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Identification and characterization of a PCD-inducing effector protein ortholog in *Ascochyta rabiei*

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Abstract

As a necrotrophic fungus, *Ascochyta rabiei* possesses various compounds that bypass host plant defense mechanisms. Understanding the genes responsible for its pathogenicity on chickpea plants is crucial for developing resistant cultivars. This study investigates the induction of programmed cell death by *A. rabiei* and its potential involvement during interaction with the chickpea plant. By tracking homologues of effector proteins from other necrotrophic pathogens, a candidate effector protein named GSh200 was identified in *A. rabiei* genomic data. The protein sequence of GSh200 was annotated using web-based tools and submitted to Gene Bank with the following accession number OR567501. Additionally, a homologue of the *GAPDH* gene (*ArGAPDH*) was identified using a homology-based search in the genome of *A. rabiei* and used as a reference gene. Specific primers were designed to confirm the presence of *A. rabiei* isolates in chickpea plants after fungal infection. Sequence analysis and alignment of the *GSh200 gene* in different *A. rabiei* pathotypes revealed identical sequences, indicating a potentially conserved role in pathogenicity. Further investigation is needed to elucidate the functional role of the GSh200 protein in inducing programmed cell death in chickpea plants.

Keywords: Ascochyta rabiei, Effector proteins, Programmed cell death, Chickpea plant, Pathogenicity mechanisms

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ردیابی و تعیین مشخصات پروتئین ارتولوگ اثرگذار در مرگ برنامه‌ریزی شده سلول در قارچ *Ascochyta rabiei*

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چکیده

قارچ ***Ascochyta rabiei*** به‌عنوان یک قارچ نکروتروف از ترکیبات مختلفی برای عبور از سدهای دفاعی گیاه میزبان استفاده می‌کند. شناسائی ژن‌های دخیل در بیماری‌زایی این قارچ روی گیاه نخود می‌تواند در پیشبرد برنامه‌های اصلاحی برای تولید ارقام مقاوم مفید باشد. این مطالعه با هدف ردیابی ارتولوگ پروتئین اثرگذار القاء کننده مرگ برنامه ریزی شده سلول گیاهی مربوط به قارچ ***Rhizoctonia solani*** به نام **AG1IA_07795** در قارچ نکروتروف ***A. rabiei*** می پردازد. آنالیز داده های ژنومی ***A. rabiei*** به شناسائی یک منطقه منتج شد. توالی پروتئین **AG1IA_07795** در پایگاه توالی های کوتاه بیانی ***A. rabiei*** به شناسائی سه ترانسکریپت با همپوشانی بین ٢٨ تا ٩٠ درصد و یکسانی بین ٣٠ تا ٣٨ درصد منتج شد. موقعیت یابی ترانسکریپت با شماره بازیابی **GDJN01012940** با همپوشانی ٩٠ درصد و یکسانی حدود ٣٠ درصد به شناسائی ژن پیش بینی شده ای در توالی ژنوم ***A. rabiei*** منتج شد که می تواند پروتئینی به طول ٤٠٣ اسید آمینه را کد نماید. این ژن به نام ***GSh200*** حاشیه‌نویسی شد. الگوی حضور دامین های کارکردی در پروتئین **GSh200** با پروتئین **AG1IA_07795** مقایسه شد. آغازگرهای اختصاصی **PSh200-F/R** برای ردیابی این ژن در ژنوم جدایه های مربوط به سه پاتوتیپ قارچ ***A. rabiei*** طراحی گردید. به‌علاوه، یک همولوگ از ژن ***GAPDH*** (***ArGAPDH***) به روش جستجوی مبتنی بر همسانی در ژنوم ***A. rabiei*** شناسایی شد و آغازگرهای اختصاصی **PSH19-F/R** برای آن طراحی گردید و به عنوان ژن مرجع مورد استفاده قرار گرفت. نتایج حضور ژن حیاتی اختصاص ***ArGAPDH*** را در هر سه جدایه مورد تایید قرار داد. همچنین تک باندهایی با اندازه مورد انتظار در الگوی الکتروفورزی، حضور ژن ***GSh200*** را در سه پاتوتیپ **PI**، **PIII** و **PIV** مربوط به ***A. rabiei*** تایید نمود. آنالیز توالی این ژن در هر سه پاتوتیپ نشان داد که این ژن ماهیت حفاظت شده ای دارد. با توجه به الگوی بیانی ژن ***GSh200*** در داده های **RNaseq** موجود در پایگاه **NCBI** القاء شوندگی ١٢ برابری این ژن در شرایط رشدی در محیط کشت در مقایسه با شرایط برهمکنش و نقش محافظت شدگی مشاهده شده در این مطالعه، می تواند نشان‌دهنده نقش بالقوه این ژن در بیماری‌زایی قارچ ***A. rabiei*** روی گیاه نخود باشد.

کلیدواژه‌ها: ***Ascochyta rabiei***، ***پروتئین های اثرگذار، مرگ برنامه‌ریزی شده سلولی، گیاه نخود، مکانیسم های بیماری‌زایی***

دبیر تخصصی: دکتر روح الله شریفی

Introduction

Legumes represent a crucial source of plant protein, with the chickpea plant (*Cicer arietinum* L.) holding a notable position within this group (Jukanti et al., 2012). Ascochyta blight, caused by the fungus *Ascochyta rabiei* (Pass.) Labrousse, poses a significant threat to chickpea cultivation worldwide (Kaiser, 1995). Cultivating resistant cultivars is as an effective strategy for reducing the damage caused by this pathogen (Shokouhifar et al., 2006). Understanding the pathogenic mechanisms employed by the pathogen the resistance mechanisms within the plant can inform targeted strategies to achieve sustainable resistance (Jayakumar et al., 2005).

Plants deploy different defense mechanisms based on the lifestyles of the pathogens that infect them (Spoel et al., 2007). When dealing with biotrophic pathogens, inducing cell death can effectively halt pathogen's spread within healthy plant tissues, thereby enhancing plant resistance (Spoel et al., 2007; Gebrie, 2016). Conversely, when a plant is confronted with necrotrophic pathogens, the mechanism of inducing cell death may not serve as an effective defense strategy similar to biotrophic pathogens. This is because necrotrophic pathogens utilize the host cells and their resources for nutrition and energy, leading to accelerated growth of the fungus through the destruction and utilization of plant tissues (Ghozlan et al., 2020). Moreover, some reports reviewed by Shao et al. (2021) indicate that certain necrotrophic pathogens can exploit the plant's defense mechanism by producing effector proteins that trigger cell death, thereby facilitating the absorption of cell contents and accelerating the pathogen's access to nutrients (Van-Kan, 2006; Verma et al., 2016; Shao et al., 2021).

In addition to pathogenicity genes that are directly involved in disease occurrence and disease severity caused by the fungal pathogen in the host plant, identification of the effector proteins belongs to the fungal pathogen is also very important. These proteins can facilitate and accelerate the fungal development in the host plant by inhibiting defense pathways or changing metabolic pathways (Shao et al., 2021). Several studies have been conducted to elucidate the pathogenic mechanisms of *A. rabiei* during the infection of chickpea plants. These studies have identified various toxins such as solanopyrone A, solanopyrone B, solanopyrone C, cytochalasin D, and proteinaceous toxins (Höhl et al., 1991; Latif et al., 1993; Chen & Strange, 1994; Kaur, 1995; Hamid & Strange, 2000; Kim et al., 2017). Additionally, virulence genes like cutinase, xylanase, and pectinase, directly implicated in disease occurrence and severity, have been reported in previous researches (Tenhaken & Barz, 1991; Tenhaken et al., 1997; Bruns, 1999; Jayakumar et al., 2005). More recently, some studies have focused on identifying effector proteins that can indirectly impact the pathogenicity of the fungus (Fondevilla et al., 2015; Verma et al., 2016; Maurya et al., 2020; Shah et al., 2020; Hasani et al., 2023; Singh et al., 2023).

