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Detection of *Ascochyta rabiei* isolates using the *GSh302* gene sequence as a molecular marker

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Abstract

The present study investigated the expression patterns of several candidate effector genes of *Ascochyta rabiei* using RNAseq data available in the Sequence Read Archive (SRA) database. Results revealed that the *GSh302* gene exhibits a distinct expression profile during the fungus-plant interaction. Analysis of the gene sequence in genomic data from 250 isolates available in the SRA database showed that the sequence was highly conserved in *A. rabiei* isolates. Further comparative analyses across different *Ascochyta* species confirmed the presence of the sequence in all examined species. Multiple sequence alignment identified both conserved and variable regions of this gene, with the variable regions subsequently used to design specific primers. The distinct regions were used to design specific primers for the detection of *A. rabiei* isolates. The sequence of this gene was tracked in *A. rabiei* isolates collected from different farms in northeastern Iran during 2020–2022, as well as isolates related to pathotypes PI, PIII, and PVI. The findings demonstrated that a specific band could be amplified using the designed differential primers in all isolates of *A. rabiei*. Moreover, the fungus was detected in inoculated plants as early as 36 hours post-inoculation, while no amplification occurred in healthy controls. The findings of this study indicated that the gene, *GSh302*, which is involved in pathogenicity and shows expression in fungal isolates during interaction with host plant, can serve as a molecular marker for the identification and population diversity analysis of pathogens such as *A. rabiei* with closely related species like *Ascochyta lentis*, *Ascochyta fabae* and *Ascochyta viciae*.

Keywords: Ascochyta spp., Genetic diversity, Pathogen detection, Specific primers

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شناسائی جدایه های قارچ *Ascochyta rabiei* با استفاده از توالی ژن *GSh302* به عنوان نشانگر مولکولی

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چکیده

در این مطالعه، الگوهای بیان چندین ژن اثرگذار نامزد در قارچ *Ascochyta rabiei* با استفاده از داده‌های RNaseq موجود در پایگاه داده Sequence Read Archive (SRA) مورد بررسی قرار گرفت. نتایج نشان داد که ژن *GSh302* در طول برهمکنش قارچ بیمارگر با گیاه میزبان، الگوی بیان متمایزی را نشان می‌دهد. واکاوی توالی این ژن در داده‌های ژنومی از ۲۵۰ جدایه موجود در پایگاه داده SRA نشان داد که توالی این ژن در جدایه‌های قارچ *A. rabiei* بسیار حفظ شده است. همچنین واکاوی‌های مقایسه‌ای بیشتر در گونه‌های مختلف جنس *Ascochyta* وجودژن مذکور را در تمام گونه‌های مورد بررسی تأیید نمود. هم‌ردیفی چندگانه توالی‌ها، منجر به شناسایی مناطق حفاظت شده و متغیر این ژن گردید و متعاقباً مناطق متغیر برای طراحی آغازگرهای اختصاصی برای تشخیص جدایه‌های *A. rabiei* استفاده شدند. توالی این ژن در جدایه‌های *A. rabiei* جمع‌آوری‌شده از مزارع مختلف در شمال شرقی ایران طی سال‌های ۲۰۲۰ و ۲۰۲۲ و همچنین جدایه‌های مربوط به پاتوتیپ‌های PI، PIII و PVI ردیابی شد. نتایج نشان داد که می‌توان با استفاده از آغازگرهای افتراقی طراحی‌شده، یک باند خاص را در تمام جدایه‌های *A. rabiei* تکثیر کرد. علاوه بر این، قارچ در گیاهان مایه‌زنی‌شده، ۳۶ ساعت پس از مایه‌زنی ردیابی گردید، در حالی که هیچ تکثیری در گیاهان شاهد سالم رخ نداد. یافته‌های این مطالعه نشان می‌دهد که ژن‌های دخیل در بیماری‌زایی می‌توانند به عنوان نشانگرهای مولکولی برای شناسایی و تجزیه و تحلیل تنوع جمعیتی عوامل بیماری‌زایی مانند *A. rabiei* عمل کنند.

کلید واژه‌ها: آغازگر اختصاصی، تشخیص بیمارگر، تنوع ژنتیکی، گونه‌های آسکوکیتا

دبیر تخصصی: دکتر نیما خالدی

Introduction

Chickpea Ascochyta blight, caused by the fungus *Ascochyta rabiei*, is a highly destructive disease that affects chickpea globally. The occurrence of genetic changes in the fungal population during sexual and asexual reproduction causes the damage of this pathogen to increase quickly under favorable conditions, with the emergence of new races and pathotypes. Understanding the pathogen population and determining the pathogenicity diversity of isolates in each region over several years is essential for the appropriate selection of chickpea varieties and making recommendations for each region. These studies can be useful in assessing the emergence of highly pathogenic pathotypes and designing a strategy to control and manage this disease. Given the special position of chickpea cultivation and the importance of Ascochyta blight damage in Iran, numerous studies have been conducted on the population of the fungus *A. rabiei* (Nourollahi et al., 2011; Vafaei et al., 2015; Farahani et al., 2021; Omer et al., 2023; Shokouhifar et al., 2003a).

Several methods (including determining the pathogenicity diversity of isolates collected from different regions on chickpea cultivars) have been considered in the study of the *A. rabiei* fungal population. The study of the damage quantity of 94 pathogenic isolates of *A. rabiei* collected from different provinces of Iran on chickpea cultivars led to the identification of different resistance levels, and the isolates were grouped based on the pathogenicity power (Virulence) required to break each level. As a result of this study, six pathotype groups, PI, PII, PIII, PIV, PV and PVI were reported by the order of pathogenicity power from lowest to highest in Iran (Shokouhifar et al., 2003a).

The use of differential chickpea cultivars to group *A. rabiei* isolates have been considered in most studies, and as a result, the population of this pathogen has been separated into different races or pathotypes. Although these methods can be considered indicators for estimating the level of diversity in the pathogen population, the results cannot be compared with each other. Several studies have been conducted to investigate the diversity of the population of this pathogen based on molecular markers and diversity at the level of genomic content of isolates (Santra et al., 2001; Phan et al., 2003; Shokouhifar et al., 2003b; Bar et al., 2021; Blake et al., 2022).

