *tritici-repentis* but differed from the ToxA sequence of *P. nodorum* (10, 34, 37, 38). This
328 evolutionary relatedness of *P. tritici-repentis* and *B. sorokiniana* sequences have also been
329 reported earlier (10, 34). The earliest report suggested that ToxA has been present in *P.*
330 *nodorum* for a long time as it exhibited greater sequence diversity and was interspecifically
331 transferred to *P. tritici-repentis* quite later. This is evident from monomorphism or lesser
332 sequence diversity in the ToxA1 sequence from *P. tritici-repentis* and the emergence of tan
333 spot symptoms more recently in the near past (38).
334 Further insights into the ToxA structural analysis were performed by predicting the 3D protein
335 structure using an amino acid sequence of ToxA1 (Fig.4). This is the first report for the 3D
336 protein structure ToxA1 haplotype from *B. sorokiniana*. The predicted protein structure was
337 similar to a single-domain protein with a β-sandwich fold of PtrToxA. This β-fold represents a
338 solvent-exposed loop containing arginyl-glycol-aspartic acid (RGD)- functionally active motif
339 (39, 40). ToxA1 has been known to resemble with fibronectin III domain which retains a
340 solvent-exposed loop containing RGD motif for cell wall-plasma membrane interaction in
341 plants (40, 41). It has been proposed that ToxA with RGD-containing motif has been known to
342 interact with wheat mesophyll cells and thus establish disease (42). The mesophyll cells have
343 a high-affinity matrix to recognize these motifs, and these all suggest the importance of the
344 RGD motif in receptor binding and internalization into wheat cells (40).

ToxA is the only known virulence gene for which a host sensitivity gene (*Tsn1*) has been known in wheat-*B. sorokiniana* pathosystem. For the first time, the present study reports a cloned and purified protein ToxA1 infiltration in both seedling and adult stages of wheat lines harboring *Tsn1/tsn1*. The severity was enhanced in the adult stage compared to the seedling stage in *Tsn1* wheat lines. The present data also reveals that *Tsn1*-carrying genotypes exhibited more disease in seedlings than in the adult stage. The toxin activity was highest in the K68 genotype (*Tsn1*) and lowest in WAMI280 (*tsn1*) at both the seedling and adult stages. This could be validated by past studies suggesting that ToxA causes severe necrotic lesions on *Tsn1,* harboring wheat genotypes.

In contrast, less or no lesion was observed for wheat genotypes lacking the sensitivity gene (43). Navathe et al. (34) also reported one such possibility of the involvement of a recessive allele of *Sb2* towards an unknown HST. *Tsn1* has been reported attributing susceptibility towards both *P. nodorum*-causing SNB (6, 44) and *P. tritici-repentis*, causing tan spots (45). However, the environmental requirements for *Bipolaris* are different from these two pathogens, which are not prevalent in the South-Asian region.

Conclusions:

Nevertheless, it would be desirable for wheat breeders to select *tsn1* for making crops resistant to *B. sorokiniana, P. tritici repentis* and *P. nodorum* in nearest future. The study in this horizon should focus on loci other than *Tsn1* and *Sb2*. It would be interesting to study other quantitative trait loci involved to understand the complexity of this *ToxA-Tsn1* interaction and spot blotch disease resistance.

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and editing, RC, PKS; supervision, RC, VKM, SS; project administration, RC; funding acquisition, RC, PKS. All authors have read and approved the manuscript.

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Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

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Figure Legends

Figure 1. Expressed ToxA1 protein showing 16.5kd band size on SDS-PAGE

Figure 2 (a.) The multiple sequence alignments with a phylogenetic tree, Colors both on the phylogenetic tree and the branch scale represent genetic distance. ToxA sequences have been coloured by four colours shown in the 'seq' column for each nucleotide. Colour changes on the aligned sequences represent nucleotide differences. (b) Based on the number of nucleotide variations between ToxA haplotypes, a heat map was created. The relevant ToxA haplotype in the matrix is represented by each branch of the phylogenetic tree. The near ties to "dark red" and the distance ties to "white" colour. (c) Neighbor-joining (NJ) tree for ToxA haplotypes based on the nucleotide sequence differences. Coloured internal nodes represent the bootstrap confidence level.

Figure 3 Phylogenetic tree using Maximum Likelihood algorithm of different ToxA1 of *Pyrenophora tritici-repentis, Bipolaris sorokiniana,* and varipus haplotypes of *Parastagnospora nodorum*. The percentage of bootstrap support (1000 replicates) is indicated at corresponding nodes. The nomenclature of the haplotypes is based on the Aboukhaddour et al. (2023)

Figure 4. Ramachandran plot and ProsSA z-score analysis of modelled ToxA1 protein from *B. sorokiniana*. Ramachandran distributions of amino acids in the model were calculated using pro check (a), and the z-score plot was plotted using ProsSA-WEB (b).

Figure 5. *ToxA1* protein structure of *B. sorokiniana* isolate MCC 1533 (Pusa2) depicting conserved RGD motif (ARG-GLY-ASP)

542 **Figure 6.** Symptom of leaf necrosis on *Tsn1/tsn1* harbouring genotypes after infiltration ToxA1
543 protein at seedling (3-5 leaf stage) and on flag leaf of adult plant (Zadoks growth stage
544 55).

545 **Table Legends**

546 **Table1.** Response of wheat genotypes (*Tsn1/tsn1*) in terms of leaf necrosis after infiltration of
547 *ToxA1* protein at both seedling and adult stage of wheat genotypes harboring
548 *Tsn1/tsn1* allele.