Through conventional methods, certain effector genes in *A. rabiei* have been predicted. By employing *in-silico* secretome analysis and computational algorithms, proteins exhibiting secretion signals (such as signal peptides or non-classical secretion motifs) and other characteristics like small size, cysteine-rich motifs, and conserved non-canonical secretion signals have been identified as potential effector proteins warranting further experimental validation (Verma et al., 2016; Shah et al., 2020; Hasani et al., 2023). Numerous proteins have been predicted to exhibit effector characteristics through various methods, but the key challenge lies in elucidating their roles and functions in host pathogenicity.

To expedite the discovery of effectors in *A. rabiei*, identifying orthologs of known effectors from other necrotrophic fungi species could prove valuable. The

identification process can be accelerated by searching for conserved effector candidates in *A. rabiei* through homology base analysis with characterized effectors from related species. Verma et al. (2016) utilized this approach to identify orthologs of effector proteins from other necrotrophic pathogens such as *Pyrenophora tritici-repentis*, *Cochliobolus heterostrophus*, and *Parastagonospora nodorum* in the genomic data of the ArD2 strain of *A. rabiei*.

Drawing from insights gained from known effectors in the necrotrophic fungus *Alternaria brassicae*, which impacts the lignin catalysis pathway during interactions with the rapeseed plant (Sharma et al., 2007), a parallel pathway was discovered in the interaction between *A. rabiei* and chickpea plant. Specifically, the effector *ArPEC25* was found to impede the lignin synthesis pathway, as reported by Singh et al. (2023). This discovery suggests that the functional mechanisms of effectors in pathogenicity pathways may be conserved across similar pathosystems belonging to the same lifestyle.

It is anticipated that *A. rabiei*, as a necrotrophic pathogen, will encounter plant cell death (PCD) during its interaction with chickpea plants. The pathogen has the ability to protect itself against oxidative compounds produced during degrading and dying host cells (Singh et al., 2012; Maurya et al., 2020). Although PCD can result from the destructive effects caused by toxins and enzymes produced by the fungal mycelium during colonization, it remains unclear whether *A. rabiei*, similar to *Rhizoctonia solani*, *Alternaria alternata*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*, can actively induce PCD in chickpea plants during colonization. Proteins such as ST47_g8388 and ST47_g3737, as reported by Fondevilla et al. (2015), and ST47_g426, as reported by Maurya et al. (2020), are predicted to play a role in inducing PCD in chickpea plants infected by *A. rabiei*. However, the functional roles of these candidate effector proteins have not been clarified yet.

The AG1IA_09161, AG1IA_07795, and AG1IA_05310 are effector proteins well-documented for their functional roles in inducing PCD. These proteins, identified in the necrotrophic fungus *R. solani*, were shown to trigger PCD 48 hours after inoculation of purified proteins on rice, corn, and soybean leaves (Zheng et al., 2013). Through the identification of conserved motifs in their protein sequences, paralogs such as RsAG8_06778 and RSAG8_03224 have been identified in other races of *R. solani* (Anderson et al., 2017). Additionally, transient expression of RsAG8_06778 in tobacco leaves was observed to strongly induce cell death at the injection site and result in a necrotic phenotype in *Nicotiana benthamiana* leaves (Li et al., 2019).

Considering the clear function and mode of action of PCD-inducing effector proteins identified in *R. solani* and their similar impact on various plant species like corn, rice, and soybean, it is plausible to anticipate the presence of their orthologs in the *A. rabiei* genome. Achieving this objective could expedite the identification of pathways involved in PCD induction by this pathogen in chickpea plants. Furthermore, the detection of orthologs of these proteins in various *R. solani* races increases the likelihood of identifying corresponding orthologs in *A. rabiei* pathotypes. The *A. rabiei* pathotypes have been categorized into six groups named PI, PII, PIII, PIV, PV, and PVI, based on their decreasing order of pathogenicity power (Shokouhifar et al., 2003).

This study aims to identify the orthologs of known cell death-inducing effectors in *R. solani* within the genomic data of *A. rabiei*. Subsequently, confirmation will be sought in the genomes of isolates associated with the pathotypes of this fungus using molecular methods. The expression pattern of the corresponding gene will be analyzed

in the expression data of *A. rabiei* fungus from the database. Following this, the gene expression will be examined in isolates cultivated on culture medium as well as under interaction conditions with resistant and sensitive chickpea cultivars. Additionally, detecting the presence of these effectors in different pathotypes can reveal the genetic diversity within the coding gene.

Materials and Methods

Bioinformatics analyses

The amino acid sequence of known effector proteins in the necrotrophic fungus *Rhizoctonia solani* includes AG1IA_07795 (Zheng et al., 2013) and RsAG8_06778 (Anderson et al., 2017) were retrieved from NCBI databases. Both of which have the Inhibitor I9 domain and cause the induction of programmed cell death in the host plant. The protein sequences of these effectors were searched in the NCBI database using the TblastN method in the genomic data of *Ascochyta rabiei* strain ME14 under the accession number GCF_004011695.1, the version released on Jan 13, 2021. In the next step, the sequences of these proteins were investigated using the TblastN method against the data in the Transcriptome Shotgun Assembly (TSA) database related to *A. rabiei* with BioProject retrieval number: PRJNA288273 and biological samples with BioSample retrieval number: SAMN03835260, SAMN03835261and SAMN03835262.

The position of the transcript sequences on the genome of *A. rabiei* strain ME14 under the accession number GCF_004011695.1, the version released on Jan 13, 2021, was determined by blasting the number of transcripts retrieved in the genome viewer web program and the hypothetical predicted genes were identified in transcript regions. The putative protein sequences of the identified regions along with the sequence of effector proteins 07795 (Zheng et al., 2013) and RsAG8_06778 (Anderson et al., 2017) were retrieved from the NCBI database and their multiple sequence alignment was done using the web based tool COBALT in the NCBI database (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi). Protein dissimilarity distances were drawn with the Gene tree sub-menu in the NCBI database based on the Grishin distance coefficient with Max seq difference = 0.85 and Neighbor Joining method. The composition and order of the domains present in the protein sequence were determined using the SMART web-based program (http://smart.embl-heidelberg.de/). The presence of transmembrane helices sequences in protein sequences was defined using the web-based program TMHMM2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/). Confirmation of the location of proteins in the extracellular space relative to the cytoplasmic membrane was investigated using the Protter web-based tool (https://wlab.ethz.ch/protter/start). The number of cytosine amino acids and the possibility of disulfide bond formation in proteins were defined using the SCRATCH web-based tool (http://scratch.proteomics.ics.uci.edu). The expression pattern of transcripts during the interaction of *A. rabiei* with chickpea plant were investigated by searching their retrieval number in the data registered in the gene bank related to *A. rabiei* with BioProject accession number: PRJNA288273 and biological samples with BioSample accession number: SAMN03835260, SAMN03835261 and SAMN03835262 (Fondevilla et al., 2015) and the graph associated with their expression level was plotted using Excel software. The probability of effector function for proteins was evaluated by analyzing their sequence in the web-based tool Effector P3 (https://effectorp.csiro.au/). Tracking of the housekeeping *GAPDH* gene in *A. rabiei* was performed based on the

blast of part of the GAPDH protein sequence reported in *A. alternata* under retrieval number QNM37764.1 in genomic data of *A. rabiei* isolate ME14 with accession number GCF_004011695.1, the version released on Jan. 13, 2021. The similar and different regions of *GAPDH* gene sequence in *A. rabiei* and *Cagapdh* sequence related to chickpea with retrieval number GeneID: 101491441 were determined by BLASTn2 tool at NCBI. The genomic sequences of the studied genes were retrieved from the gene bank and analyzed by the SnapGene program. The schematic map of the genes and their features was drawn afterwards. All primers used in this study were designed by SnapGene program.

