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GSh302 :A candidate effector protein in *Ascochyta rabiei*, induced during host interaction, with a promoter structure harboring a bZIP transcription factor binding site

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Abstract

Effector proteins secreted by necrotrophic fungi have been considered essential to identify and understand their functions to develop effective defenses and breed resistant plant varieties. In recent years, bioinformatics-based analysis tools have increased the likelihood of identifying these effector genes by providing genomic and transcriptomic data on the stages of pathogen development within the host. In this study, a combination of in silico methods were used to identify the effectors of the fungus *Ascochyta rabiei*, focusing on the structural features of effector proteins, including secretion, short length, and disulfide-bond-forming ability. Also, the promoter sequence analysis of the gene encoding the effector protein and the presence of regulatory sites for transcription factors involved in the pathogen-host interaction were used to confirm the functional relationship of the candidate effector. Moreover, the candidate gene was tracked at the genome and transcriptome levels in *A. rabiei* under growth conditions in culture medium and during interactions with resistant and susceptible chickpea genotypes. A 159-amino-acid protein, GSh302, was identified as a candidate effector, and its coding sequence (accession number OR875943) and protein sequence (accession number WPV74376) were registered in GenBank. Analysis of the upstream sequence of the gene confirmed the presence of regulatory elements, including transcription factor binding sites and the Sko1 (bZIP_1) transcription factor, which plays a key role in activating the expression of secretory genes during the fungus-plant interaction. The gene sequence was tracked in *A. rabiei* isolates grown in culture medium, but it was not detectable at the transcript level. This gene was discernible at the genome and transcriptome levels of the fungus in resistant and susceptible chickpea cultivars 96 hours after inoculation. The combined method used in this study identified the GSh302 protein as a candidate effector in *A. rabiei*, expressed during the fungus-chickpea interaction.

Keywords: Computational Analyses, Gene Expression Tracking, In Silico Predictions, Molecular Fungal-Plant Interaction, Secretome

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GSh302: پروتئین نامزد افکتوری در قارچ *Ascochyta rabiei*، القاشونده در شرایط برهمکنش با گیاه میزبان، با ساختار پیشبری حامل جایگاه اتصال فاکتور رونویسی bZIP

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چکیده

شناسائی و درک کارکرد پروتئین‌های افکتوری ترشح‌شده توسط قارچ‌های نکروتروف برای توسعه روش‌های دفاعی مؤثر در گیاهان و اصلاح ارقام مقاوم مورد توجه قرار می‌گیرد. فراهم شدن داده‌های ژنومی و ترانسکریپتومی مربوط به مراحل توسعه بیمارگر روی میزبان می‌تواند با پیشرفت‌هایی که در ابزارهای واکاوی مبتنی بر روش‌های بیوانفورماتیک انجام شده‌است امکان شناسائی این ژن‌های افکتوری را بطور چشمگیری افزایش دهد. در مطالعه حاضر با هدف شناسائی افکتورهای قارچ *Ascochyta rabiei* از ترکیبی از روش‌های این سیلیکو با تمرکز بر ویژگی‌های ساختاری پروتئین‌های افکتوری از جمله ترشحی بودن، طول کوتاه، توانائی تشکیل باندهای دی‌سولفیدی استفاده شده است. همچنین به منظور تایید ارتباط کارکردی افکتور نامزد شده از روش واکاوی توالی پیشبر ژن کدکننده پروتئین افکتوری و بررسی حضور جایگاه‌های تنظیمی مرتبط با فاکتورهای نسخه برداری دخیل در برهمکنش بیمارگر و میزبان استفاده شده است. همچین ردیابی ژن نامزد شده در سطح ژنوم و ترانسکریپتوم قارچ *A. rabiei* در شرایط رشد در محیط کشت و همچنین در شرایط برهمکنش با گیاهان مقاوم و حساس نخود مورد بررسی قرار گرفته‌است. در این بررسی‌ها یک پروتئین ۱۵۹ اسیدآمینه‌ای به عنوان نامزد افکتوری شناسائی شد و تحت نام GSh302 موقعیت توالی کدکننده و توالی پروتئین آن به ترتیب با شماره بازیابی OR875943 و WPV74376 در بانک ژن ثبت شد. واکاوی توالی بالادست ژن، حضور عناصر تنظیمی جایگاه اتصال فاکتورهای نسخه-برداری از جمله Sko1 (bZIP_1) را تایید نمود که در فعال‌سازی بیان ژن‌های ترشحی در مراحل برهمکنش قارچ با گیاه نقش کلیدی دارد. ردیابی توالی ژن در جدایه‌های قارچ *A. rabiei* رشد یافته در محیط کشت تایید گردید ولی در سطح رونوشت آن‌ها قابل ردیابی نبود. این ژن در گیاهان مقاوم و حساس نخود ۹۶ ساعت پس از مایه زنی در سطح ژنوم و ترانسکریپتوم قارچ قابل ردیابی بود. روش ترکیبی بکارگرفته شده در این مطالعه، پروتئین GSh302 را به‌عنوان یک پروتئین نامزد افکتوری در قارچ *A. rabiei* که در زمان برهمکنش قارچ با گیاه نخود بیان می شود معرفی نمود.

کلید واژه‌ها: برهمکنش مولکولی قارچ و گیاه، پروتئوم ترشحی، پیش‌بینی‌های این‌سیلیکو، ردیابی بیان ژن، واکاوی بیوانفورماتیک

دبیر تخصصی: دکتر روح الله شریفی

Introduction

Ascochyta blight, caused by the necrotrophic fungus *Ascochyta rabiei,* is a devastating disease of chickpea that leads to significant yield losses worldwide. Controlling this disease is a major challenge, requiring the development of chickpea cultivars that are resistant to it. A key to achieving durable resistance lies in understanding the molecular basis of pathogenicity, particularly the role of fungal effector proteins. These small, secreted proteins are crucial virulence factors that manipulate host plant processes to facilitate infection. Effector detection in plant pathogens leverages a multifaceted approach that integrates bioinformatic predictions and experimental validation. A foundational strategy relies on sequence-based features, primarily the prediction of N-terminal secretion signals using tools like SignalP, which is crucial for eukaryotic pathogens and bacteria lacking specialized secretion systems. Genomic analyses provide another critical layer by exploiting the tendency of effector genes to be located in repeat-rich regions, on accessory chromosomes, or in genomic islands. Furthermore, transcriptomic profiles during infection are invaluable, as induced expression *in planta* or co-expression with known virulence genes can pinpoint active effectors, with promoter analysis of co-regulated genes revealing regulatory motifs (Lovelace et al., 2023).