549 **Supplementary Materials:**

550 **Figure S1.** Correlation coefficients estimated by point biserial correlation for phenotypic traits
551 associated with*Tsn1/tsn1* gene effect in wheat genotypes with ToxA1 isolate
552 (MCC1533/Pusa2)**.**
553 **Figure S2.** The necrotrophic area developed due to ToxA1–Tsn1 interaction at seedling (3-5
554 leaf stage) and adult stage (Zadoks growth stage 55) of 11 wheat genotypes with *Tsn1*
555 and 7 wheat genotypes with *tsn1***.**
556 **Figure S3**. Symptom of leaf necrosis after infiltration ToxA1 protein at both seedling (3-5 leaf
557 stage) and adult stage (Zadoks growth stage 55) of wheat genotypes harbouring *Tsn1*.
558 **Figure S4**. Symptom of leaf necrosis after infiltration ToxA1 protein at both seedling (3-5 leaf
559 stage) and adult stage (Zadoks growth stage 55) of wheat genotypes harbouring *tsn1*.
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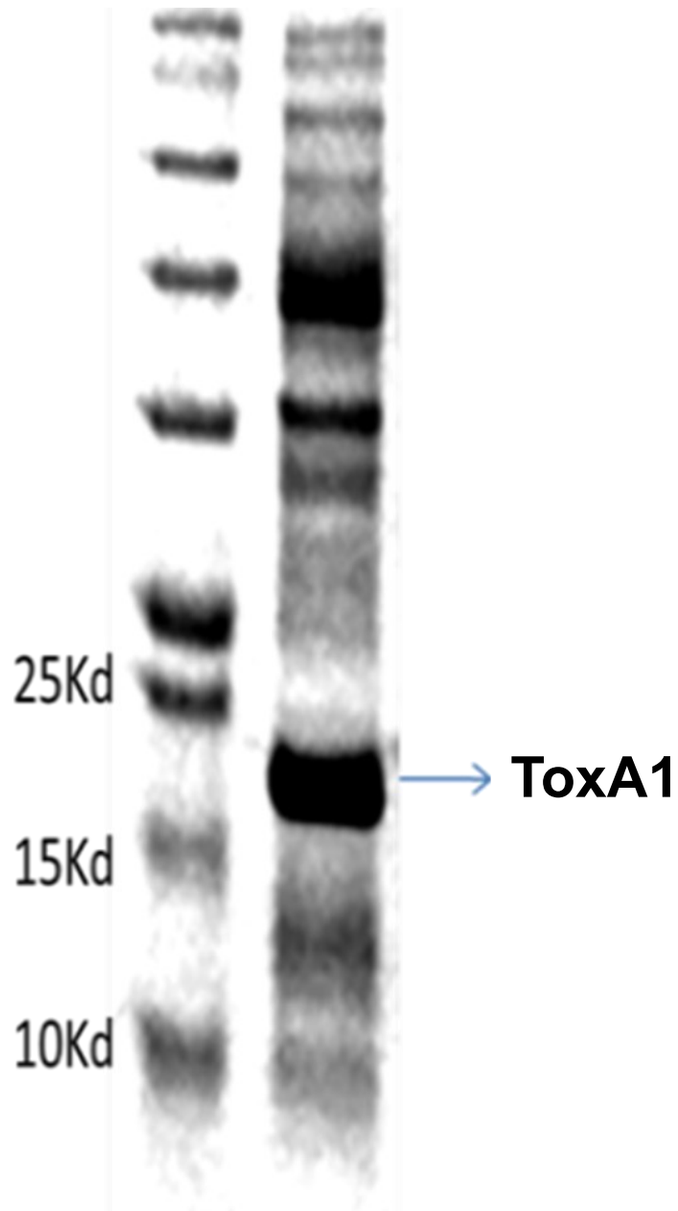

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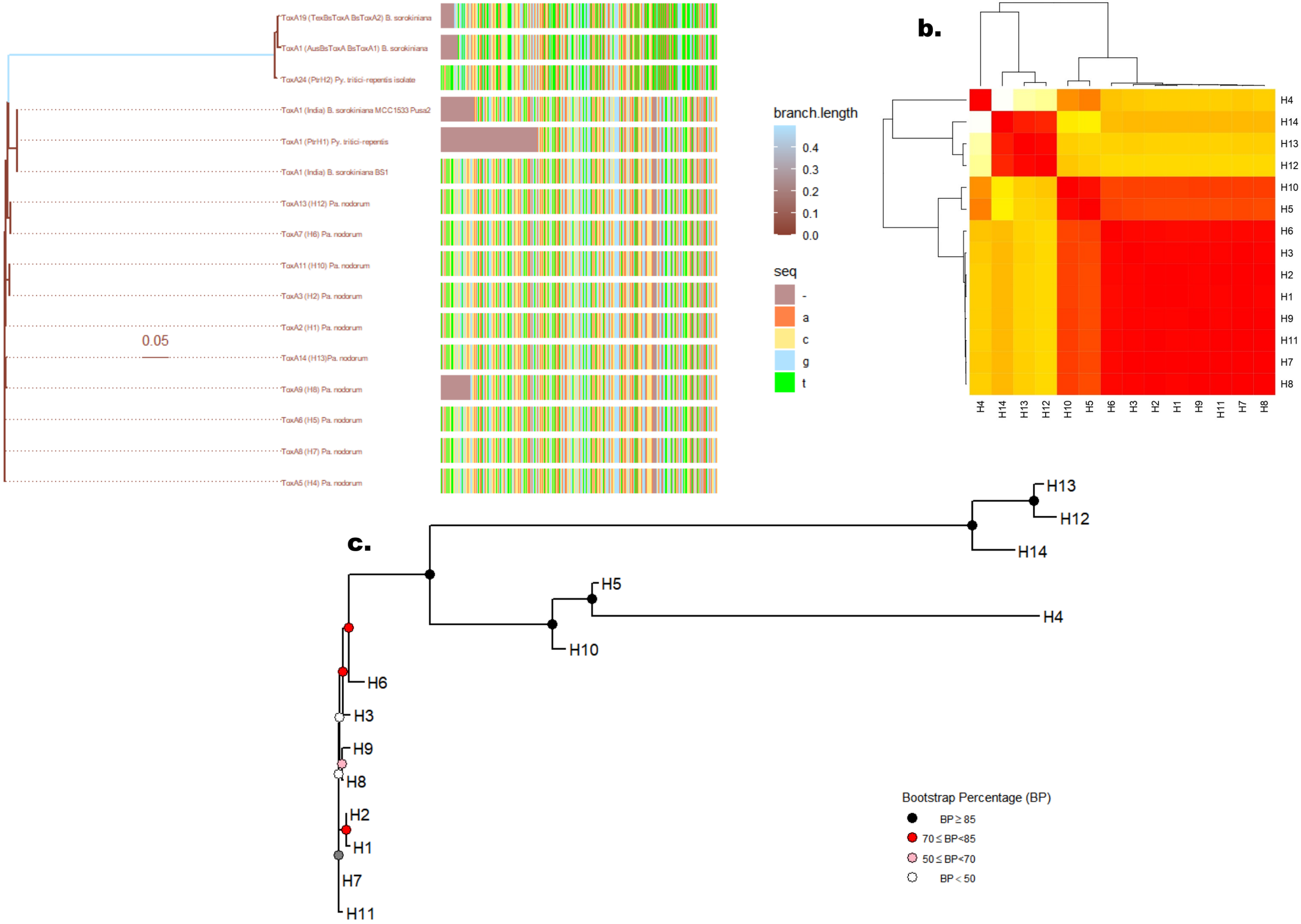