Fungal pathotypes cultivation and DNA extraction

Three pathotypes of *A. rabiei*, classified in a previous study (Shokouhifar et al., 2003), with the following strain/code number; FUM 1001/ASR001 (Pathotype I), FUM 1003/ASR003 (Pathotype III) and FUM 1006/ASR006 (Pathotype VI) were provided by the Microorganisms Collection of Ferdowsi University of Mashhad (WDCM 1207), Iran. These pathotypes were cultivated on PDA (Potato Dextrose Agar, Merck, Germany) in completely sterile conditions and kept for ten days at 25 ˚C in a shaker with 125 rpm under light condition to prepare fresh mycelium for DNA extraction. The Grown mycelium was collected from the surface of petri dishes by adding sterile following 5 minutes of centrifugation at 8000 rpm. The collected mycelium was powdered using a mortar and pestle in the presence of liquid nitrogen. DNA extraction was done by CTAB method (Clarke, 2009) and its quality and quantity were tested using electrophoresis on 1% agarose gel.

Tracking and amplification of *A. rabiei* housekeeping gene at the genome level

The contents of PCR (polymerase chain reaction) mixtures consisted of 5 µl PCR master mix 10X (Ampliqon, Denmark), one µl of genomic DNA, one µl (5 pmol) of each following specific primers PSh119-F1 (5'- CCT TCA TCG AGC CCC ACT AC-3') and PSh119-R1 (5'- GCC TCC CTT CAA GTG AGC C-3') and 2 µl sterile water to make the volume up to 10 µl. PCR was conducted in a thermocycler (MWG personal, Germany) with following thermal protocol. An initial denaturation at 93 ˚C for 3 min, followed by 35 amplification cycles consisting of 92 ˚C for 45 s for DNA denaturation, 62 ˚C for 40 s for primers annealing, and 72 ˚C for one min for DNA extension. Also, a final extension step at 72 ˚C for 5 min was included. The PCR product was analyzed and visualized on a 1% electrophoresis gel.

PCR amplification of the target gene and confirming its presence in different pathotypes

Tracking and amplification of the fragment containing the target SNP in genomic DNA extracted from ILC263 and MCC133 chickpea lines were performed using specific primers PSh200-F1 (5'- TCC ACC TGA ATC ATG CAG CTC CA -3') and PSh200-R1 (5'- GCG CCG AGC AAA CCA ATG TAG A -3') by PCR. The contents of PCR mixtures consisted of 5 µl PCR master mix 10X (Ampliqon, Denmark), one µl of genomic DNA, one µl (5 pmol) of each specific primer (PSh200-F1/R1) and 2 µl sterile water to make the volume up to 10 µl. PCR was conducted in a thermocycler (MWG personal, Germany) with following thermal protocol. An initial denaturation at 93 ˚C for 3 min, followed by 35 amplification cycles consisting of 92 ˚C for 45 s for DNA denaturation, 60 ˚C for 40 s for primers annealing, and 72 ˚C for one min for DNA extension. A final extension step at 72 ˚C for 5 min was also included. The PCR product was analyzed and visualized on a 1% electrophoresis gel. Amplified fragments were bi-directionally sequenced using primers PSh118.2-F and PSh118.2-R (CinnaGen Company, Iran).

Sequencing of the target gene amplified fragment from the genome of different pathotypes

Amplified fragments of the genome of different *A. rabiei* pathotypes were sent to CinnaGen Company for sequencing using specific PSh200-F1/R1 primers and were sequenced uni-directionally using PSh200-R1 primers.

Multiple sequence alignment analysis of the sequences belongs to different pathotypes with the sequence retrieved from the gene bank

The sequencing results quality was evaluated using DNA Baser V4 software and the confirmed regions of the bidirectional sequence from each fragment were assembled together. Multiple alignment of sequencing results of each pathotype was done using CLC Genome Workbench 21 software and nucleotide diversity was identified.

Results

Genomic and transcriptomic data analysis to track homologues of effector genes in *A. rabiei*

Tracking of the effector protein AG1IA_07795 sequence (inducer of programmed cell death in the host related to the necrotrophic fungus *R. solani*), in the genomic data of *A. rabiei* at the revision of *A. rabiei*_Me14 RefSeq assembly under retrieval number [GCF_004011695.1] in NCBI showed about 30% identity using TblastN method with over 90% overlap with the predicted gene under retrieval number NW_024066183.1. Furthermore, sequence tracking of this protein in the gene expression pattern data of *A. rabiei* with BioProject retrieval number: PRJNA288273 and biological samples with BioSample retrieval number: SAMN03835260, SAMN03835261 and SAMN03835262 in the Transcriptome Shotgun Assembly (TSA) database related to *A. rabiei* in the NCBI, showed about 30% identity with 90% overlap to the transcript with retrieval number GDJN01012940.1 (Table 1).

The genomic sequence containing these transcripts was not positioned on any of the *A. rabiei* chromosomes in the studied genomic version. Investigation of the sequence alignment results of these transcripts (Figure 1 A-F) on the genomic sequence of *A. rabiei* isolate ME14 with accession number GCF_004011695.1, the released version on Jan 13, 2021, showed that they can be similar with three hypothetical proteins.

Localization of this transcript on the genomic sequence of *A. rabiei* strain ME14 with accession number GCF_004011695.1, released version on Jan 13, 2021, showed that it contained a putative gene encoding an undescribed protein with retrieval number EKO05_005870 (Figure 1A and C). The gene that encodes this protein has been designated as GSh200 in this study.

The second transcript, showing 80% coverage and over 31% identity to AG1A-07795, was the transcript with accession number GDJN01007218.1. It is localized in the region of A. rabiei ‘s genome that encodes an uncharacterized protein, EKO05_004848 (accession number: XP_038799043) (Figure 1B).

Table 1- BLAST results of effector protein sequence AG1IA_07795 related to the necrotrophic fungus *Rhizoctonia solani* versus short reads database of *A. rabiei* transcripts in NCBI

Accession number	Total Score	Query Cover (%)	E-value	Identity (%)	Accession Length
GDJN01012940.1	120	90	6e-27	29.31	1581
GDJN01007218.1	149	81	9e-37	31.50	2351
GDJN01008718.1	129	53	2e-10	34.88	2036
GDJN01000472.1	93.6	28	4e-18	38.89	1418

The third transcript showed about 51% overlap and more than 31% identity with AG1A-07795 protein. The blast of this transcript under retrieval number GDJN01008718.1 with the genome sequence of *A. rabiei* strain ME14 with accession number GCF_004011695.1, the version released on Jan 13, 2021, was related to the identification of a region containing a predicted gene that encodes an undescribed protein with the retrieval number EKO05_010600 (Figure 1C and F).

The constructed gene tree showed that the sequences of EKO05_005870 (GSh200) and EKO05_004848 proteins have a higher similarity to the effector protein AG1A-07795, and EKO05_010600 protein has relatively more differences with them (Figure 2).