Accurate molecular methods can facilitate the identification of pathogens and are very useful in studying pathogen populations. The use of DNA-based molecular markers can be remarkably efficient in monitoring the genetic diversity of these agents. The molecular markers used are mostly selected from the conserved regions of the fungal genome, which can be referred to as the ITS (Internal Transcribed Spacer) regions, which include the ITS1 and ITS2 regions, the boundaries of the ribosomal rRNA genes encoding the 18S, 5.8S and 28S ribosomal subunits (Schoch et al., 2012). These regions are suitable for differentiating fungal species. Furthermore, the sequences of genes encoding the large ribosomal subunit 28S rRNA are used in the differentiation of higher taxonomic levels, such as genus and family (Bradshaw et al., 2023). Differentiation of closely related fungal species is performed using single-copy protein genes such as β-tubulin (TUB2), which can be used to differentiate the genera *Fusarium* and *Ascochyta*.

Although several molecular markers have been used to study the genetic diversity of *A. rabiei* isolates, no relationship has been identified between the diversity and pathogenicity. Recent advances in sequencing have led to the development of sequencing-based genotyping methods and the identification of single-nucleotide polymorphisms (SNPs) aiming to find

genetic diversity related to pathogenicity in *A. rabiei* isolates.

In Australia, a study of the pathogen population was conducted to provide a molecular pathotyping tool for determining pathotypes based on molecular markers (Bar et al., 2021). The present study indicated no direct relationship between genetic diversity and the pathogenicity pattern of isolates. However, studies to determine genetic groups associated with pathogenicity have been introduced as a promising and pioneering potential in the development of molecular markers for the rapid detection of pathogenic isolates (Bar et al., 2021).

Numerous virulence and effector genes have been identified and reported in a large number of plant pathogens (Stergiopoulos & de Wit, 2009; Uhse & Djamei, 2018; Shao et al., 2021; Lovelace et al., 2023). Tracking and determining the diversity of virulence genes in many pathogens has been used as a tool for distinguishing strains and grouping isolates based on pathogenicity (Oliva et al., 2015; Sokolova et al., 2016; Qi et al., 2024).

Studies have also been conducted on *A. rabiei* to identify virulence and effector genes (Verma et al., 2016; Shah et al., 2020; Lee et al., 2021; Hasani et al., 2023; Singh et al., 2023). A study conducted on genomic data of *A. rabiei* reported the *GSh303* gene encoding a protein with effector characteristics, detected in the PI, PIII and PIV pathotypes of this fungus at the genome level. Additionally, the study provided data confirming its expression during interaction with chickpea plants (Hasani et al., 2023). In a similar study, the effector gene *GSh302* was identified in *A. rabiei*, which is expressed in resistant and susceptible genotypes of chickpea under fungal development conditions, and its sequence diversity was observed among the PI, PIII, and PIV pathotypes (Hasani et al., 2025b). Studies conducted to identify virulence and effector genes in *A. rabiei* have suggested the possibility of determining the genotype of fungal isolates based on the sequence of virulence genes. Therefore, it can be expected that the genotype of pathogenic isolates can be grouped based on their pathogenicity.

The advent of high-throughput DNA sequencing technologies has made a huge amount of genomic and transcriptomic data publicly available in databases, including the Sequence Read Archive (SRA). Such data represent underutilized information that can be used in *in silico* studies to identify and validate virulence and effector genes in plant pathogens. Analysis of such data can be used to investigate the expression patterns of candidate genes under various pathogen growth conditions at different time intervals after infection and to predict their role in pathogen pathogenicity on different plant tissues and at different times after infection.

The lack of known domains in the sequences of candidate virulence or effector proteins has caused predicting their role in pathogenicity to require costly and time-consuming methods, typically expressing their heterologous proteins permanently or transiently. However, using transcriptomic data, the expression pattern of candidate genes can be examined at different stages of pathogen-plant interaction and the prediction of their role in the interaction between the host and the pathogen can be strengthened (Qi et al., 2018). Whole-genome sequencing has been used to sequence approximately 230 isolates of diverse pathogenicity from the fungus *A. rabiei* using the Illumina Nextera library preparation and NextSeq sequencing platform to identify regions of the genome that play a role in pathogenicity (Vaghefi et al., 2024). Additionally, mutagenesis and sequencing methods have been used to identify gene loci that distinguish isolates belonging to the species *A. rabiei* and *Ascochyta lentis* (Debler et al., 2025). Data from pathogen population studies can also be useful in selecting virulence or pathogen-

specific effector genes. Based on the data, genes strongly associated with pathogen pathogenicity and highly conserved across collected pathogenic isolates can be identified in the pathogen population. Studies have been conducted to develop molecular methods for determining pathotypes in *A. rabiei* populations using genotyping by sequencing methods such as the Diversity Array Technologies sequencing (DArTseq) method (Bar et al., 2021).

Sequencing-based methods can provide a significant amount of information; however, if specific genes with a conserved pathogenic role are identified within the *A. rabiei* species and distinguished from other species, including *A. lentis*, they can be notably effective in the specific diagnosis of this pathogen on plant samples.

In this study, using in silico experiments, the DNA level diversity and expression pattern of a number of candidate effector genes identified in *A. rabiei* (Hasani et al., 2023; Shokouhifar et al., 2024; Debler et al., 2025) were examined in the transcriptomic data of the PRJNA288273 and PRJNA1247000 biological projects. Genes that showed expression under pathogen-plant interaction were identified. The sequence diversity of the genes was studied in the genomic sequence of *A. rabiei* isolates related to the PRJNA1175002 biological project. Genes expressed under plant pathogen interaction and with conserved sequences in *A. rabiei* isolates were identified compared to those in *Ascochyta* species. In the field stage, plants showing symptoms of Ascochyta blight were collected from chickpea fields in the northeastern regions of Iran during 2020 and 2022. Forty isolates of *A. rabiei* were purified and identified based on morphological and microscopic characteristics. The pattern of candidate gene presence in the collected isolates was examined alongside isolates related to the PI, PIII, and PVI pathotypes identified in previous studies (Shokouhifar et al., 2003a).

Materials and Methods

Evaluation of Candida effector gene sequences

The genomic sequences of several candidate effector genes (Table 1) reported in various studies were retrieved from the NCBI. The expression pattern of the candidate genes in the transcriptome data of *A. rabiei* isolates from the Bioproject: PRJNA1247000 and PRJNA1175002 was examined using the BLAST tool at (https://blast.ncbi.nlm.nih.gov/blast). In order to analyze the expression pattern of target genes, RNAseq data from the PRJNA1175002 bioproject were retrieved using the sratoolkit.3.1.1-win64 tool and a heatmap of normalized expression data (based on the expression of the housekeeping gene ArGAPDH) was plotted using script 1 in RStudio software. Furthermore, the expression levels of the studied genes were plotted relative to the in vitro expression conditions using Excel software.