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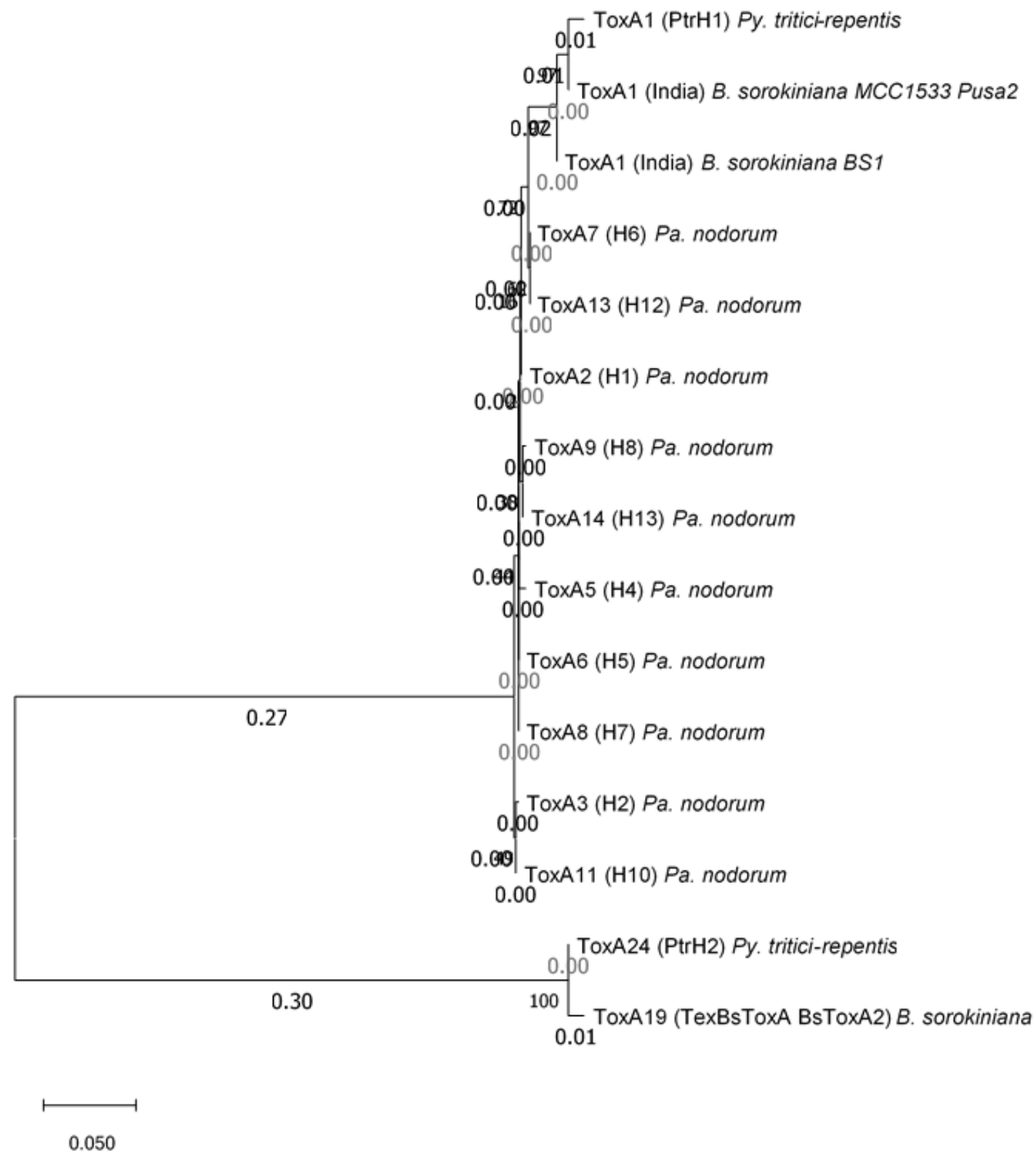