Based on previous studies, several effector proteins have been identified in the necrotrophic fungus *Ascochyta rabiei* using various molecular and bioinformatic approaches. For instance, the ARPEC25 effector was characterized for its role in suppressing host lignin biosynthesis, thereby enhancing pathogen virulence (Singh et al., 2023). More recently, a programmed cell death (PCD)-inducing effector originally reported in *Rhizoctonia solani* was also detected in *A. rabiei* isolates through homology-based searches (Shokouhifar et al., 2024). Additionally, homologues of the ECP6 effector protein that have been reported first in *Cladosporium fulvum (De Jonge & Thomma, 2009) and* a candidate effector named ArLysM1 was identified in the genomic data of *A. rabiei* using homology search approaches (Firouzmand et al., 2023a). A transcriptomic study by comparing *A. rabiei* isolates with contrasting pathogenicity levels identified 105 differentially expressed genes (DEGs), which showed the upregulation levels in a highly pathogenic isolate that involves CoNIS1 (a suppressor of plant immunity), PsGIP2/PsGIP1 (a glucanase inhibitor), and PesCDA/VdPDA1 (a chitin-binding effector involved in immune evasion) (Monsur et al., 2025).

While conventional in silico methods provide a valuable starting point for predicting small, cysteine-rich secreted proteins, they can also result in many candidates with unknown functions. To refine these predictions, alternative strategies were used that accounted for regulatory sequences within the promoter. They can facilitate targeted identification of effector proteins by leveraging signaling pathways in which the corresponding transcription factors are also involved. Transcription factors like bZIP, which are induced during plant pathogen interaction, can regulate the expression of pathogenicity genes. Screening effector candidates for binding motifs for such factors (e.g., the wtGTAGTAAkr sequence for bZIP in *A. rabiei*) provides a powerful layer of validation, as demonstrated by Verma et al. (2017) and John et al. (2021).

This combined bioinformatic approach has been successful. For instance, the effector gene *GSh303* was identified in *A. rabiei* using classical in silico tools and further validated by the presence of relevant regulatory motifs in its promoter. Subsequent laboratory analysis confirmed its presence

across different pathotypes at the genomic level and tracked its expression pattern *in vitro* and during interaction with resistant and susceptible chickpea plants (Hasani et al., 2023b). The present study was designed to characterize a novel effector candidate protein in *Ascochyta rabiei by* implementing a refined *in silico* pipeline involving sequence-based feature analysis, regulatory sequence analysis, and transcriptomic profiling based on publicly available SRA data for both the effector gene and its correlated transcription factor. Finally, the selected candidate was subjected to experimental validation, during which its expression profile was quantified across both aggressive and weak pathotypes of *A. rabiei*.

Materials and Methods

Obtaining biological data

To select candidate effector genes, the complete *A. rabiei* fungal protein sequence was retrieved from the NCBI database using the genome version GCA_004011695.2 and imported into CLC Genomics Workbench for further analysis. Candidate effector proteins were then identified and selected using various bioinformatics tools.

Secretory protein identification

Proteins with a length of fewer than 250 amino acids were selected amongst the protein sequences retrieved from the NCBI database.

The TargetP 2.0 tool (https://services.healthtech.dtu.dk/service.php?TargetP-2.0) and its AI-based version DeepLoc 2.1 (https://services.healthtech.dtu.dk/services/DeepLoc-2.1/) were used to predict eukaryotic protein subcellular localization. DeepLoc 2.1 is a multi-label predictor that can differentiate between localizations such as nucleus, cytoplasm, extracellular, mitochondrion, cell membrane, endoplasmic reticulum, chloroplast, Golgi apparatus, lysosome/vacuole, and peroxisome. The extracellular class peptides were selected for the next step of analysis. Transmembrane peptides are well defined as peripheral, transmembrane, lipid-anchored, and are distinguished from soluble peptides (non-membrane). The tools SignalP 4.1 (https://www.cbs.dtu.dk/services/SignalP/) and SecretomeP 1.0 (https://services.healthtech.dtu.dk/service.php?SecretomeP-1.0) were applied to select classical and non-classical secreted proteins, respectively. The TMHMM tool (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) was utilized to confirm elimination of transmembrane proteins from the selected peptides.

Prediction of cysteine-rich proteins

The DiANNA tool (http://clavius.bc.edu/clotelab/DiANNA/) was used to predict the presence of cysteine-rich sequences and the ability to form disulfide bonds.

Effector protein prediction using the EffectorP

Effector proteins were selected from the chosen proteins in the previous steps using the EffectorP 3.0 (https://effectorp.csiro.au/) tool (Sperschneider & Dodds 2022), which analyzes amino acid sequences based on their characteristics, including molecular weight, net charge, amino acid composition, and the presence of cysteine, serine, and tryptophan.

Prediction of candidate effector protein functional domains

The protein sequences selected by EffectorP were further checked for the presence or absence of a functional polypeptide chain using tools available in the Pfam database (http://pfam.xfam.org/).

Cis-regulatory elements analysis

From the upstream part of the candidate gene, 1000 bp was retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov). Conserved motifs in the target sequences were identified using the MEME database (https://meme-suite.org/meme/db/motifs)

with default options. The Tomtom v 5.4.1 tool (https://meme-suite.org/meme/tools/tomtom) was used to identify known cis-regulatory elements based on the JASPAR CORE 2020 fungal database, with E-value > 5, and choosing the family of *Saccharomycetaceae* as the default microorganism in the tool (Fornes et al., 2020). The GOMO tool (https://memesuite.org/meme/tools/gomo) was used for additional checks and to predict the possible functions of each feature.

Primer design for candidate effector genes

The sequence of the *GSh302* gene with the accession number (ST47_g6850) was selected and retrieved as an effector gene from the reported transcriptomic data in the NCBI database (Verma et al., 2016). The SnapGene software was used to design specific primers. The designed primers were PSh302-F (5'-ATGCGCTTCGTACTCGCTGC-3') and PSh302-R (5'-ACATGCTCGTTCTCCTCTGCCTA-3'), synthesized by Sinaclon Company, Iran. Moreover, PSh19F/R primers (Zangene et al., 2022), which belongs to the ArGAPDH housekeeping gene family, was used as a reference gene (EKO05-007398).

Fungal pathotypes cultivation and DNA extraction

Three pathotypes of *A. rabiei* named PI (Pathotype No. 1), PIII (Pathotype No. 3), and PVI (Pathotype No. 6), and following strain numbers: FUM 1001, FUM 1003, and FUM 1006, respectively, were obtained from the Microorganisms Collection of Ferdowsi University of Mashhad (WDCM 1207), Iran. These pathotypes had been classified in previous studies (Shokouhifar et al., 2003) and used to track candidate effector genes. Fungal pathotypes were grown for DNA extraction in fresh potato dextrose broth (PDB) at 24±2°C for two weeks. DNA extraction was performed using the CTAB method after fungal growth (Murray & Thompson 1980).