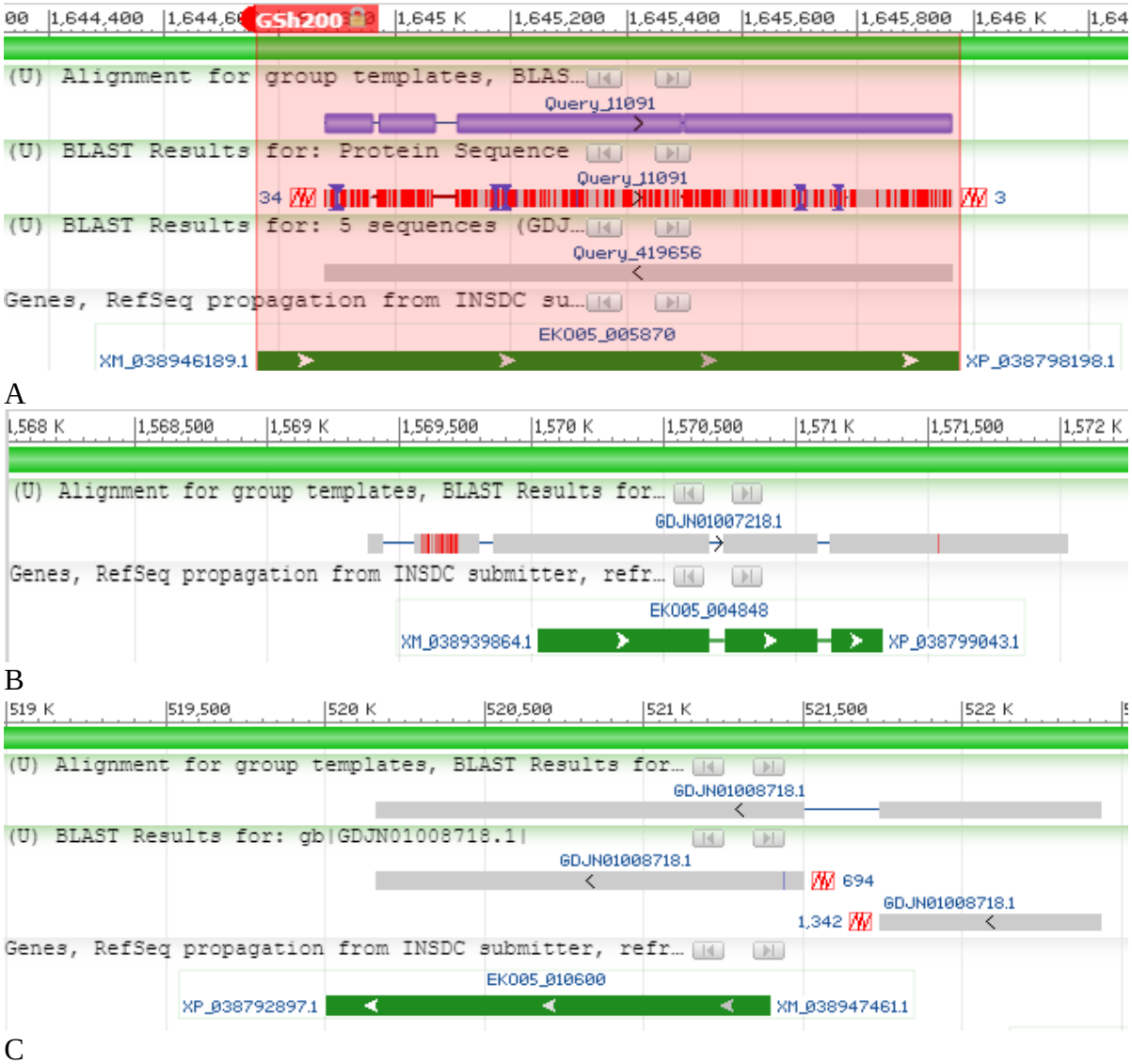


Figure 1. The positions of GDJN01012940.1 (A), GDJN01007218.1 (B) and GDJN01008718.1 (C) transcripts on the genomic sequence of *Ascochyta rabiei* isolate ME14 with accession number GCF_004011695.1 (the version released on Jan 13, 2021) which include three predicted putative genes encoding uncharacterized proteins with retrieval number EKO05_005870 (A), EKO05_004848 (B) and EKO05_010600 (C), respectively. The first protein sequence (A) was named as GSh200 in the present study.

In order to identify the presence of these similarities and differences in the functional regions of these proteins, the arrangement of their domains was also compared. The results of the sequence analysis of these proteins in the SMART web-based tool showed that all three protein sequences have a signal peptide unit which can be concluded that they are secretory proteins. Moreover, all three proteins have Peptidase S8 domain similar to effector proteins reported from *R. solani* (Table 2). The important point is that among these three proteins, only the EKO05_004848 protein possesses the Inhibitor I9 domain, similar to the effector proteins AG1A-07795 and RsAG8_06778 from *R. solani*. This is while the highest level of coverage and identity is observed between the two proteins AG1A-07795 and EKO05_5870; however, based on the domain pattern comparison, the EKO05_004848 protein is likely to be more functionally similar to AG1A-07795.

More investigation on the sequence of this protein in TMHMM2.0 web-based program confirmed the absence of membrane related sequence and it was found that this protein is a secretory protein like effector proteins reported in *R. solani*. Comparing the position of GSh200 protein relative to the cytoplasmic membrane with two effector proteins AG1IA_07795 and RsAG8_06778 using the Protter web-based tool confirmed that all three proteins are secreted. It was also found that GSh200 protein carries three glycosylation sites, while the other two proteins have only one site each (Figure 3)

Two cysteines were observed in the domain region of peptidase S8 (Subtilase) in the protein sequence encoded by *GSh200 gene*, while the only cysteine observed in the effector protein AG1IA_07795 is located between the two domains of inhibitor I9 and peptidase S8 (Subtilase). Investigation on the possibility of disulfide bond formation in GSh200 protein using the SCRATCH tool showed that a disulfide bond can be formed between amino acid cysteine at position 156 and 247, but this possibility does not exist in homologous proteins in *R. solani*.

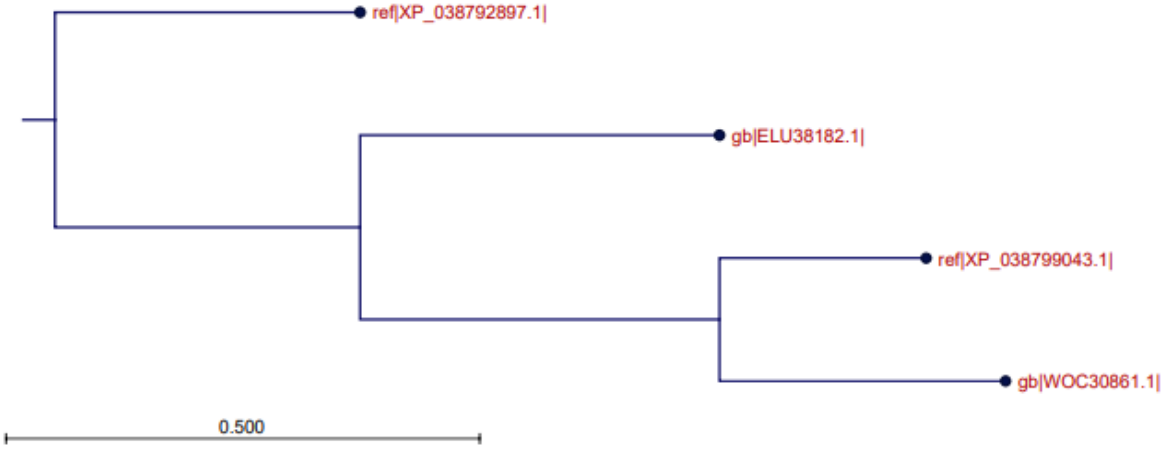
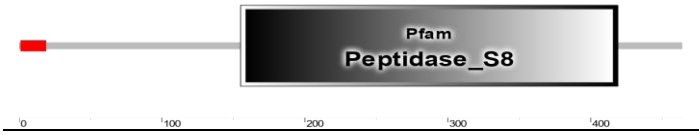


Figure 2. The gene tree illustrates the relationship of the effector protein sequence of AG1IA_07795 (accession number: ELU38182.1 from *Rhizoctonia solani*), with three proteins encoded by the adjacent matched regions of transcripts GDJN01012940.1, GDJN01007218.1, and GDJN01008718.1 on the genomic sequence of *Ascochyta rabiei* isolate ME14, which has the accession number GCF_004011695.1 (released on Jan. 13, 2021). The corresponding coded proteins related to the transcripts are EKO05_005870 (accession number: WOC30861.1), EKO05_004848 (accession number: XP_038799043), and EKO05_010600 (accession number: XP_038792897.1), respectively. These were plotted based on the distance measured by the Jukes-Cantor coefficient and the Neighbor Joining method using the Gene Tree tool in CLC Genomics Workbench, Version 21.0.5, the index line is equal to 0.5. Bootstrap = 100 Replicate.