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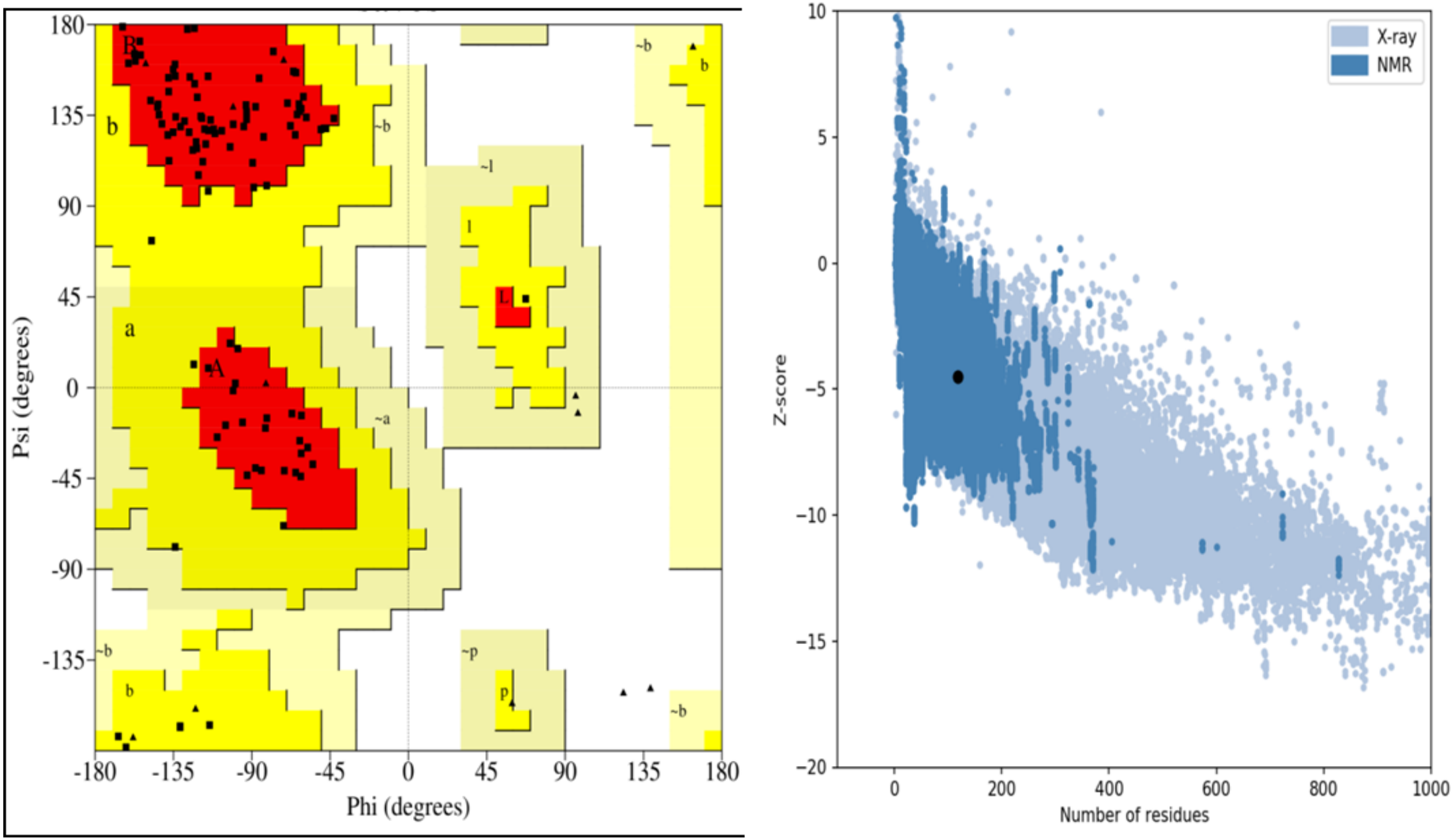