Tracking and amplification of candidate effector gene in the fungal pathotypes grown under *in vitro* conditions

The PCR (polymerase chain reaction) mixtures contained 5 µl of 2X PCR master mix (Sinaclon Company, Iran), 50 ng of genomic DNA, 1 µl (5 pmol) of each PSh302-F/R-specific primer, and distilled water to a final volume of 10 µl. PCR was conducted in a thermocycler (Eppendorf, Germany) with the following thermal protocol: an initial denaturation at 94 °C for 3 min, followed by 35 amplification cycles consisting of 93 °C for 45 s for DNA denaturation, 60 °C for 40 s for primer annealing, and 72 °C for 90 s for DNA extension. Also, a final extension step at 72 °C for 5 min was included. Three µL of PCR product was imaged on a 1% agarose gel containing 10% GreenView dye.

RNA extraction and transcriptome-based gene detection under *in vitro* conditions

Total RNA was extracted from the fungal mycelium using the Column RNA Isolation kit (Dena Biotech Co., Iran). The quantity and quality of the extracted RNA were checked by electrophoresis on an agarose gel. The cDNA was constructed using a cDNA preparation kit (Parstous, Iran) according to the manufacturer's instructions. The constructed cDNA was used as a template in a PCR reaction to amplify the target gene with PSh302-F/R primers (Table 1). The PCR protocol was the same as the conditions described above.

Inoculation of detached chickpea leaves by *Ascochyta* pathotypes

The seeds of two chickpea genotypes, MCC133 and ILC1929, respectively, as resistant and sensitive lines to *A. rabiei*, were obtained from the seed bank of Ferdowsi University of Mashhad, Iran. The resistance level of these genotypes had been determined in previous studies under field, greenhouse,

and laboratory conditions (Shokoohifar et al., 2003; Shokouhifar et al., 2016; Firouzmand et al., 2023b; Ramezani Khozestani et al., 2023). The pathogenicity of three *A. rabiei* pathotypes named PI, PIII, and PVI was reported in our previous study (Shokoohifar et al., 2003), which were provided by the microorganism bank of Ferdowsi University of Mashhad with the following reference numbers: FUM 1001/ASR001, FUM 1003/ASR003, and FUM 1006/ASR006, respectively. Plant sample preparation steps, including cultivation and preparation of leaf samples from two chickpea genotypes, and also preparation of fungal inoculum, were carried out under *in vitro* conditions using the method suggested by (Firouzmand et al., 2023b; Ramezani Khozestani et al., 2023). The leaf samples of each chickpea genotype were inoculated with sterile water as a negative control.

Tracking of *A. rabiei* in inoculated chickpea under interaction conditions at the genome and transcript level

Inoculated leaves were collected 96 h after inoculation, immediately placed in liquid nitrogen, and stored for further experiments. Genomic DNA was extracted from the samples using the CTAB method. Total RNA was extracted using the Column RNA Isolation kit (Dena Biotech Company, Iran). The cDNA was synthesized using a cDNA synthesis kit (Parstous, Iran). The quality and quantity of DNA and RNA were assessed on a 1% agarose gel, and fungal tracking was performed at the genomic and transcriptomic levels. The efficiency of the constructed cDNA was evaluated using specific primers PSh19-F (5´- CCT TCA TCG AGC CCC ACT AC -3´) and PSh19-R (5´- GCC TCC CTT CAA GTG AGCC -3´) designed for the reference gene *ArGAPDH* of *A. rabiei*. In addition, specific primers PABCT-F (5´-TCA CAG GTT GTGATG GAG TCTG-3´) and PABCT-R (5´-CCT CAA ATC TTG TTG GGG TGTC -3´) (Reddy et al., 2016), related to the housekeeping gene *ABCT* of the chickpea plant (accession number LOC101505577), were applied to confirm the construction of cDNA in control plants. The PCR was performed for both primers in a volume of 10 µl with the same conditions as stated above.

Tracking of the *GSh302* gene under interaction conditions at the genome and transcript level

To track the GSh302 gene during interaction between the chickpea plant and *A. rabiei*, PCR amplification of the *GSh302* gene was performed using specific primers (PSh302F/R). The extracted DNA and constructed cDNA under interaction conditions were used as templates, respectively.

Results

Genomic and transcriptomic data on *A. rabiei*, along with general characteristics of effector proteins, were used to identify effector proteins of this fungus. The presence of regulatory sequences associated with transcription factors involved in pathogenicity was confirmed based on the diagram shown in Figure 1.

In the first step, 2,347 proteins with fewer than 250 amino acids in their sequences were selected from 10,596 protein sequences related to *A. rabiei* available in the NCBI database (Figure 1-step1). More attention and investigation were paid to this selected set of proteins.

The Target-P 2.0 tool predicted 330 secretory proteins among 2347 selected small proteins. The tools Secretom-P and Signal-P 6.0 were used to identify 319 classical secretory proteins (with N-terminal signal peptide) and 1139 non-classical secretory proteins (without N-terminal signal peptide) among 2347 proteins. To confirm secreted proteins, we evaluate the selected proteins using DeepLoc-2.1, a Prediction of eukaryotic protein subcellular localization through deep learning (Figure 1 – step 2).

The secretory proteins with at least four cysteine residues (outside the signal peptide sequence) and two disulfide bonds were analyzed, and 113 sequences belonging to classical proteins and 231 to non-classical proteins were subsequently selected. A total of 344 small secretory proteins were identified, which were rich in cysteine amino acids (Figure 1 – step 3). Analysis of this collection using the TMHMM tool identified 1282 secretory proteins lacking transmembrane domains, of which 224 were classical, and 1058 were non-classical (Figure 1 – step 4).

The results of sequence analysis for 344 proteins, which were selected at this step, were analyzed by the Effector-P v3.0. 82 candidate effector proteins were identified among the classical secreted proteins, including 15 as cytoplasmic effectors and 67 as apoplastic effectors. Moreover, 175 non-classical secreted proteins were predicted, which contained 155 cytoplasmic and 20 apoplastic sequences as effectors. By this step, 257 secreted proteins had been identified as effectors in *A. rabiei* (Figure 1 – step 3). Searching these proteins in the Pfam database showed that 108 proteins lacked any functional polypeptide domains (Figure 1 - step 3).

Although many transcription factors can induce effector proteins, based on expression data for the bZIP factor, this study focused only on secreted proteins carrying regulatory elements in the promoter sequence upstream of the gene coding sequence. Accordingly, the proteins carrying this motif were identified (Figure 1- Step 4). *GSh302* was selected for further laboratory investigation among the proteins with bZIP-related motifs.