The variation in the *GSh30* gene sequence in the genomic sequences of 230 *A. rabiei* isolates (originating in Australia) and 20 isolates from the World Gene Bank, related to the biological project PRJNA1175002 (Vaghefi et al., 2024), was examined using the Sequence Read Archive Nucleotide BLAST tool. The alignment results of the reads related to the target gene were downloaded. The mapping of the reads on the target gene sequence and the determination of the consensus sequence of each isolate were performed using the mapping to reference and Consensus tools in the CLC genomics workbench software. The variant detection tool in the CLC genomics workbench software was used to identify the variants, and the alignment result of the consensus sequences of each isolate was prepared using the alignment tool in the CLC genomics workbench software.

Specific primers were designed for the identified distinct regions using Primer-BLAST software at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The *GSh302* gene sequence was inserted into the software, and appropriate regions were identified. In the target organism section, *A. rabiei* was included as the target and other species of the genus *Ascochyta* were excluded, and the length of the amplified fragment was defined between 470 and 520.

Sampling

Symptomatic chickpea plants were collected from chickpea fields in different regions of Khorasan Razavi and North Khorasan provinces between 2020 and 2022. Sampling of infected fields was carried out based on a quasi-hierarchical manner, in which infected plants were collected from the four corners and the midpoint of the field (five plants from each field) by collecting the entire plants and placing them in paper bags, recording the location of collection and the sampling date. All samples were stored in a dry and dark place at a temperature of 4 °C after being transferred to the laboratory. Three isolates of *A. rabiei* originating from Iran, with the reference numbers FUM 1001/ASR001, FUM 1003/ASR003 and FUM 1006/ASR006, related to previous studies (Shokouhifar et al., 2003a) with different levels of pathogenicity, were used in molecular analyses. Table 2 presents information on isolates, including the collection area, province, city, and region, as well as the collection year.

Isolation and purification of *A. rabiei* from infected samples

Within 48 hours after sampling, fungi were isolated from infected plant tissues. Tissue samples were obtained from the interface of the symptomatic and healthy areas and cut into 5 to 10 mm pieces. Surface disinfection was performed by treatment with 70% ethanol for 30 seconds, followed by three times washing in sterile distilled water. Tissues were dried on sterile paper towels and were cultured in a 9 cm Petri dish containing CSDA culture medium (containing 4 g of chickpea seed powder, 3 g of dextrose, 1.8 g of agar in 100 mL of distilled water, autoclaved for 15 minutes at 121 °C and addition of 5 mg of streptomycin after cooling to 50 °C), so that the wounds were in contact with the culture medium. The Petri dishes were kept upside down in a dark incubator at 20 °C for one week (Farahani et al., 2019). The isolates were sub-cultured onto PDA (Potato Dextrose Agar) and incubated at 20 °C for 14 days with a 16-hour light cycle. The colonies grown on the surface of the medium were compared with the color indices and growth patterns of *A. rabiei* isolates previously reported (Bahr et al., 2016; Crociara et al., 2022). Isolation of

Table 1. Characterization of candidate effector genes in Ascochyta rabiei reported in different studies

Genes	Accessions	Functions	References
GSh301	Unsubmitted	Effector Candidate	(Hasani et al., 2023)
GSh302	OR875943 757	Effector Candidate	(Hasani et al., 2025b)
GSh303	OR567502 591	Effector Candidate	(Hasani et al., 2023)
GSh304	Unsubmitted	Effector Candidate	(Hasani et al., 2025a)
GSh200	OR567501 1212	Effector Candidate	(Shokouhifar et al., 2024)
GSh201	Unsubmitted	Effector Candidate	(Shokouhifar et al., unpublished data)
GSh210	Unsubmitted	Effector Candidate	(Firouzmand et al., 2020)
ArGAPDH	Unsubmitted	Hose keeping gene	(Zangeneh et al., 2022)

Table 2. Characteristics of different collected isolates of Ascochyta rabiei including their sampling date/location and plant organs

Row	City	Latitude	Longitude	Organs	Isolate No	Year
1	Quchan	37.055623	58.553123	Leaf	Ar001	2020
2	Quchan	37.055623	58.553123	Stem	Ar002	2020
3	Sabzevar	36.111509	57.624191	Leaf	Ar003	2020
4	Sabzevar	36.111509	57.624191	Stem	Ar004	2020
5	Firuzah	37.355711	57.246916	Leaf	Ar005	2020
6	Firuzah	37.355711	57.246916	Leaf	Ar006	2020
7	Firuzah	37.355711	57.246916	Leaf	Ar007	2022
8	Firuzah	37.355711	57.246916	Stem	Ar008	2022
9	Mashhad	36.204335	59.683799	Leaf	Ar009	2020
10	Mashhad	36.204335	59.683799	Stem	Ar011	2020
11	Mashhad	36.204335	59.683799	Leaf	Ar012	2022
12	Khoshab	36.540798	58.145721	Leaf	Ar013	2020
13	Khoshab	36.540798	58.145721	Stem	Ar014	2020
14	Khoshab	36.540798	58.145721	Leaf	Ar015	2022
15	Nishapur	36.172703	58.928878	Leaf	Ar016	2020
16	Zebarkhan	36.048234	59.138325	Leaf	Ar017	2022
17	Nishapur	36.172703	58.928878	Leaf	Ar018	2020
18	Nishapur	36.172703	58.928878	Leaf	Ar019	2020
19	Nishapur	36.172703	58.928878	Stem	Ar020	2022
20	Salehabad	36.405159	58.038687	Leaf	Ar021	2020
21	Salehabad	36.405159	58.038687	Leaf	Ar022	2020
22	Salehabad	36.405159	58.038687	Stem	Ar023	2022
23	Farooj	37.231735	58.219286	Leaf	Ar025	2020
24	Farooj	37.231735	58.219286	Stem	Ar026	2022
25	Farooj	37.231735	58.219286	Leaf	Ar027	2022
26	Joghatai	36.638031	57.074372	Leaf	Ar028	2020
27	Shashtmad	35.968523	57.765520	Leaf	Ar031	2020
28	Chenaran	36.648396	59.118501	Leaf	Ar032	2020
29	Esfarayen	37.075098	57.509617	Leaf	Ar033	2020
30	Esfarayen	37.075098	57.509617	Stem	Ar034	2022
31	Jovin	36.725493	57.174309	Leaf	Ar035	2020
32	Jovin	36.725493	57.174309	Stem	Ar037	2020
33	Malekabad	36.010602	59.536704	Leaf	Ar038	2020
34	Jajarm	36.928186	56.379294	Leaf	Ar039	2020
35	Jajarm	36.928186	56.379294	Stem	Ar041	2020
36	Binalud	36.427552	58.845053	Leaf	Ar042	2020
37	Tabadkan	36.645147	59.452417	Leaf	Ar043	2020
38	Jovin	36.725493	57.174309	Leaf	Ar044	2020
39	Jovin	36.725493	57.174309	Stem	Ar045	2020
40	Chenaran	36.648396	59.118501	Leaf	Ar048	2020
41	Karaj	***	***	Leaf	PI	2000
42	Golestan	***	***	Leaf	PIII	2000
43	Hamedan	***	***	Leaf	PVI	2000