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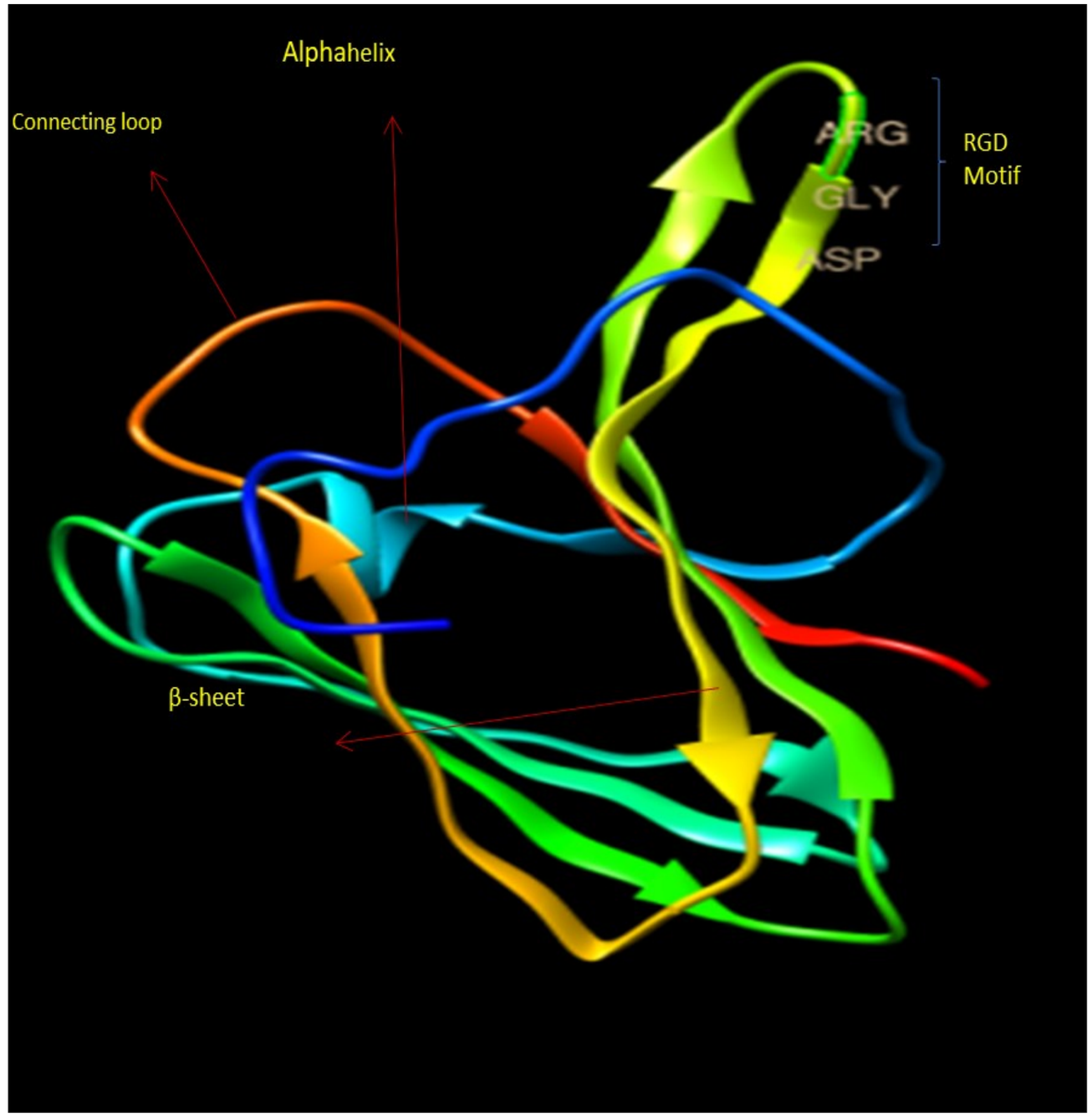

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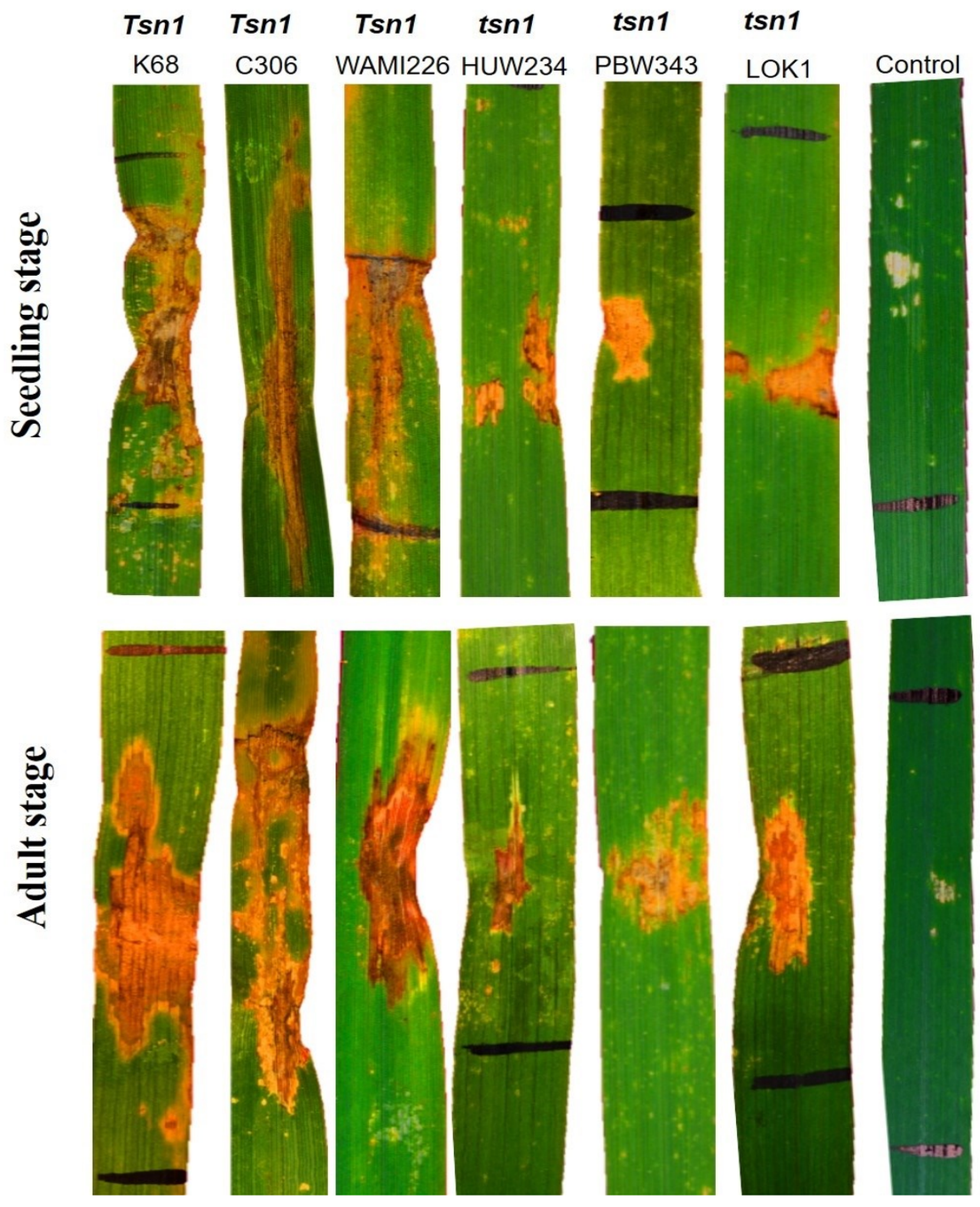


Fig. 6 Symptom of leaf necrosis on Tsn1/tsn1 harbouring genotypes after infiltration ToxA1 protein at seedling (3-5 leaf stage) and on flag leaf of adult plant (Zadoks growth stage 55).