Based on analyses performed according to the diagram above, among the proteins expressed in *A. rabiei*, four candidate effector proteins were identified, each with an upstream regulatory site associated with a bZIP transcription factor. A number of these four peptides, including GSh301 (Hasani et al., 2023a), GSh303 (Hasani et al., 2023b), GSh304 (Hasani et al., 2025) have been previously reported individually. However, only the peptide *GSh302*, whose final analyses have recently been completed, has been fully introduced in the present study due to its unique characteristics. Recently, the expression pattern of this peptide in multiple *A. rabiei* isolates has been confirmed to be highly conserved, and its expression in fungal isolates has also been shown to be induced during interactions between the isolates and the plant (Bahrabadi et al., 2025). Based on these features, the present study provides complete information on this effector protein, including its structural features and the presence of regulatory elements associated with transcription factors involved in fungal pathogenicity. The effector protein's transcript has also been examined under conditions of interaction between isolates with different pathogenicity of the fungus in resistant and susceptible chickpea plants in varying hours after inoculation, and the relevant results are presented in this study.

Detection of cis-regulatory elements in the promoter sequence of the *GSh302* gene

From the upstream of the *GSh302* gene, a 1000 bp sequence was considered to identify conserved motifs and cis-regulatory elements using JASPAR 2024. The core collection under the Fungi taxon was used to scan 1,000 nucleotides upstream of the transcription initiation site in GSh302, using a 90% relative profile score threshold. Among the 26 profiles of fundamental leucine zipper factors (bZIP) reported from *Saccharomyces cerevisiae* in the JASPAR databases, 18 sequences belonging to 7 motifs were detected, with around 6 to 10 nucleotides identified in the promoter region of the *GSh302* gene (Table 1). Computational analysis of the promoter sequence revealed several high-confidence bZIP transcription factor binding sites with potential

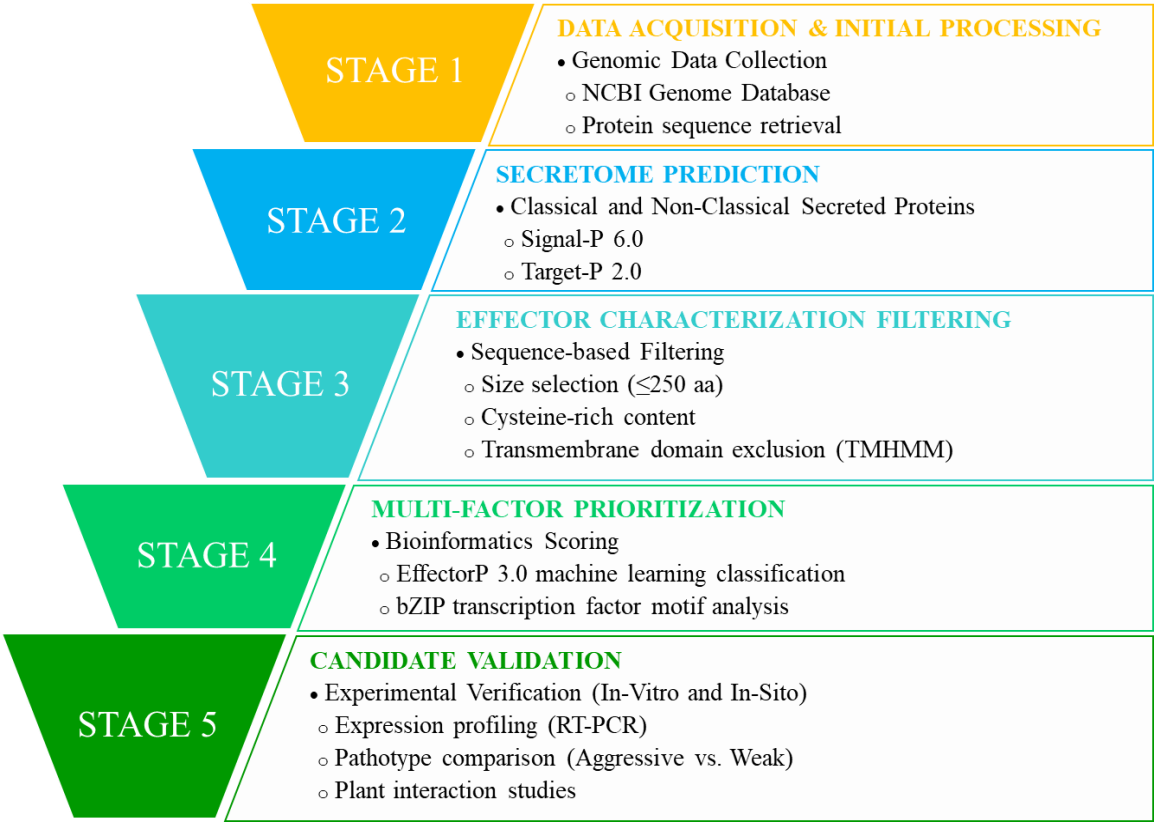


Figure 1. Integrated bioinformatics pipeline for effector protein identification in *Ascochyta rabiei*

Stage 1: Data acquisition and initial processing

- **Genomic data collection: Retrieving *Ascochyta rabiei* genomic data from the NCBI genome database.**
- **Protein sequence retrieval: Extracting and compiling all predicted proteins sequences from the annotated genome.**

Stage 2: Secretome prediction

- **Signalp: Identifying proteins containing N-terminal secretion signal peptides using SignalP 4.1 or 6.0.**
- **Secretomep: Applying Secretomep 2.0 to predict non-classical proteins secretion pathways and expand the secretome candidate list.**

Stage 3: Effector characterization filtering

- **Sequence-based filtering: Selecting proteins with a length ≤250 amino acids.**
- **Cysteine-rich content: Analyzing and filtering for proteins with elevated cysteine content, ≥4 cysteines.**
- **Transmembrane domain exclusion: Using TMHMM 2.0 to exclude proteins containing transmembrane helices (except the signal peptide).**

Stage 4: Multi-factor prioritization

- **Effectorp 3.0: Employing machine learning-based classification to predict effector probability.**

Bzip transcription factor motif analysis: Screening for the presence of bzip transcription factor binding motifs, which may be associated with regulation during infection.