*** Provided by the Microorganism collection of Ferdowsi University of Mashhad, Mashhad, IRAN (WDCM 1207).

single spores was performed according to the method reported by Udupa and Weigand (1997). A volume of 2 mL sterile distilled water was added to the surface of the colonies to release the pycnidiospores from the pycnidia. The distilled water from the surface of the Petri dishes was collected after 10 minutes, and volumes of 50, 100 and 200 µl were spread on the surface of Petri dishes containing water-agar and kept for 24 hours at 20 °C. The culture surface was transferred to a slide in 2 cm pieces and cut into 2 mm squares in a checkerboard pattern using a sterile blade. Pieces carrying germinated single spores were selected using a microscope and transferred to Petri dishes containing CSMA medium.

Pathogenicity

The pathogenicity test of 40 isolates was performed using the *in vitro* pathogenicity test method on the susceptible cultivar ILC1929 (Ramezani Khozestani et al., 2023). In order to prepare the required plant materials, chickpea seedlings of the chickpea line ILC1929 susceptible to Ascochyta blight (with gene bank number MCC507) were obtained from the Legume Seed Bank of Ferdowsi University of Mashhad. After surface disinfection and cultivation in sterile Petri dishes, the germinated seeds were cultivated in pots containing a mixture of cocopeat and perlite (1:1 ratio), sterilized and washed, and kept in a growth chamber until they reached the 5 to 10 leaf stage at a temperature of 16 °C at night and 24 °C in light with a 16-hour light period. Completely developed leaves of the same age were harvested and used for inoculation with the spore suspension of the isolates after surface disinfection.

Single-spored isolates were used to prepare spore suspensions according to the method described by Udupa & Weigand (1997). Spore suspensions of isolates were prepared at a concentration of 1.6×10^5/mL using a hemocytometer. Inoculation was done by selecting three disinfected leaves and immersing them in the spore suspension of each isolate. Excess surface moisture of the leaves was removed with sterile tissue and then placed in a jar containing sterile cotton and sealed with parafilm. Three leaves were kept as controls by immersion in distilled water inside a jar. Furthermore, three leaves were placed on a glass as a control in a spore suspension prepared from the pathotype I (PI) isolate of *A. rabiei* (Shokouhifar et al., 2003a) with reference number FUM 1001/ASR001 obtained from the Ferdowsi University of Mashhad Microorganism Bank (WDCM 1207). Symptoms appearing on the leaves were photographed after five days, and the extent of damage was determined based on the *in vitro* Ascochyta blight damage scoring system (Firouzmand et al., 2023).

DNA extraction

From each field, one pathogenic isolate in a total of 40 isolates (Table 2), along with three isolates related to the PI, PIII and PVI pathotypes of *A. rabiei* with reference numbers FUM 1001/ASR001, FUM 1003/ASR003, and FUM 1006/ASR006 were selected for molecular analyses. DNA extraction was performed based on the CTAB (Cetyl Trimethyl Ammonium Bromide) method with minor modifications (Shokouhifar et al., 2003b). In this method, mycelium grown on the surface of the Petri dish from the purified culture of the isolates was collected and transferred to 100 mL vials containing 25 mL of PDB (Potato Dextrose Broth) liquid culture medium and kept for two weeks at 20±2 °C in the dark on a shaker at a speed of 120 rpm.

The grown mycelium was separated by passing the contents of each vial through two layers of sterile gauze. A small amount of sterile distilled water was added to the mycelium to remove the culture medium. The mycelium tissue was wrapped in gauze, transferred to a 50 mL Falcon containing about 20 mL dry cotton swabs, and

centrifuged for 5 minutes at 5,000 rpm to extract water. The dehydrated mycelium tissue was cut into small pieces with a sterile scalpel, and about 200 mg of the resulting pieces were transferred to a 1.5 mL microtube. A total of 500 µL of CTAB extraction buffer (containing 2% CTAB, 100 mM Tris-HCl, 20 mM EDTA and 1.4 M NaCl, pH ~ 8.2) was added and completely homogenized using a micro-grinder (Aitoserlea Co, China). The extracted DNA was electrophoresed on a 1% agarose gel containing Green Viewer dye (1 µL/mL) to check the quality.

Molecular confirmation of *GSh302* presence at the DNA level of isolates

The *GSh302* gene, recently identified and reported as a candidate effector gene in *A. rabiei* (Hasani et al., 2025b), was studied in the genome of the collected isolates. For this purpose, PCR amplification was performed with specific primers PSh302-F3 with the sequence (5'-caacggtgtaggcggttgca-3') and PSh302-R3 with the sequence (5'-gcccttggtgtcaacaagt-3') (Sinaclon Bioscience Company, Tehran, Iran) based on the contents of the amplification reaction and with an annealing temperature of 40 seconds at 60 °C for specific primers. PCR was conducted in a thermocycler (Eppendorf, Germany), and the master mix was provided by amplicon, Denmark. The PCR product was separated on a 1% gel electrophoresis and imaged with a GeDoc device.

Results

The results of transcriptomic data analysis available in the SRA database available in NCBI, stored under the biological project number PRJNA288273, showed that all the candidate genes were expressed in the conditions of fungal growth on the culture medium, and among the studied genes, the *GSH302* gene was the only one not expressed *in vitro*. Additionally, the investigation of the expression pattern of the housekeeping gene ArGAPDH in transcriptomic data related to the fungal samples grown in the culture medium and during interaction with the host plant at 12, 36 and 96 hours after inoculation showed that this gene is expressed under all conditions. The expression level of the genes is presented relative to the ArGAPDH gene in the heat map. As shown in Figure 1, the expression level is presented by a color index from red (highest expression level) to dark blue (lowest expression level), and white indicates the average expression. The expression pattern of the studied genes during the interaction of *A. rabiei* with the chickpea plant is different at different times after inoculation. The number of reads associated with the studied genes increased over time after inoculation, and in all cases, the highest expression was observed at 96 hours after inoculation. Among the studied genes, the expression pattern of the *GSh302* gene was quite distinct. The very low expression of the gene under growth conditions on the culture medium was different from the expression pattern during plant interaction. The expression pattern of this gene under plant interactions increased significantly over time after inoculation.