Table 2- Schematic representation of the position of functional domains in the effector proteins AG1A-07795 and RsAG8_06778 related to *Rhizoctonia solani* compared to known hypothetical proteins in the genomic region encoding the transcripts with the highest homology with them in *Ascochyta rabiei* based on the results of their protein sequence analysis obtained in the SMART web-based tool.

Domains	Protein lengths	Protein name
	405 aa	AG1A-07795
	381 aa	RsAG8_06778
	403 aa	EKO05_005870
	396 aa	EKO05_004848
	464 aa	EKO05_010600

Investigation on the expression pattern of transcripts GDJN01012940.1, GDJN01007218.1 and GDJN01008718.1 during plant-pathogen interaction in the gene expression pattern data of *A. rabiei* with BioProject accession number: PRJNA288273 and biological samples with BioSample accession number: SAMN03835260. SAMN03835261, SAMN03835262 in the Transcriptome Shotgun Assembly (TSA) database at NCBI showed that only GDJN01012940.1 transcript was differentially expressed at different times after fungal inoculation on the plant compared to the fungal growth conditions on culture medium. These data showed that the expression level of this transcript significantly increases at 12 hours after inoculation and gradually decreases at 36 and 96 hours after inoculation (Figure 4).

These results indicate that the expression of the gene *GSh200* was significantly higher at 12 hours post-fungal inoculation on the chickpea compared to 36 and 96 hours after inoculation, with its expression gradually decreasing thereafter. However, its expression was significantly elevated at all three time points compared to in vitro conditions. Based on this, it can be concluded that the expression of this gene is induced by exposure to plant factors. Additionally, it is noteworthy that shortly after inoculation, its expression increased rapidly, reaching a peak before 12 hours.

Sequence analysis of the GSh200 protein from *A. rabiei* and the effector proteins AG1IA_07795 and RsAG8_06778 of *R. solani*, using the Effector P3 web-based tool, revealed that none of these three proteins exhibit effector functions.

This tool can differentiate between cytoplasmic and apoplastic effectors, but as it can be seen in the presented results, none of the three proteins can have a chance to have effector function in fungi and *oomycetes*. The noteworthy point is that this tool is based on the

data of the function of effectors in biotrophic pathosystems, and regarding the necrotrophic pathosystem of *A. rabiei* on chickpea, it is expected to be similar to the necrotrophic pathosystem of *R. solani* on rice, which effector function has been identified for two proteins AG1IA_07795 and RsAG8_06778, and here it is also possible to have similar function. Therefore, the important question is to find evidence for the effector role of protein coded by *GSh200 gene* during the interaction between *A. rabiei* and chickpea. For this purpose, the presence of this gene was tracked in the genomic sequences of different pathotype of *A. rabiei* and their differences were investigated afterwards.

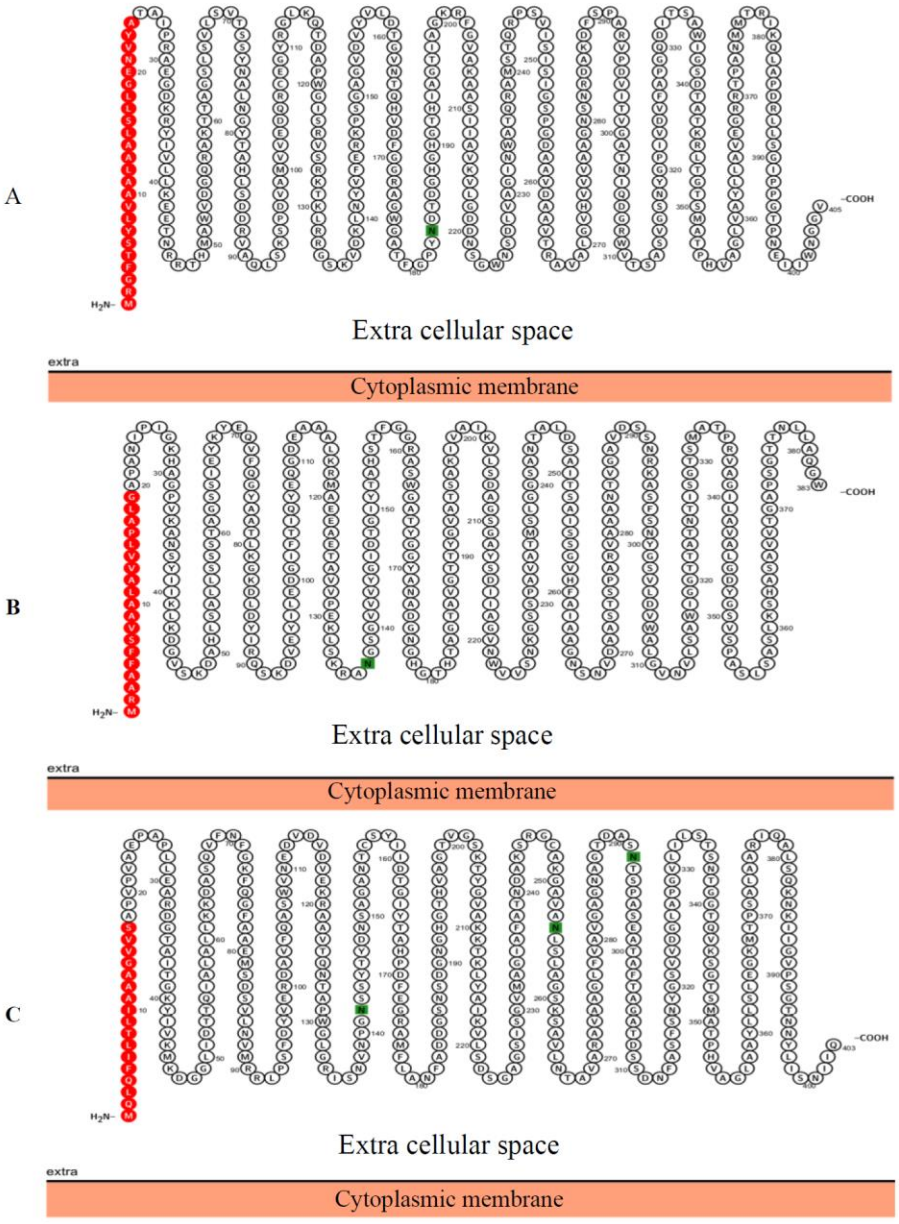


Figure 3. Schematic representation of the locations of effector proteins AG1IA_07795 (A) and RsAG8_06778 (B) from *Rhizoctonia solani*, along with the GSh200 protein (C) from *Ascochyta rabiei*, in relation to the cytoplasmic membrane. Topological structures were generated using Protter v1 (https://wlab.ethz.ch/protter/start/). Circles and numbers indicate amino acids and their positions, while the red-colored N-terminal regions represent putative signal peptides. The green squares denote the positions of N-glyco motifs. The red box illustrates a schematic representation of the plasma membrane, with the extracellular regions specified.

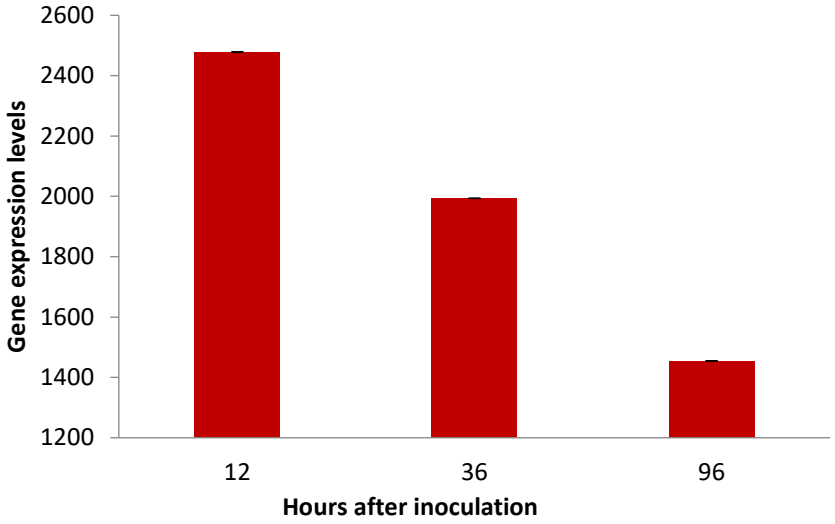


Figure 4. *GSh200* gene expression level during fungal development of *Ascochyta rabiei* on the chickpea plant in the hours after inoculation compared to the growth conditions on culture medium, plotted based on the RNaseq data recorded in the gene bank (Fondevilla et al. 2015).