- **SRA expression data integration: Integrating RNA-seq data from the public SRA datasets to assess expression levels during infection or under stress conditions.**

Stage 5: Candidate validation

- **Experimental verification**
- **Pathotype comparison: Comparing candidate effectors between aggressive and weak pathotypes to identify virulence-associated factors.**
- **Expression profiling (rt-PCR): Validating gene expression patterns via rt- PCR during host infection.**
- **In vitro mycelium profiling: Assessing baseline expression levels in mycelium grown in culture.**
- **Plant-pathogen interaction profiling: Conducting functional assays (e.g., transient expression in plants) to confirm the role in virulence and plant immune suppression.**

significance in the pathogenicity of *A. rabiei* (Table 1). Among the identified sites, MET28 (relative score = 1.00), YAP3 (relative score = 0.952), and HAC1 (relative score = 0.949) emerged as the most promising candidates due to their high binding scores and established roles in fungal stress response mechanisms. These transcription factors are implicated in critical processes, including oxidative stress defense (YAP family), ER stress management during effector secretion (HAC1), and sulfur metabolism for detoxification (MET28). Based on the clustering of these regulatory sites in specific genomic regions, it is likely that these genes are coordinately regulated, making them prime targets for further experimental validation and for the development of targeted control strategies for chickpea blight. bZIP family members, including MET28, YAP3, SKO1, and HAC1, have been reported to be engaged in the regulation of stress response pathways in necrotrophic fungi (Verma et al., 2016; Gai et al., 2022).

Sequence logo analysis revealed distinct conservation patterns for each motif (Figure 2). The MET28 binding site exhibited strong conservation at core positions, while YAP3 and HAC1 sites showed characteristic bZIP dimerization signatures. These motifs represented potential key regulatory elements in the pathogenicity network of *A. rabiei*.

The schematic depicts the positions of computationally identified cis-regulatory motifs within the -1000 to transcription start site (TIS) region (Figure 3). Key predicted binding sites for transcription factors Yap5, Sko1, Met28, and Hac1as are highlighted in Table 1. Notably, multiple binding motifs for Yap5 (MA0417.1.YAP5) and Sko1 (MA0382.3.SKO1 / bZIP_1) were distributed throughout the promoter, suggesting their potential roles as major regulators. Core promoter elements, including a CAAT box and a TATA box, were located proximal to the TIS. The recurrence of specific motifs indicated a complex regulatory landscape for *GSh302* expression. The promoter analysis revealed the presence of Motif 8, a *cis*-regulatory element previously identified as one of the ten most frequent motifs in the promoters of *A. rabiei* secretome-encoding genes (Verma et al., 2016). This motif was discovered through a systematic scan of 1 kb upstream regions of 758 putative secretory protein genes using the RSAT platform. Identifying Motif 8 in the promoter of *GSh302* suggested that this gene belongs to the co-regulated secretome of *A. rabiei* and is potentially controlled by the same transcriptional regulatory network that coordinates the expression of virulence factors in this necrotrophic pathogen. The presence of these *cis*-regulatory motifs, specifically matching the Sko1 (bZIP_1) profile, indicated that the expression of this gene is potentially under the direct regulatory control of this bZIP transcription factor. Computational analysis of the *GSh302* promoter revealed the presence of two specific cis-regulatory elements, which were identified as Sko1 (bZIP_1) binding sites (Figure 3). This finding was highly significant, since the identical motif (wtGTAGTAAkr) was previously reported as one of the ten most enriched elements in the promoter sequences of *A. rabiei*'s secretome—a key set of virulence-related genes (Verma et al., 2016).

Furthermore, transcriptomic data from *A. rabiei* during chickpea infection showed a distinct expression pattern for a putative bZIP transcription factor, ST47_g1688 (Fondevilla et al., 2015). This TF was not expressed during vegetative growth but was significantly upregulated at 12, 36, and 96 hours post-inoculation (HPI), with expression levels peaking at 36 HPI (FPKM 3,875*) (Verma et al., 2016). The concurrent presence of its cognate binding motif in the *GSH* promoter and the induced expression of this bZIP factor during host invasion strongly suggested a direct regulatory relationship.

Table 1. High-confidence bZIP transcription factor binding sites identified in the *Ascochyta rabiei* promoter region and their potential roles in pathogenicity.

JASPER ID	TF Name	Relative Score	Strand	Predicted Sequence	GO annotations
MA0332.1	MET28	1.00	+	ctgtgg	Sulfur metabolism and detoxification
MA0416.2	YAP3	0.952	-	cttacgt	Oxidative stress response and host defense survival
MA0382.3	SKO1	0.801978	+	atgacatagg	cellular response to osmotic stress
MA0310.1	HAC1	0.936	+	tacacgta	ER stress response and protein folding
MA0279.1	CAD1	0.914	-	gttaataatc	Environmental stress adaptation
MA0417.1	YAP5	0.980	-	aaacat	Iron homeostasis and survival under iron limitation



MA0332.1



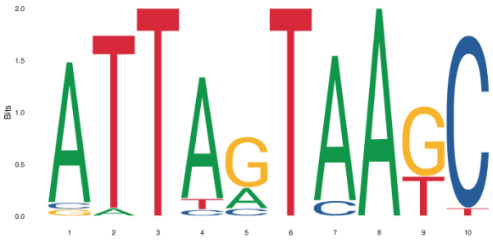
MA0416.2



MA0382.3



MA0310.1



MA0279.1



MA0417.1

Figure 2. The logo sequence of high-confidence bZIP transcription factor binding sites identified in the *Ascochyta rabiei* promoter region.

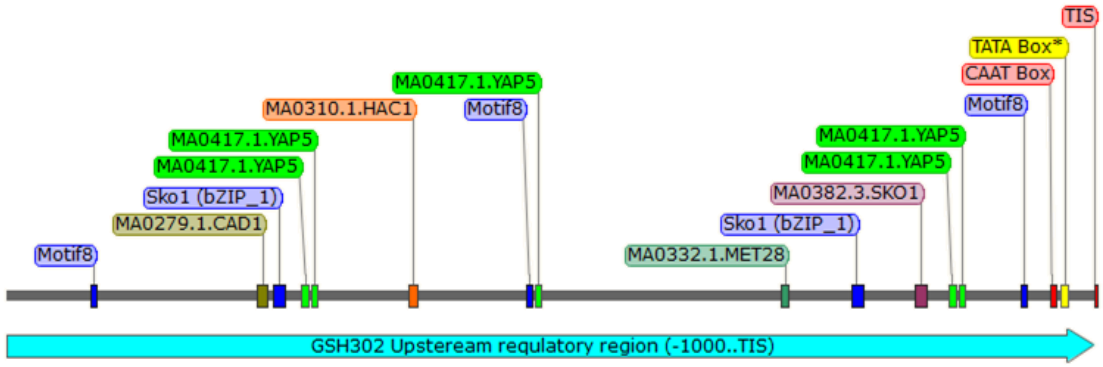


Figure 3. Schematic representation of predicted transcription factor binding sites in the upstream regulatory region (-1000 to TIS) of the *GSh302* gene.