The sequencing reads from each isolate were mapped onto the *GSh302* gene sequence to verify the accuracy of the sequencing and analysis of the *GSh302* gene region. Multiple sequence alignment analysis was performed for all isolates with high coverage and read depth. Figure 2 demonstrates the results of mapping the reads of isolate IsoAR0038 (with number SRR31046207) onto the *GSh302* gene sequence. As presented, the position of the reference sequence length from 1 to 680 bp is shown at the top of Figure 2, and the curve below it indicates the depth of reads at each position, showing the number of reads that overlap at each genomic position. The red curve corresponds to the depth of reads in the antisense direction, and the green curve indicates the depth of reads in the sense

direction. The depth of the red and green curves along the sequence indicates the high depth of reads throughout the sequence and confirms the quality of sequencing throughout the gene sequence. The area below the curve shows the results of aligning the length and position of each read with respect to the *GSh302* gene sequence. The absence of different colors in each read indicates that the read sequence matches the *GSh302* gene sequence. This uniformity indicates that no point mutation (SNP) was observed in any of the reads, and the continuity and overlap of the reads along the target gene sequence also confirm the absence of additions or deletions along the gene sequence. The mentioned results indicate that the *GSh302* gene sequence in isolate Ar0038 was highly conserved compared to the reference sequence.

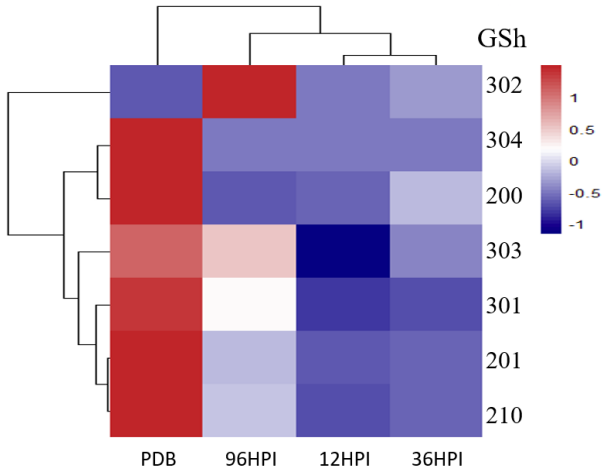


Figure 1. Expression patterns of candidate effector genes in *Ascochyta rabiei* under growth conditions in PDB medium (*in vitro*) and during interaction with chickpea plant (*in planta*) based on the SRA database under the bioproject PRJNA288273. Heatmap depicting Z-score normalized expression values of genes *GSh301, GSh302, GSh303, GSh304, GSh200, GSh201* and *GSh210* (rows) across different experimental conditions (columns). Experimental conditions included cultivating the fungus in PDB liquid medium as well as chickpea leaves inoculated with *A. rabiei* at 12 (L12), 36 (L36), and 96 (L96) hours after inoculation. Expression values are normalized as Z-scores represented by a color scale from blue (expression below the mean) to red (expression above the mean). Row clustering was performed using hierarchical clustering with Pearson correlation and the complete linkage method.

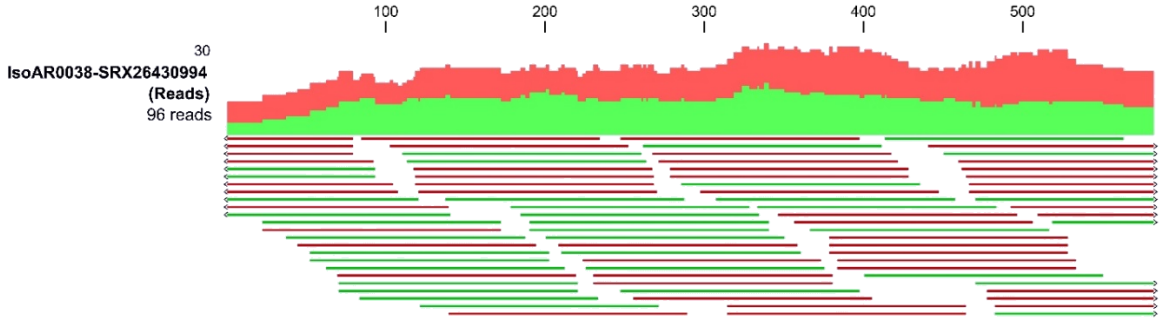


Figure 2. Representative read mapping related to the isolate Ar0038 onto the *GSh302* gene sequence. The upper region shows the coverage plot, confirming the uniform and high depth of sequencing reads across the entire length of the gene (682 bp). The lower region shows the nucleotide alignment of the obtained consensus sequence (middle) with the reference sequence (top). As shown, the consensus sequence matches the reference sequence perfectly, and no single-nucleotide variant (SNP) or indel (insertion-deletion) was detected in the genome region of this *Ascochyta rabiei* isolate.

The results of read mapping of each isolate to the *GSh302* gene sequence showed a complete overlap of high-depth reads of nearly 100 reads on the gene sequence in each isolate, and the sequencing and consensus sequence results obtained for each isolate were acceptable with high probability. This step was investigated for all 230 isolates reported from Australia and 20 isolates reported from the World Microorganism Bank, all of which are presented in the biological data with the number PRJNA1175002 available in the RAS database at NCBI.

The conserved sequence obtained from read mapping of each isolate was further investigated to identify possible mutations and variants among different isolates. The alignment results showed that the conserved sequences of the isolates were entirely identical, and no mutation was present between the sequences. The alignment results of a number of isolates are presented as examples in Figure 3.

Overall, the results of these analyses showed that the sequence of the *GSh302* gene is completely conserved in all the studied isolates. Therefore, the sequence of the *GSh302* gene can be used to identify isolates related to the fungus *A. rabiei*. The important point is to determine the extent to which the sequence of this gene is conserved in other *Ascochyta* species, so it is necessary to investigate the presence and diversity of this gene in other species of this fungus.

Tracking the *GSh302* gene sequence in economically pathogenic *Ascochyta* species

For the first time in this study, tracking the *GSh302* gene sequence in the genomic sequences of the fungi *Ascochyta fabae*, which causes Ascochyta leaf and pod spot disease in fabae beans, *Ascochyta lentis*, which causes Ascochyta blight on lentil, and *Ascochyta pisi*, which causes Ascochyta blight on pea, was performed by sequencing BLAST on the genomic data of these species. Alignment of the sequences identified by the CLC genomics workbench software showed that there were completely conserved regions along the sequence of this gene, as well as regions with nucleotide diversity (Figure 4). The presence of the sequence of this gene in the studied species indicates the vital importance of this gene in this genus, which can be considered as an indicator for discriminating and performing phylogenetic analyses among these species.