Tracking of *ArGAPDH* gene in genomic data of *A. rabiei*

Tracking of the *GAPDH* housekeeping gene in *A. rabiei* was performed based on the blast of a part of the GAPDH protein sequence reported in the fungus *A. alternata* with retrieval number QNM37764.1 (Figure 5-A) in the genomic data of *A. rabiei* isolate ME14 with accession number GCF_004011695.1, the version released on Jan 13, 2021. It was resulted in the identification of an unknown hypothetical protein with retrieval number EKO05_007298 (Figure 5-B) termed ArGAPDH in the present study. The coding sequence of *ArGAPDH* gene has about 1564 base pairs length and includes 5 exons (Figure 5-C).

In order to use the coding sequence of the *ArGAPDH* gene as a reference for the molecular identification of *A. rabiei* in chickpea plants inoculated with this fungus, seven similar regions, including three regions with a degree of homology less than 40 and four regions with a degree of homology between 50 and 80, were identified based on the nucleotide sequence alignment of *ArGAPDH* gene from *A. rabiei* with *CaGAPDH* from chickpea with GeneID: 101491441 (Shown in supplementary file 1). In addition, in order to specifically track of *ArGAPDH* gene related to *A. rabiei* and also prevent interference with *CaGAPDH* gene from chickpea, specific primers named PSh19-F/R were designed based on the sequence of distinct and specific regions of *ArGAPDH* to amplify a specific fragment from *A. rabiei* genome sequence.

PSh19-F and PSh19-R specific primers were designed on exon No. 4 and No. 5, respectively, and are expected to amplify a fragment of about 331 bp from the *A. rabiei* genomic sequence and a fragment of about 239 bp from the cDNA (Figure 6 -A). Based on this difference in size, it is possible to distinguish fragments amplified from cDNA from possible amplified fragments caused by genomic contamination.

Based on the sequence extracted from the genome data of *A. rabiei* from the NCBI database, a pair of specific primers named PSh200-F1/R1 were designed at the beginning and end of the coding sequence of the *GSh200 gene*, which are expected to amplify a fragment with 1277 bp length from the genome of different pathotypes of this fungus.

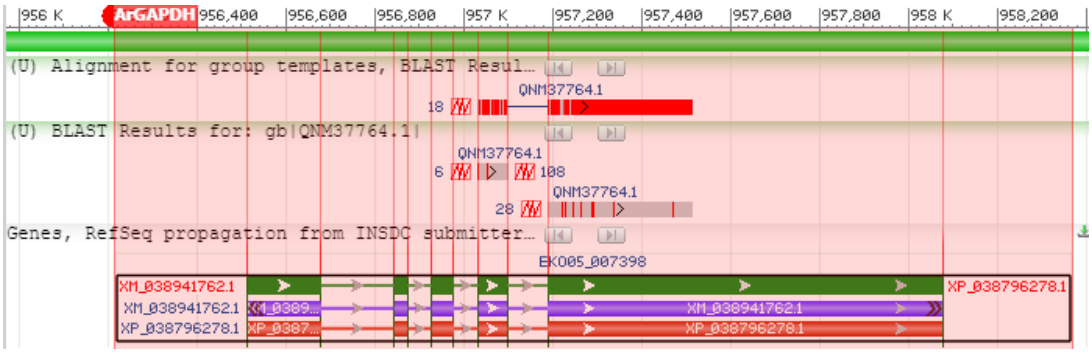
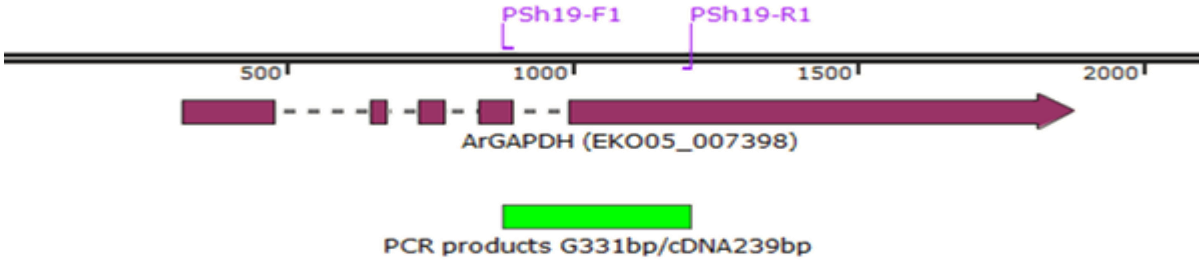
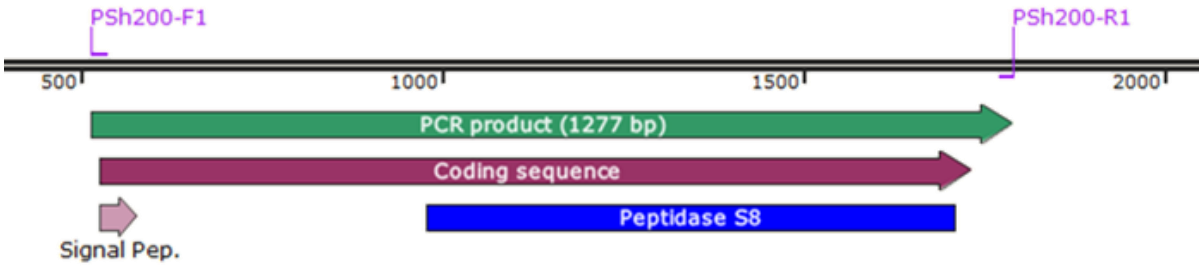


Figure 5. The schematic genome position of *Ascochyta rabiei*, which exhibited a higher hit to the EKO05_007298 protein (GAPDH protein sequence from *Alternaria alternata*), is located in the unlocalized region of the chromosomal sequences within the genomic sequence of *Ascochyta rabiei* isolate ME14, with accession number GCF_004011695.1 (version released on Jan 13, 2021).



A



B

Figure 6. Schematic representation of the genes *ArGAPDH* and *GSh200* and the position of the designed primers and the expected amplified fragment based on the sequences extracted from the genomic data of *Ascochyta rabiei* in the NCBI database. A fragment of the *ArGAPDH* gene with 331 bp length is amplified by specific primers PSh19-F/R (A) and a fragment of the *GSh200* gene with 1277 bp length is amplified by specific primers PSh200-F1/R1 (B). The figures were drawn with SnapGene V5.2 software. Pink arrow: Signal peptide from amino acid position 1 to17, Blue Box: Peptidase_S8: from amino acid position 152 to 395. Green arrow: PCR product amplified by PSh200-F1/R1 primers, red arrow: gene coding sequence area.

Confirming the viability of *A. rabiei* pathotypes and testing the quality of extracted DNA

Different isolates of *A. rabiei* related to pathotypes PI, PIII and PVI, which were cultivated on PDA to check the viability and purity of their stocks, formed 3-4 cm diameter colonies within two weeks (Shown in supplementary file 2). The viability of the isolates was confirmed. The uniformity of the growth of the clones indicated the purity of the original stock at -80. The marginal growth

of the fungal colonies was used to fungal cultivation in liquid culture for DNA and RNA extraction.