Figure 3 shows the positions of key identified motifs relative to the Transcription Start Site (TIS, designated as +1). The MA0417.1.YAP5 motif was found at six distinct positions: -923 to -918, -729 to -724, -720 to -715, -515 to -510, -134 to -129, and -125 to -120. Motifs for MA0382.3.SKO1 and Sko1 (bZIP_1) were identified at multiple locations, including -755 to -745, -224 to -214, and -165 to -156. A single binding site for MA0332.1.MET28 was detected at position -288 to -283, and for MA0310.1.HAC1 at position -631 to -624. Core promoter elements, including a CAAT Box (-41 to -37) and a TATA Box (-31 to -26), were located proximal to the Transcription Initiation Site (TIS).

The co-occurrence of the Sko1 (bZIP_1) motif in both the *GSH* promoter and the coregulated secretome of *A. rabiei* provides compelling evidence for the integration of *GSh302* into the fungal pathogenicity program. We hypothesize that the product of the differentially expressed gene ST47_g1688, a bZIP transcription factor, acts as the key regulator binding to this motif. As a result of its significant induction during infection (12-96 HPI), rather than in culture, this TF is a critical node for orchestrating virulence genes, including those encoding secretory proteins and potentially *GSh312*. This regulatory mechanism would ensure the synchronized production of this gene alongside other necrotrophic effectors, possibly to mitigate host-derived reactive oxygen species (ROS) generated during the plant's defense response. Thus, the Sko1 motif directly links GSh302 expression to fungal infection strategies, highlighting its potential role in basic metabolism and in oxidative stress management necessary for necrotrophy.

Detection of candidate effector genes in three pathotypes of *A. rabiei* under *in vitro* conditions

The candidate putative effector gene is located in the *A. rabiei* genomic data in the NCBI database under accession OR875943. The coding gene is 582 bp and has three exons and two introns. Based on software prediction, this gene encodes a hypothetical protein of 156 amino acids, which has been registered in NCBI under accession number WPV74376. No function has yet been reported for this protein. This gene, named GSh302, was investigated for its presence across different pathotypes of *A. rabiei*. The presence of the *GSh302* gene in three pathotypes of *A. rabiei* was confirmed using PSH302-F/R primers designed for the upstream and downstream regions of this gene (Figure 4-A). The PCR results showed a single specific band within the 500-750 bp range of the ladder, corresponding to the expected size of 645 bp. This indicated that the desired fragment was present in the

genomes of the *A. rabiei* pathotypes PI, PIII, and PVI (Figure 4-B).

Total RNA was extracted from the mycelium of the pathotype PI of *A. rabiei* to determine whether GSh302 was expressed during growth on PDA, and the corresponding cDNA was subsequently constructed. PCR was performed on the cDNA using specific Ar*GAPDH* primers as a reference gene (Figure 5-A) and as a positive control to confirm the quality of the cDNA. *GSh302* was tracked in *Ascochyta* pathotypes PI at the cDNA level. Specific primers (PSh19-F1/PSh19-R1) amplified two distinct fragments associated with the ArGAPDH housekeeping gene: 331 bp at the genomic level and 239 bp at the transcript level (Figure 5B). Specific primers (PSh302-F1/PSh302-R1) amplified the gene GSh302 at the DNA level, yielding a 645 bp fragment, whereas this gene was not detected at the cDNA level under in vitro growth conditions (Figure 5-B).

Tracking of *GSh302* gene in infected chickpea detached leaves with *A. rabiei*

Symptoms of *Ascochyta* blight were observed 96 hours after inoculation with *A. rabiei* pathotype PI on the detached leaves of ILC1929 (susceptible) plant (Figure 6-A). Disease symptoms were clearly visible on inoculated leaves compared to the control samples inoculated with distilled water. No disease symptoms were observed on the detached leaves of the MCC133 (resistant) plant inoculated with the pathotype PI (Figure 6-A). However, minimal disease symptoms were observed on the leaves of the inoculated resistant plant under a stereomicroscope (data not shown). Molecular evaluation was performed by the extraction of genomic DNA and total RNA from the inoculated and uninoculated chickpea leaves of resistant (MCC133) and susceptible (ILC1929) chickpea genotypes to *Ascochyta* blight. The quality and accuracy of the constructed cDNA were checked using specific primers designed to amplify the ABCT gene, a reference gene for chickpea (data not shown). The gene *GSh302* was tracked in infected (In) and uninfected (U-In) detached leaves at the beginning of the experiment (T0) and 96 hours after infection (T96) in resistant (MCC133) and susceptible (ILC1929) chickpea genotypes at the DNA and cDNA levels. The lack of DNA contamination in the constructed cDNA was confirmed in both infected MCC133 and ILC1929 genotypes. Therefore, electrophoretic pattern results can be assumed only from cDNA prepared from RNA expressed by the fungus (Figure 6-B).

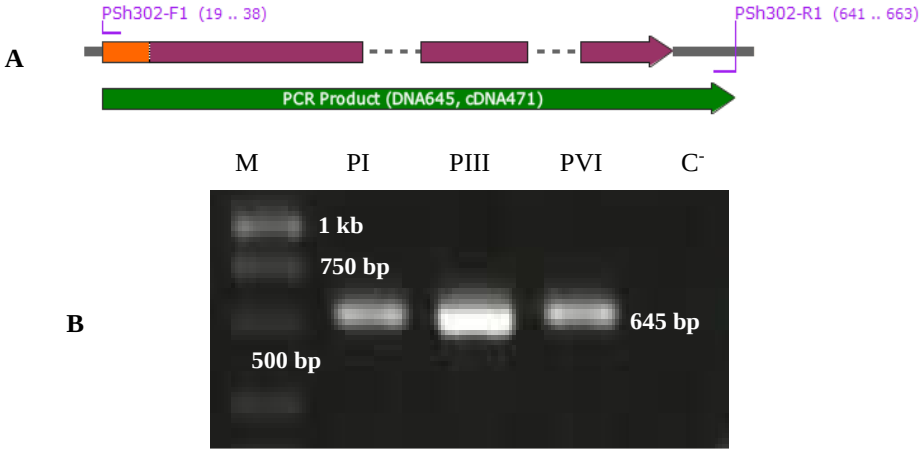


Figure 4. (A) Schematic representation of *the GSh302* gene showing the position of primers and the length of the amplified fragment from both DNA and cDNA levels. (B) Electrophoretic pattern of the *GSh302* amplified band from genomic DNA of three pathotypes (PI, PIII, and PVI) of *Ascochyta rabiei*. C⁻: negative control, L: 1Kb DNA size marker.