Table1: Response of wheat genotypes (*Tsn1/tsn1*) in terms of leaf necrosis after infiltration of ToxA1 protein at both seedling and adult stage of wheat genotypes harboring Tsn1/tsn1 allele.

Genotypes	**Length(cm)**		**Width(cm)**		**Area(cm2)**		**Tsn1/tsn1**
	Seedling	Adult	Seedling	Adult	Seedling	Adult	
K 68	2.872	4.613	0.404	0.530	0.977	2.326	*Tsn1*
C 306	1.921	4.226	0.329	0.633	0.678	1.737	*Tsn1*
HUW468	2.057	3.472	0.402	0.438	0.718	1.407	*Tsn1*
Yangmai 6	0.860	1.406	0.311	0.381	0.316	0.450	*Tsn1*
K65	1.513	2.582	0.380	0.566	0.388	1.096	*Tsn1*
WAMI160	1.615	3.461	0.345	0.506	0.646	1.521	*Tsn1*
WAMI188	1.693	2.582	0.310	0.500	0.585	1.036	*Tsn1*
WAMI224	1.643	2.857	0.330	0.488	0.476	1.298	*Tsn1*
WAMI226	1.782	2.320	0.405	0.503	0.628	1.090	*Tsn1*
WAMI277	1.464	2.174	0.360	0.485	0.464	1.024	*Tsn1*
WAMI288	1.591	2.054	0.432	0.575	0.622	1.017	*Tsn1*
Max.	2.872	4.613	0.432	0.633	0.977	2.326	
Min.	0.860	1.406	0.310	0.381	0.316	0.450	
HUW234	0.964	1.894	0.335	0.408	0.345	0.561	*tsn1*
PBW343	0.860	1.446	0.349	0.408	0.319	0.423	*tsn1*
LOK1	1.098	1.761	0.307	0.432	0.278	0.493	*tsn1*
Sonalika	1.919	2.923	0.361	0.492	0.584	1.321	*tsn1*
WAMI280	0.972	1.305	0.319	0.327	0.265	0.411	*tsn1*
WAMI289	1.065	1.471	0.319	0.344	0.342	0.529	*tsn1*
WAMI290	1.206	1.467	0.327	0.360	0.365	0.493	*tsn1*
Max.	1.919	2.923	0.361	0.492	0.584	1.321	
Min.	0.860	1.305	0.307	0.327	0.265	0.411	

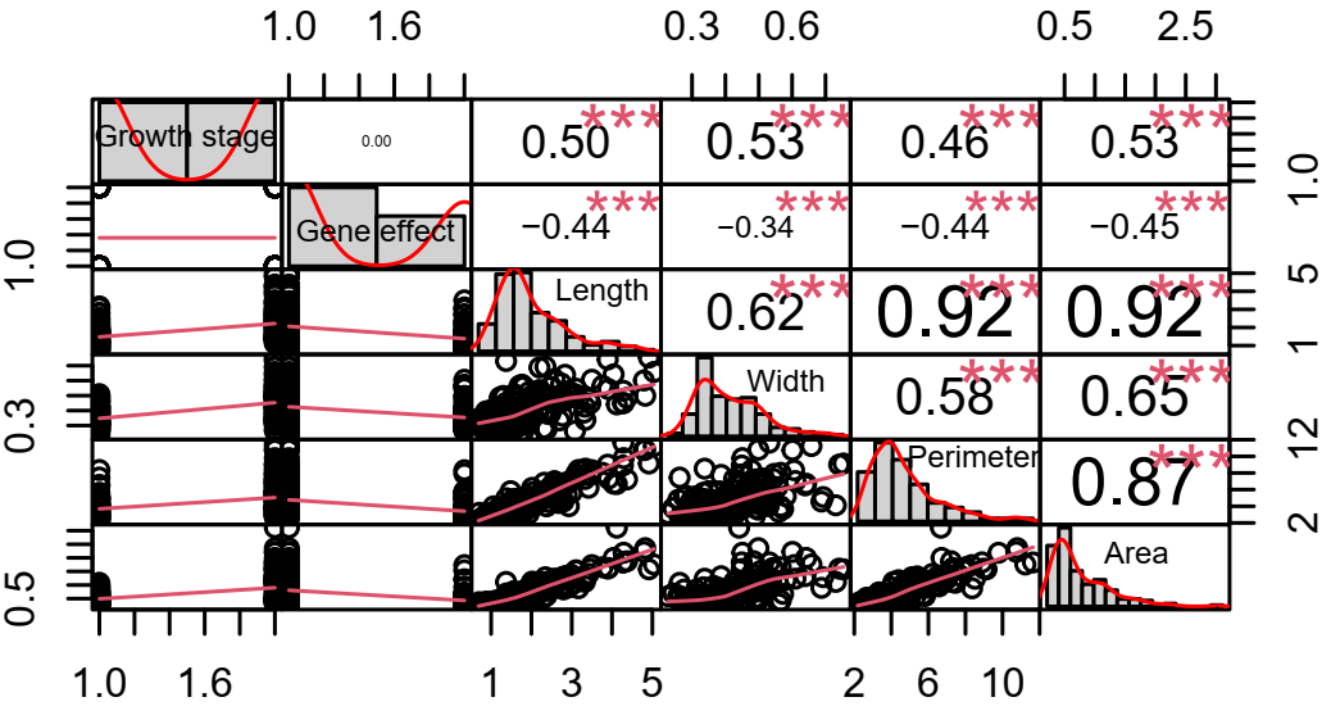
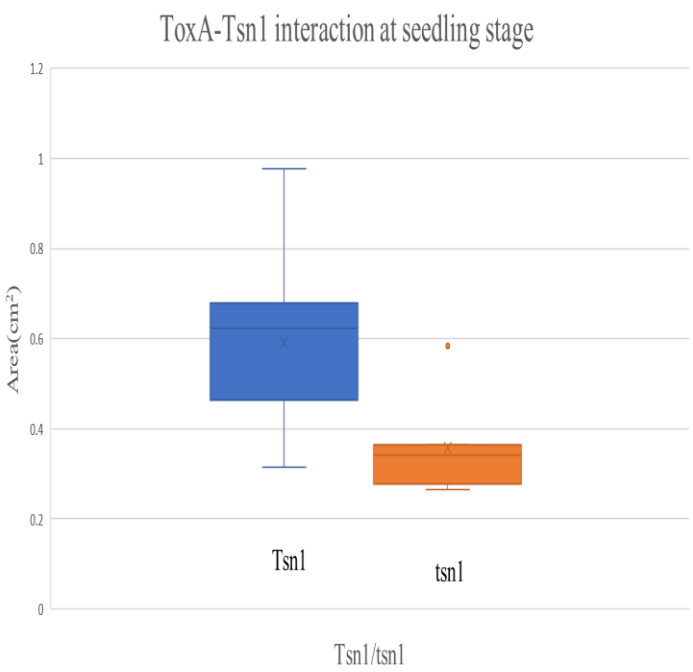
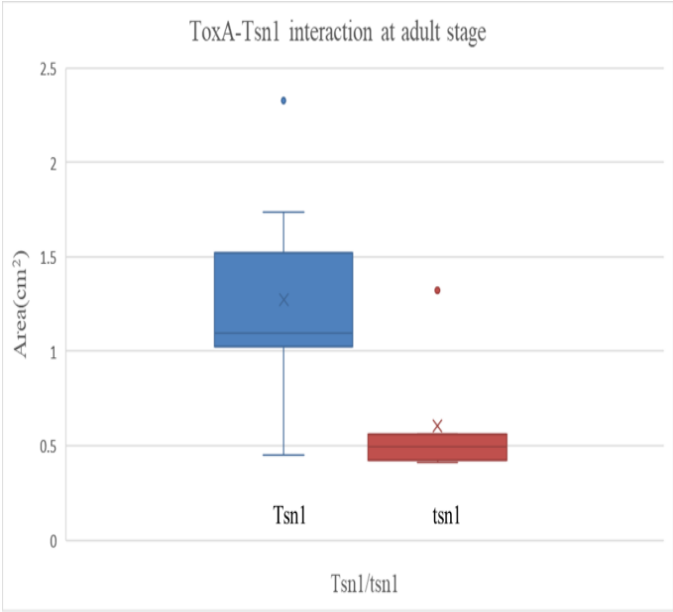