A genomic thick band was visible in the electrophoretic pattern of extracted DNA from *A. rabiei* pathotypes I, III and VI. Many fractures were observed in the sample extracted from pathotype I at the end of the gel while in the extracted sample from pathotypes III and VI, a large amount of RNA was seen in the electrophoretic pattern. However, based on the intensity of the genomic band and its comparison to the size marker bands, the concentration of each extracted genomic DNA was estimated to be at least 40 ng/μL (Shown in supplementary file 3).

Tracking of *ArGAPDH* gene in genomic content of *A. rabiei* pathotypes

The electrophoretic pattern of the PCR product with PSh19-F/R primers showed that a single band was amplified in the range of 250-500 bp in all three pathotypes, which corresponded to the expected product size (330 bp) (Figure 7A). This result firstly showed that the quality and quantity of DNA extracted from different pathotypes was appropriate for PCR. Secondly, the lack of amplification of the same fragment in the negative control confirmed that there was no DNA contamination in the samples. In other words, the amplified single fragment corresponds to the DNA added as a template, which belongs to *A. rabiei* and is amplified independently in each reaction. Considering the presence of the housekeeping *GAPDH* gene in different species and also its conserved nature, it is better to investigate on the amplified product sequence in order to know the possibility of distinguishing *A. rabiei* from other fungi in supplementary experiments.

Tracking of *GSh200 gene* in the genomic content of *A. rabiei* pathotypes

The electrophoretic pattern of the PCR product showed that the *GSh200 gene* could be tracked in all three pathotypes of *A. rabiei* and the amplification of the specific fragment in the range of 1 to 1.5Kb was consistent with the expected product size (Figure 7B). The results also showed that the binding site of the designed primers in the genome of *A. rabiei* pathotypes was correctly recognized by the primers and there was no difference in this aspect among the pathotypes. However, the size of amplified fragments in different pathotypes showed minor differences in the electrophoretic pattern. Therefore, it is necessary to perform sequencing in order to ensure the presence or absence of differences in the sequence of the amplified fragments. Again, the absence of the amplified product with same size in the negative control sample showed that the reactions were independently accomplished in each sample and the absence of DNA contamination was confirmed among the samples.

***GSh200 gene* sequence analysis in *A. rabiei* pathotypes**

Multiple sequence alignment results of *GSh200 gene* sequence amplified from PI, PIII and PVI pathotypes of *A. rabiei* using specific primers PSh200-F1/R1 in comparison with the extracted sequence related to the genomic data of *A. rabiei* isolate ME14 with retrieval number GCF_004011695.1, the version released on Jan 13, 2021, showed that from position 370 bp to around 1232 bp, the sequence of amplified fragments with the genome sequence retrieved from the gene bank related to *GSh200 gene* are completely matched. As seen in all three pathotypes, the nucleotide sequences in most regions were identical with the reference sequence (Figure 8).

The results of sequencing and multiple sequence alignment analysis show that the amplified fragment of *GSh200 gene* is conserved in all three pathotypes. Based on these results, it can also be concluded that the DNA sequence of *GSh200 gene* in pathotypes I, III and VI is identical to the reference sequenc

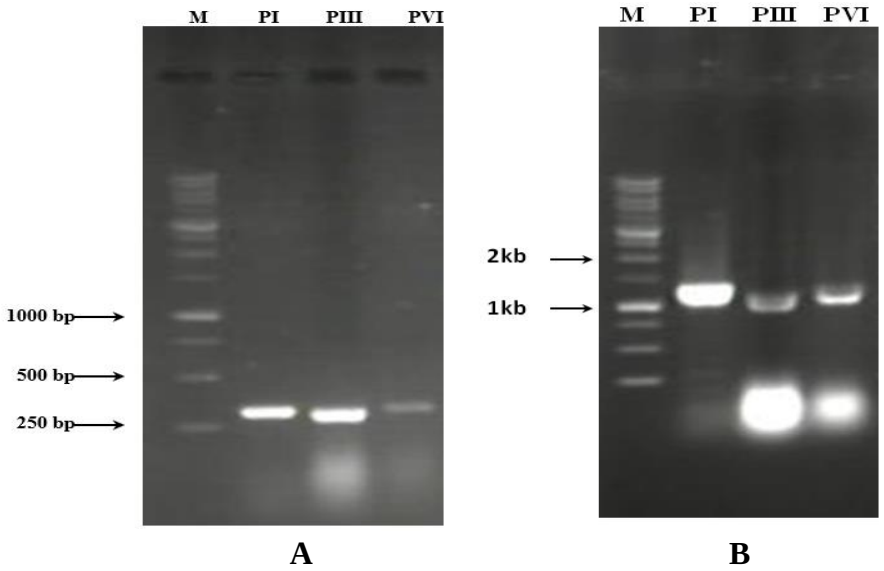


Figure 7. The result of electrophoretic analysis of the PCR product using specific primers PSh19-F1/R1 to track the sequence related to the *ArGAPDH* gene (A) and the PCR product using specific primers PSh200-F1/R1 to track the sequence related to *GSh200* gene (B) in the genome of *Ascochyta rabiei* pathotypes I, III and VI. M is 1 Kb molecular size marker.

Discussion

Identification of effector proteins in necrotrophic pathosystems, such as chickpea Ascochyta blight caused by the interaction of *A. rabiei* and chickpea plant, can be effective in our more complete understanding of the pathogenicity mechanism of pathogens and targeted planning in the breeding of resistant cultivars. Finding the orthologs of well functionally characterized effector proteins from necrotrophic phytopathogens such as *R. solani*, which induce plant cell death during host infections, in the necrotrophic phytopathogen *A. rabiei* could provide valuable insights into the mechanisms of plant cell death induction and pathogenicity of this important pathogen.

Although the possibility of *A. rabiei* ability to induce programmed cell death was already mentioned in chickpea plants, there has been no report on the identification of effector proteins that can play a role in this process. For this purpose and to study this possibility, the present study has investigated the homologues of the effector protein AG1IA_07795 reported in *R. solani* in different pathotypes of the *A. rabiei*. In the first step, the homologue of this protein was analyzed in the genomic and transcriptomic data of *A. rabiei*. The sequence analysis of effector protein AG1IA_07795 reported in *R. solani* showed the presence of inhibitor I9 and peptidase S8 (Subtilase) functional domains. Moreover, the presence of signal peptide, which confirms the secretion of this effector protein, was considered as another important feature in the identification and confirmation of possible homologues of this protein in *A. rabiei*. The blast results of the effector protein sequence AG1IA_07795 related to *R. solani* against the genomic data of *A. rabiei* version *A. rabiei*_Me14 RefSeq assembly with the retrieval number [GCF_004011695.1] in NCBI showing over 90% overlap and about 30% identity with the gene corresponds to the retrieval number NW_024066183.1.