Comparison of the *GSh302* gene sequence among different species showed that the highly conserved regions are recognizable among all four species. For example, the nucleotide positions of the beginning of the gene up to position 80 are very similar among the species, making them suitable for designing general primers for the detection of the four species. Furthermore, it is possible to identify distinctive regions along the sequence to distinguish *A. rabiei* from the other three species. The most important regions are nucleotide positions 85 to 100, as well as the region 155 to 180, which can be used for the design of specific primers. The presence of highly specific sequences for *A. rabiei* in the ranges 286 to 300 and 462 to 474 can also be considered in the design of reverse primers.

Fungal isolation and morphological observation

Leaflets and infected stem fragments from chickpea plants carrying single lesions of Ascochyta blight cultured on CDA medium containing the antibiotic streptomycin showed initial signs of fungal growth after 48 hours. The fungal mycelia were growing well on the culture medium and could be isolated from the fifth day from both the leaflets and infected stem fragments. The Fungal isolates were purified afterwards.

Pathogenicity test

The pathogenicity of 40 isolates was confirmed using the *in vitro* pathogenicity test method (Figure 5). The results revealed

that leaves of the susceptible line ILC1929, inoculated with spore suspensions of the isolates, exhibited disease symptoms within one week after inoculation. As shown, although different disease severities were observed on the leaves, isolates capable of causing disease and symptoms on the chickpea line ILC1929 leaves susceptible to Ascochyta blight were selected for further experiments.

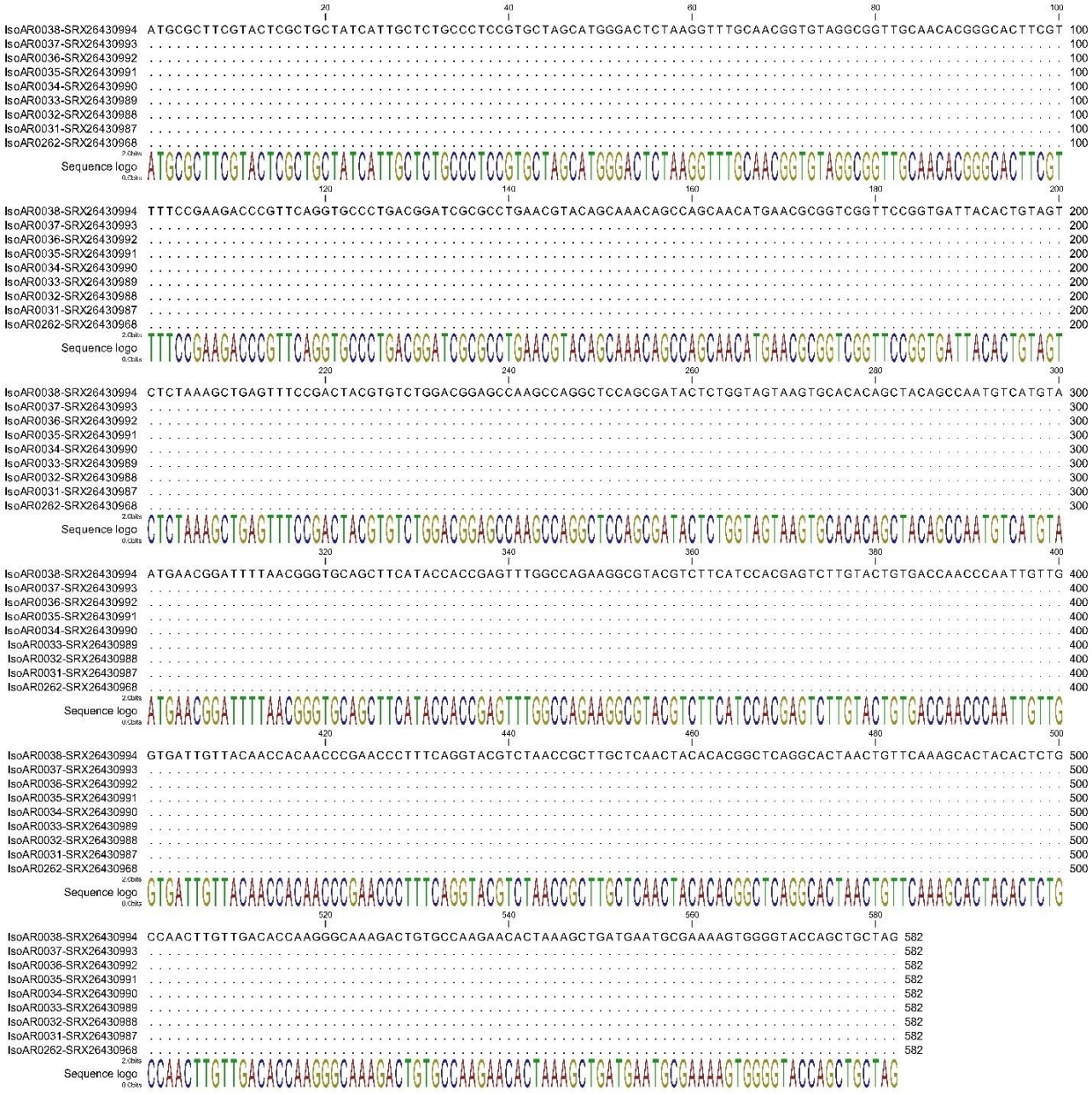


Figure 3. Multiple sequence alignment of consensus sequences related to different isolates of *Ascochyta rabiei*, each drift indicates the name of the isolate and the accession number of the data of the studied reads. The sequence logo indicates the lack of variation in nucleotides along the *GSh302* gene sequence.