Figure S1: Correlation coefficients estimated by point biserial correlation for phenotypic traits associated with*Tsn1/tsn1* gene effect in wheat genotypes with ToxA1 isolate (MCC1533/Pusa2)



A. Seedling Stage



B. Adult stage

Figure S2: The necrotrophic area developed due to ToxA1–Tsn1 interaction at seedling (3-5 leaf stage) and adult stage (Zadoks growth stage 55) of 11 wheat genotypes with *Tsn1* and 7 wheat genotypes with *tsn1*.

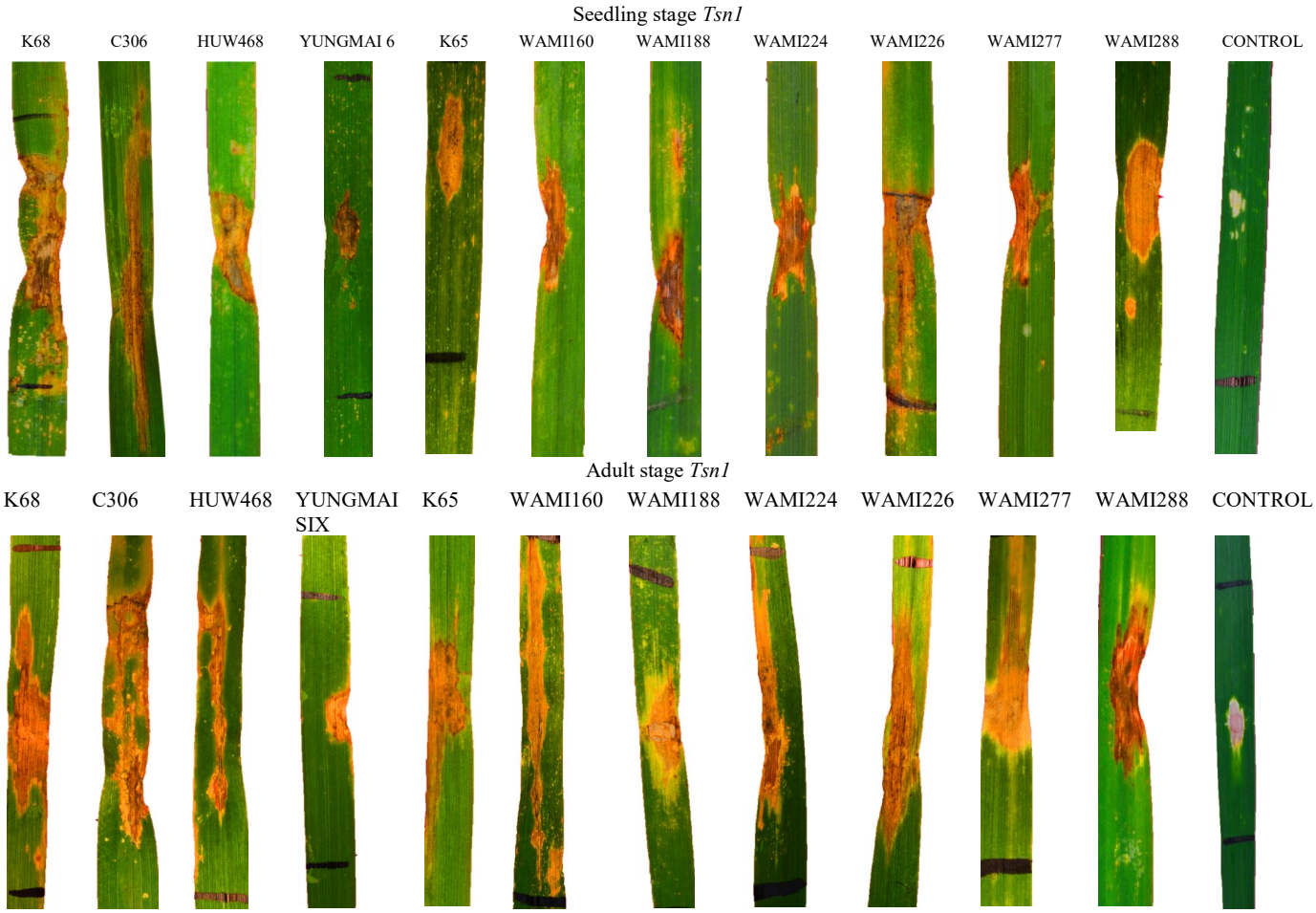
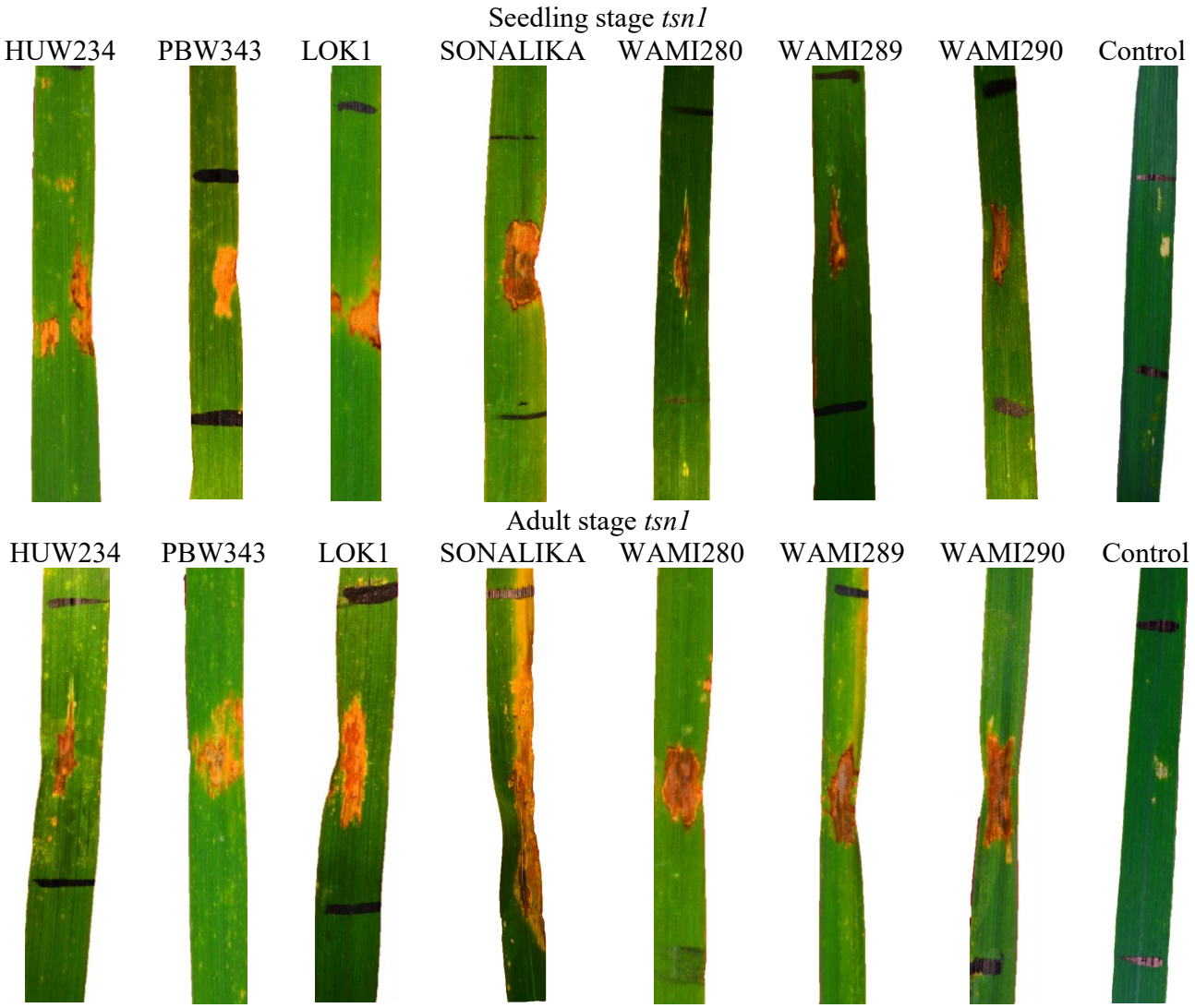


Figure S3. Symptom of leaf necrosis after infiltration ToxA1 protein at both seedling (3-5 leaf stage) and adult stage (Zadoks growth stage 55) of wheat genotypes harbouring *Tsn1*.



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