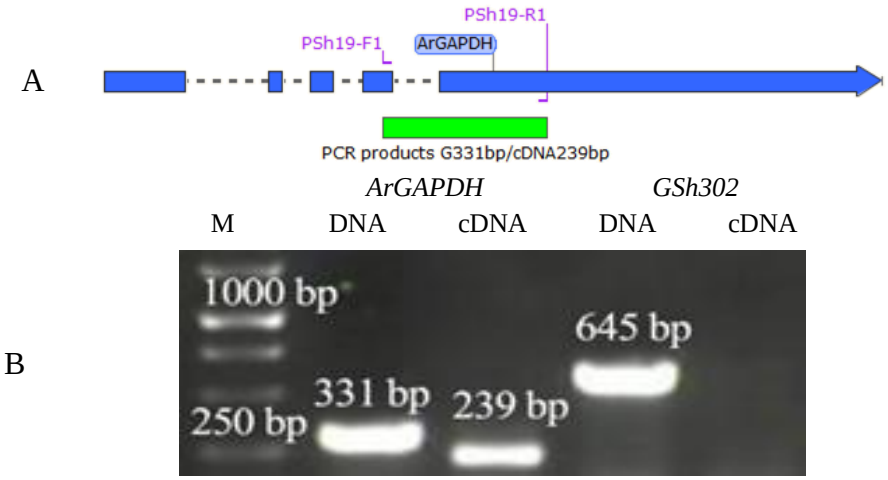


Figure 5. (A) Schematic representation of the *ArGAPDH* gene showing the position of primers and the length of the amplified fragment based on the sequence of the *ArGAPDH* gene in both DNA and cDNA levels (B) Electrophoretic pattern of the *GSh302* and *ArGAPDH* (positive control) genes with two DNA and cDNA samples related to pathotype PI of *Ascochyta rabiei*. C^-: negative control, L: 1Kb DNA size marker.

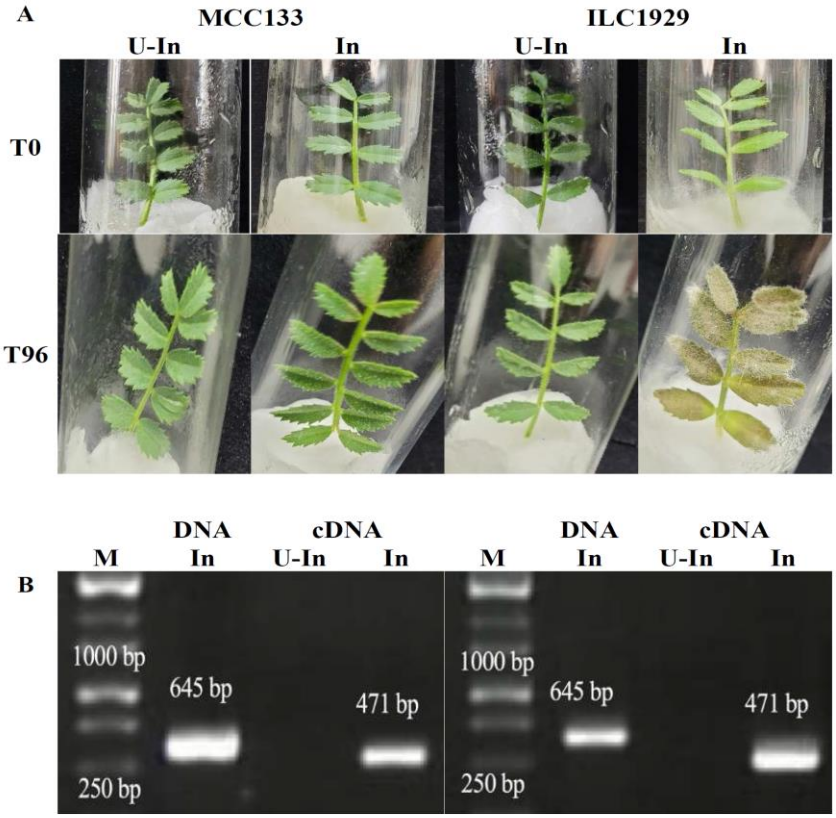


Figure 6. (A) Disease symptoms caused by *Ascochyta rabiei* pathotypes PI on two chickpea genotypes resistant (MCC133) and susceptible (ILC1929) to *Ascochyta* blight. Infected (In) and Un-Infected (U-In) detached leaves at the beginning of the experiment (T0) and 96 hours after infection (T96) on resistant (MCC133) and susceptible (ILC1929) chickpea genotypes to *Ascochyta* blight. The pictures were taken at 0 and 96 hours after inoculation. (B) The electrophoresis pattern of the *GSh302* gene using cDNA samples from resistant (MCC133) and susceptible (ILC1929) chickpea genotypes inoculated with pathotypes PI of *Ascochyta rabiei*. Lanes' DNA is extracted from MCC133 and ILC1929 chickpea plants inoculated by pathotype PI, used as a control. U-In and In are constructed cDNAs from MCC133 and ILC1929 chickpea plants inoculated with water (as the mock treatment) and *Ascochyta rabiei* PI, respectively. M is a 1 Kb DNA size marker.

The electrophoretic pattern of the amplified PCR products in the expected size (645 bp for genomic DNA and 471 bp for cDNA) confirmed that this gene was detectable at both genomic and transcript levels in resistant (MCC133) and sensitive (ILC1929) chickpea genotypes 96 hours after inoculation with pathotype PI of *A. rabiei* (Figure 6-B). These results also confirmed the expression and inducibility of the *GSh302* gene during *A. rabiei*-chickpea interactions.

Discussion

Until a few years ago, necrotrophic fungal pathogens were thought to cause cell wall degradation and eventual cell death through simple mechanisms of releasing secondary metabolites, such as phytotoxins (Rep, 2005; Shao et al., 2021). Effector genes are often involved in interactions with host organisms, ranging from the suppression of host defense responses to the manipulation of host metabolism or nutrient acquisition. In non-pathogenic conditions, these genes may have roles in other biological processes, such as symbiotic interactions, interactions with other microorganisms, or even cellular homeostasis (Agrawal et al., 2010).

The development of bioinformatics tools and their use in identifying molecules involved in pathogenicity and in functional studies have led to the discovery of new virulence factors, such as small secreted protein effectors and sRNA effectors, in these pathogens. Necrotrophic effectors are usually a diverse group of small molecules that cause programmed cell death in the host, and these small molecules can be part of proteins or secondary metabolites. These effective factors are used by necrotrophic pathogens in a wide variety of virulence mechanisms during the pathogenicity process of their host (Rep, 2005; Denton-Giles et al., 2020; Kanja & Hammond-Kosack, 2020; Shao et al., 2021).

A large number of predicted proteins in the necrotrophic fungus *A. rabiei* are qualified to be studied as effector proteins. We used bioinformatics analyses in the present study to identify motifs associated with specific transcription factors in the regulatory sequences of secretory proteins, which allowed us to select a limited number of effector proteins due to the large number of these proteins and some laboratory limitations.