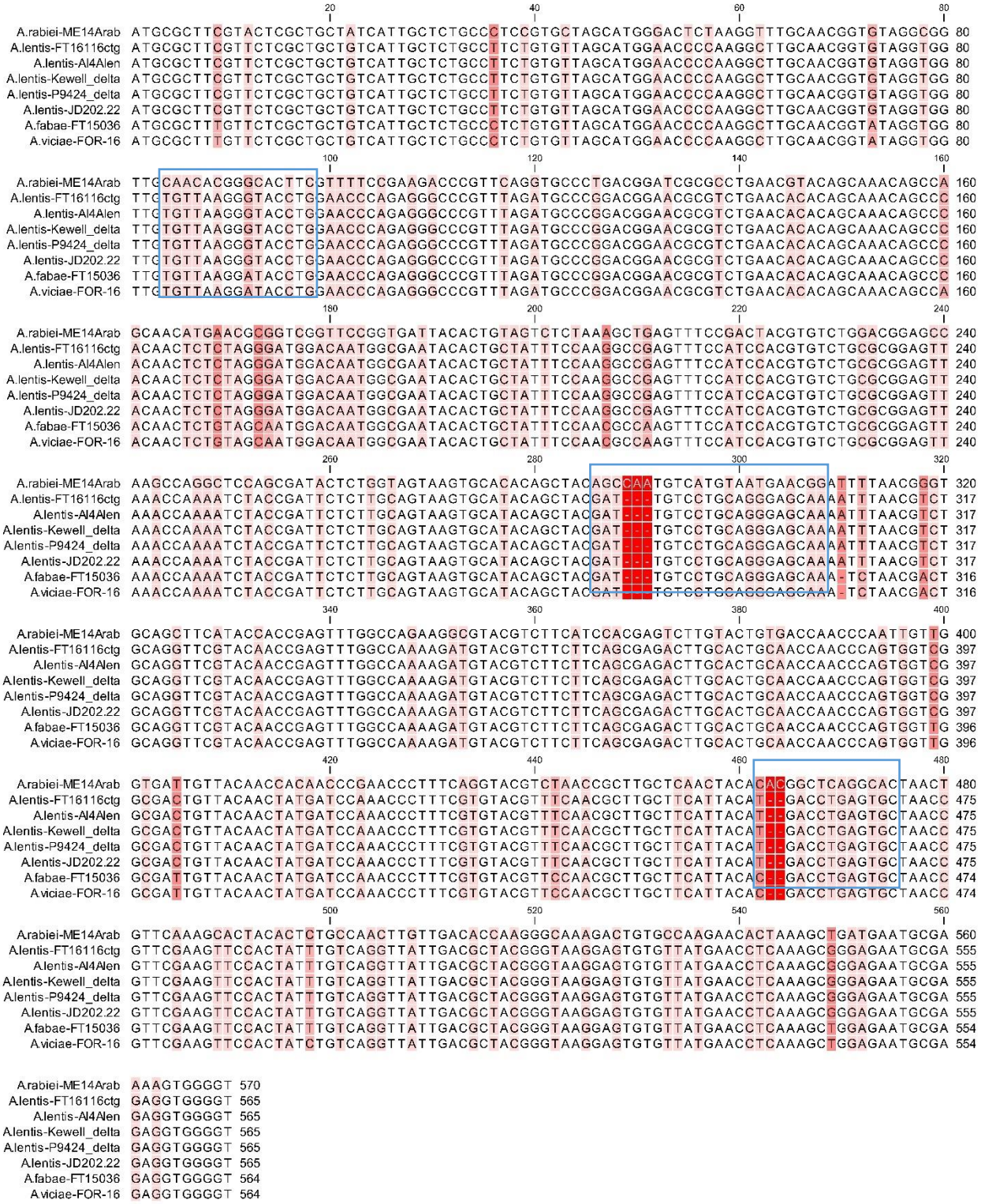


Figure 4. Multiple sequence alignment of the *GSh302* gene across different *Ascochyta* species. The names of *Ascochyta rabiei*, *Ascochyta lentis*, *Ascochyta fabae*, and *Ascochyta viciae* isolates are shown on the aligned sequences. The colored background with different color intensity indicates the amount of nucleotide diversity at the positions. Conserved regions are shown with a light background. The blue box indicates the distinct regions for designing specific PCR primers to distinguish *A. rabiei* from other species.

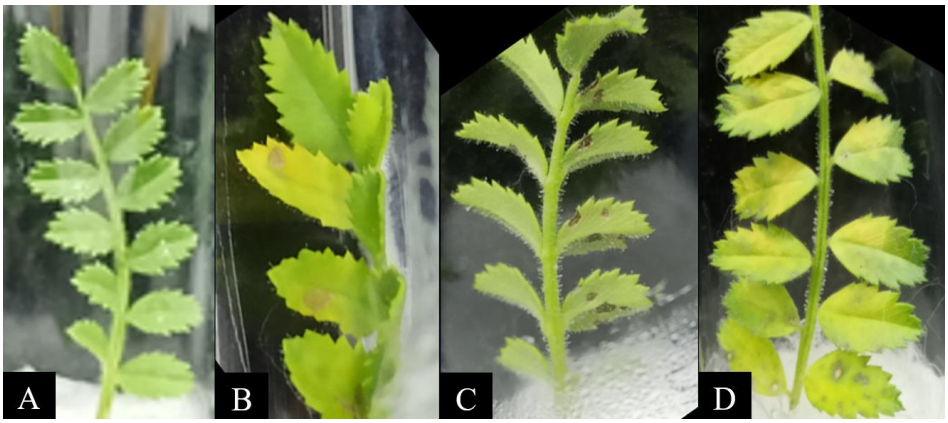


Figure 5. Results of *in vitro* pathogenicity test of a number of *Ascochyta rabiei* isolates by detached leaves method on ILC1929 as a susceptible line, 8 days after immersion in spore suspension at 18 °C with 12 hours light period under fluorescent light (1000 lux) in a test tube kept containing sterile cotton. A: Control leaf without inoculation, B, C, D: Leaves inoculated with isolates A011, A019, A044, respectively.

Molecular detection of *A. rabiei* isolates using the *GSh302* gene

Given the highly conserved nature of the *GSh302* gene in the genome of *A. rabiei* isolates, as well as confirming the expression pattern of this gene related to the interaction of the fungus with the host plant, and also identifying distinct regions between the *A. rabiei* species and other species of the genus *Ascochyta*, the present studyconducted sequence tracking of this gene using specifically designed primers to confirm the purified isolates. The evaluation of DNA extracted from mycelial isolates cultured in PDB liquid medium on gel electrophoresis showed that the quality and quantity of DNA were suitable for molecular analyses. Figure 6-A presents a schematic representation of the *GSh302* gene and the location of the GSh302-F3/R3 specific primers. Thus, detection of the *GSh302* gene was performed in the genomic DNA of *A. rabiei* isolates (Figure 6-B). As shown, a similar specific single band related to the *GSh302* gene can be detected from *A. rabiei* isolates. A similar single band can also be detected to a similar extent from all pathotypes of this fungus isolated from different regions of Iran in 2003. These results indicate that this gene can be detected with a conserved nature in all *A. rabiei* isolates.

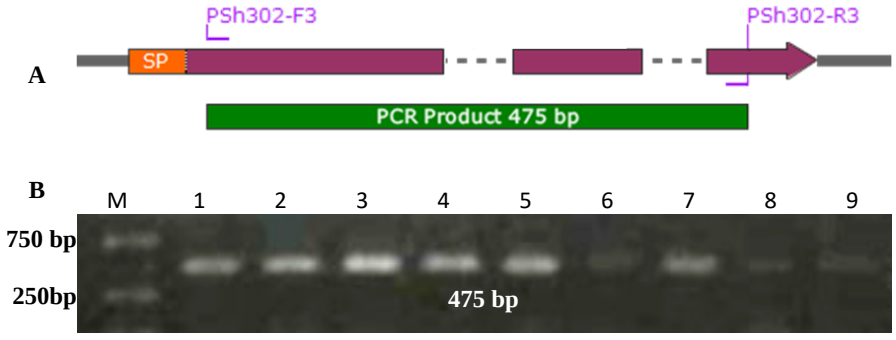


Figure 6. Schematic representation of the *GSh302* gene and the location of the GSh302-F3/R3 specific primers and expected size of the amplicon 475bp (A), the same unique bands in expected size are amplified from genomic DNA of *Ascochyta rabiei* isolates collected from different regions of North Khorasan in (2020 and 2022) in comparison with Pathotypes PI, PIII, PVI collected from Iran in 2000. Well numbers 1-6 correspond to isolates Ar001, Ar011, Ar012, Ar019, Ar020, and Ar043, respectively. Well numbers 7-9 correspond to pathotypes PI, PIII, and PVI, respectively.

Tracking *A. rabiei* in infected chickpea plants

Detection of the *GSh302* gene in chickpea plants infected with *A. rabiei* resulted in the expected specific band amplification, while no band was amplified in healthy plants (Figure 7). Findings showed that *A. rabiei* could be effectively identified in infected plants by detecting the *GSh302* gene using specific primers PSh302-F3/R3.

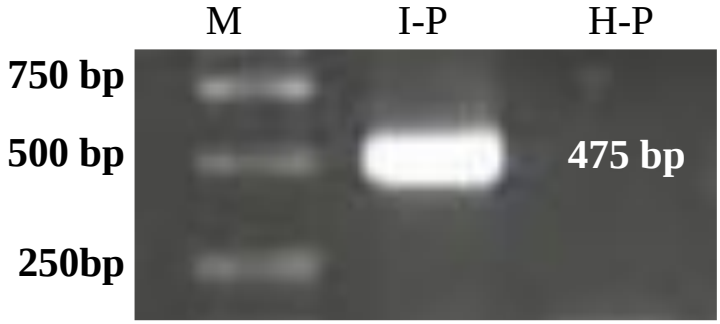


Figure 7. Tracking the *GSh302* gene in the leaf of a chickpea plant (ILC1929) 96 hours after inoculation of the unique amplified band in the expected size in well I-P and no detection of the gene in a healthy plant in well H-P. M is a 1KB DNA size marker.

Discussion

So far, different molecular markers have been used to identify *A. rabiei*, including markers related to the ITS region and the β-tubulin gene (TUB2), which have been used to differentiate between species of the genus *Ascochyta*. Mating type markers have also been used to study the population of this fungus (Barve et al., 2003). Comparison of the genome of *A. rabiei* and its close relatives has resulted in the identification of specific regions, such as the CytB gene region, which can be used to differentiate *A. rabiei* with high accuracy by designing specific primers (Bayraktar et al., 2016). Techniques such as RAPD (Random Amplified Polymorphic DNA) and SCAR (Sequence Characterized Amplified Region) have been used to study genetic diversity among *A. rabiei* isolates (Shokouhifar et al., 2003b).

The primary aim of the present study was to investigate the population of *A. rabiei* and provide a molecular marker based on pathogenicity and effector genes. The expression pattern of a number of candidate effector genes was investigated using RNAseq data available in the SRA database. Clustering of the studied genes based on expression pattern demonstrated that the *GSh302* gene showed a completely distinct pattern compared to the other studied genes (Figure 1). In this regard, the two genes *GSh201* and *GSh210* showed a very similar expression pattern. Previous studies have shown that the *GSh210* gene (Firouzmand et al., ۲۰۲۰) encodes a protein similar to ECP6 (Bolton et al., 2008). This protein has an effector role and Lysine Repeat Domains (LysMD) and is required by fungi to coat chitin in their walls, and also binds to chitin oligomers, preventing the induction of resistance in plants (Gust et al., 2012). The similar expression pattern and their placement in the same expression groups indicate the relationship between the biological functions of genes. This distinction between the expression pattern of the *GSh302* gene and other genes studied may indicate a completely different biological role of this gene. The *GSh302* gene was introduced as a candidate effector gene in a previous study, and it has been shown that the protein it encodes is a secreted protein with the characteristics of effector proteins. Furthermore, the expression of this

gene at the transcriptome level has been confirmed in resistant and susceptible chickpea plants during interaction with *A. rabiei* pathotypes (Hasani et al., 2025b). The lowest expression level of the studied genes was observed 12 hours after fungal inoculation on the plant. Grouping of different samples based on the expression pattern of the studied genes showed that during the interaction of *A. rabiei* with the chickpea plant, 12 and 36 hours after inoculation, the expression pattern of most genes is similar. The most significant difference is related to the expression pattern of genes under conditions of fungal growth on the culture medium. As can be seen, *GSh200* and *GSh201* genes show a significant increase in expression *in vivo* (host plant leaf) and especially at late times (L96), which probably indicates the role of these genes in overcoming the host plant defense response.

On the other hand, comparing the sequence of the gene *GSh302* in *A. rabiei* isolates with the analysis of genomic data of 250 isolates of this fungus available in the SRA database showed that the sequence of this gene is completely conserved. In order to design specific primers that differentiate *A. rabiei* from other species of the genus *Ascochyta*, the sequence of this gene was tracked in other species, and its diversity was studied as well. The results revealed that the complete sequence of this gene can be tracked in other species despite nucleotide variations. The presence of nucleotide variations provided the possibility of designing specific primers to differentiate *A. rabiei*.

In this study, isolates of *A. rabiei* were collected from different farms in the northeast of Iran during 2020 and 2022, and after performing the isolation, purification, and pathogenicity tests, molecular examinations were conducted on them to confirm the presence of the *GSh302* gene using specifically designed primers. The results showed that the desired fragment can be detected in all *A. rabiei* isolates from farms in the northeast of Iran. Moreover, the *GSh302* gene can be used as a suitable marker for identifying and confirming *A. rabiei*. Evaluation of this marker, along with other common markers, to evaluate its efficiency and accuracy in complementary studies is recommended. Furthermore, the design of specific primers for the identification of other *Ascochyta* species based on the *GSh302* gene sequence and specific regions of each species can result in the provision of multiplex markers for the detection of different *Ascochyta* species.

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