Around 10,596 hypothetical proteins encoded by the *A. rabiei* genome were screened to identify effector proteins. The result led to the identification of 1282 secretory proteins, which is more than the 758 secreted proteins identified by previous studies (Verma et al., 2016).

Cysteine-rich effector proteins are secreted proteins with a role in microbial infection and disease promotion in plant pathogens. These proteins are diverse and typically dissimilar to known protein families and functional domains. Understanding the function of these effector proteins is essential for studying plant-pathogen interactions (Zhang et al., 2017). Fusarium graminearum, the primary cause of Fusarium head blight in wheat, was found to contain 190 small secreted cysteine-rich proteins (SSCPs) in its genome, of which 25 were confirmed as true extracellular proteins in a comprehensive analysis of its small secreted cysteine-rich proteins (SSCPs) (Lu & Edwards 2016). They are mainly secreted, cysteine-rich proteins (Stergiopoulos & de Wit 2009). Based on this and analysis of selected secretory proteins, we selected 344 cysteine-rich secretory proteins for further analysis.

Effector protein prediction faces several challenges in developing a more accurate method. One challenge is the lack of consensus over the annotation and functionality of effector proteins, leading to an overestimation of the number of proteins involved in plant-pathogen interactions (Sonah et al., 2016). EffectorP 3.0 is a machine learning classifier that accurately

predicts known apoplastic and cytoplasmic effectors in the secretome of fungi and Oomycetes, with a low estimated false positive rate of 3% and 8%, respectively (Sperschneider & Dodds 2022). In the current study, 108 proteins met the criteria using all available filters.

Identifying effector genes based on transcription factor cis-regulatory elements in pathogenic fungi involves analyzing the DNA sequences upstream of effector gene coding regions for the presence of specific transcription factor binding sites.

Another example can be observed in the study on rice blast disease caused by the fungus *Magnaporthe oryzae* (Mat Razali et al., 2019). They have used a combination of transcriptomic data and DNA sequence analysis to identify effector genes. They also discovered conserved binding sites for known rice transcription factors by searching DNA motifs in the promoter regions of co-regulated effector genes. This approach helped them to find potential regulatory networks controlling effector gene expression in *M. oryzae* (Mat Razali et al., 2019).

The expression levels of transcription factors and virulence genes were studied under conditions of interaction between *A. rabiei* and chickpea plants in culture medium (Fondevilla et al., 2015). The study examining the expression pattern of the bZIP transcription factor in *A. rabiei* during interaction with the chickpea plant also showed an increase in expression 12 hours after inoculation. Based on reports, several secreted proteins, including transcription factors, have been expressed 96 hours after inoculation under interaction conditions (Verma et al., 2017). There were only five proteins among this extensive set that contained regulatory sequences that were directly associated with the bZIP transcription factor; some of these proteins were previously described (Hasani et al 2023a), GSh303 (Hasani et al 2023b), Gsh304 (Hasani et al 2025). Interestingly, the use of regulatory sequences within promoter regions of secretory proteins has been suggested in previous studies, leading to the identification of several other virulence genes (Markstein et al., 2002; Zhang et al., 2005; Seidl et al., 2012). Among the proteins that met the required qualifications, a single protein (GSh302) was selected as a potential subject for laboratory investigation. Therefore, the expression of the *GSh302* gene is expected to follow a similar pattern due to the presence of sequences associated with this transcript.

According to a hypothetical effector gene candidate, the GSh302 gene was confirmed at the DNA level in all three pathotypes of *A. rabiei* cultivated under *in vitro* conditions. The sequencing results of amplified fragments showed that the GSh302 gene sequence is conserved across all three *Ascochyta* pathotypes.

Effector genes are generally associated with the pathogenicity of microorganisms, including necrotrophic fungi. However, effector genes can also be expressed in non-pathogenic conditions, possibly to a lesser extent under different circumstances. In non-pathogenic conditions, the expression of effector genes may be influenced by various factors, such as environmental conditions, nutrient availability, and the growth stage of the microorganism, or during interactions with other organisms. Although the expression of effector genes in non-pathogenic conditions may be lower compared to pathogenic conditions, it does not necessarily mean that they have no function (Win et al., 2012; Todd et al., 2022; Singh et al., 2023).

The expression of the *GSh302* gene at the transcript level was not proved in the *Ascochyta* pathotype PI cultivated in the cultural medium (*in vitro*), so that the pathotype PI amplified no fragment.

Simultaneously, the expression of the *ArGAPDH* housekeeping gene confirmed the lack of impurity in the constructed cDNA. The presence of this gene in previous studies has been investigated only in the ARDII strain, based on in silico genomic analyses (Verma et al., 2016). Recently, Bahrabadi and coworkers analyzed RNA-seq data from *A. rabiei* under growth conditions on cultural medium and during interaction with chickpea plants. They have revealed that the gene GSh302 shows a significant increase in expression during interaction with the chickpea plant, with expression levels increasing incrementally up to 96 hours after inoculation (Bahrabadi et al., 2025). Confirmation of the infection process was achieved by tracking the fungal housekeeping gene in inoculated chickpeas. The quality of the constructed cDNA was also confirmed by observing specific fragments amplified to the anticipated size. Tracking the fungus within infected plants at the genomic and transcript levels demonstrates that the RNA obtained from infected plants was adequate for detection.

The findings above demonstrated that the GSh302 gene can be detected in pathotype I of *A. rabiei* 96 hours after inoculation. These findings confirmed the presence of this gene and its capacity to be induced under interaction conditions, compared with its growth in the culture medium.

Moreover, the results of this study showed a high level of resistance in the MCC133 genotype against different *A. rabiei* pathotypes and the appearance of disease symptoms was consistent with previous studies (Shokouhifar et al., 2006). Disease symptoms on the susceptible genotype (ILC1929) were also confirmed in the *Ascochyta* pathotype PI. However, the results of the present study were consistent with the results reported under *in vitro* conditions (Firouzmand et al., 2023b; Ramezani Khozestani et al., 2023).

In conclusion, inoculation of susceptible and resistant chickpea genotypes with pathotypes of *A. rabiei* demonstrated that the in vitro pathogenicity test method can be used in molecular interaction studies and that the fungus can grow and reproduce on leaves under *in vitro* conditions. Also, there was a significant difference in the amount of damage on sensitive and resistant genotypes, which was consistent with previously reported results (Firouzmand et al., 2023b). The results of this study confirmed that PSh19-F/R-specific primers, designed based on the ArGAPDH gene sequence, can detect *A. rabiei* in infected plants with high specificity at both DNA and cDNA levels. The gene *GSh302* was detectable during plant pathogen interaction at both the genomic and transcript levels in resistant (MCC133) and sensitive (ILC1929) chickpea genotypes.

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