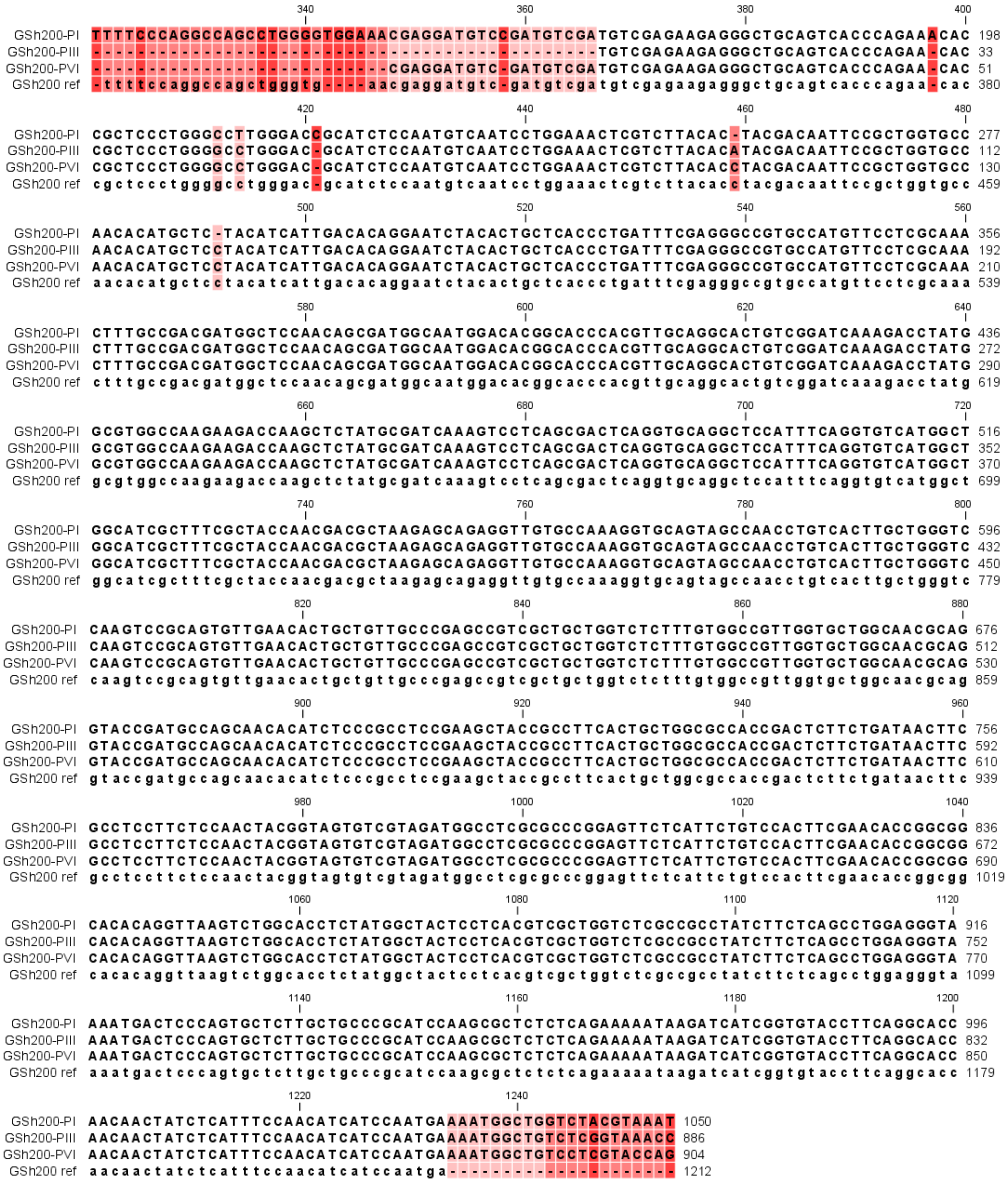


Figure 8. Multiple sequence alignment of *GSh200* gene and PCR product sequences belong to different pathotypes of *A. rabiei* using CLC program.

Moreover, the sequence blast result of this protein with the transcript data of *A. rabiei* with the BioProject retrieval number PRJNA288273 in the Transcriptome Shotgun Assembly (TSA) database at NCBI showed the highest overlap (90%) with the transcript retrieval number GDJN01012940.1. In addition, the results of the expression data analysis confirm the inducible expression pattern in the transcript with retrieval number GDJN01012940.1 during the interaction of *A. rabiei* with chickpea plant. Although the results of the domains pattern investigation show the similarity between the transcript of retrieval number GDJN01012940.1 with the two effector proteins AG1IA_07795 and RsAG8_06778 less than the transcript of GDJN01007218.1, the differential induction expression pattern is only identified for the transcript with retrieval number GDJN01012940.1 in the reported data (Fondevilla et al., 2015). Observing the

expression data related to this transcript with the two effector proteins AG1IA_07795 and RsAG8_06778 related to the *R. solani* confirmed that all three proteins are involved in the early stages of the interaction between the fungus and the plant in the pathosystem of rice and *R. solani* (Anderson et al., 2017) and the pathosystem of chickpea and *A. rabiei* (Fondevilla et al., 2015) and show a significant increase in expression compared to the growth conditions in the culture medium. Therefore, the main analyzes were focused on the gene corresponding to this transcript with retrieval number GDJN01012940.1 which named *GSh200* in this study.

Cellular localization analysis of the proteins encoded by the *GSh200* gene in *A. rabiei*, along with the two effector proteins AG1IA_07795 and RsAG8_06778 from *R. solani*, confirmed their extracellular presence. Furthermore, assessment of their potential to be effector was produced similar results using the Effector P web-based tool and none of them being identified as possessing effector characteristics based on key indicators in Oomycetes. Genomic sequence analysis showed that the coding sequence of the GSh200 protein lacks introns, while the coding sequences of the effector proteins AG1IA_07795 and RsAG8_06778 in *R. solani* do contain introns. Additional observed differences between GSh200 and AG1IA_07795 and RsAG8_06778 included the number of cysteine amino acids and their ability to form disulfide bonds.

The step of analyzing the extracted genomic content of *A. rabiei* pathotypes was carried out in order to ensure the quality of the extracted DNA and also to confirm the way of tracking the genes. First, the housekeeping *GAPDH* gene was identified in the genome of *A. rabiei* and named as *ArGAPDH*. Specific PCR results with specific primers designed for all three pathotypes of *A. rabiei* confirmed the possibility of its detection. The specific primers PSh19-F/R can be used in additional studies to distinguish *ArGAPDH* gene transcripts from genomic content due to the generation of distinct bands.

Specific PCR results using specific primers of *GSh200 gene* showed that the sequence of this gene can be tracked in all three pathotypes of *A. rabiei* and amplify distinct fragments with similar size.

Identification of orthologous effectors of other fungi was also recently reported in *A. rabiei* (Singh et al., 2023). They were able to identify a similar pathway and a similar effector with a similar function in *A. rabiei* based on the similar pathway used by the *Alternaria* fungus in lignin degradation in rapeseed (Singh et al., 2023). Similar studies conducted on *GSh200* ortholog in other plant pathogenic fungi including *R. solani* AG1 IB, *R. solani* AG1 IC and *R. solani* AG3 have shown that in all these pathogens, this gene encodes a protein with a length of about 300 amino acids which carries a 22 amino acid signal peptide at its amino terminus (Wei et al., 2020). A similar function for effector proteins has also been reported in other studies (Lee & Rose, 2012; Neu & Debener, 2019; Kanja & Hammond-Kosack, 2020; Lovelace et al., 2023). Recently, two effector proteins named AsCFEM6 and AsCFEM12 have been reported from *Alternaria solani* which their transient expression induces cell death in several plant species and they have been considered as target proteins to control this pathogen in the early stages of development (Qiu et al., 2024). However, based on the reports about the similarity in the function of effector proteins, GSh200 protein is expected to show a similar function to effector proteins AG1IA_07795 and RsAG8_06778. Anyway, it is necessary to investigate the effect of inducing cell death in the model plant in the additional experiments and in the next steps to investigate its role in

the pathogenicity of the fungus on the host plant (chickpea).

The sequencing results and multiple sequence alignment analysis of these fragments showed that the sequence of the *GSh200 gene* in all three pathotypes PI, PIII and PVI is completely identical to the reference sequence of *A. rabiei* isolate ME14 with retrieval number GCF_004011695.1. The version released on Jan 13, 2021. Several functional mutations have been identified in the sequence of effector proteins AG1IA_07795 and RsAG8_06778 in *R. solani*, and their effects on inducing programmed cell death have been proven (Anderson et al., 2017; Li et al., 2019; Wei 2020). The study of the sequence of the RsIA_NP8 effector coding gene in the genome of 25 different races of *R. solani* has led to the identification of natural nucleotide diversity that can induce PCD in the tobacco model plant as a non-host.

Among the three identified transcripts (GDJN01012940.1, GDJN01007218.1 and GDJN01008718.1) in *A. rabiei*, only GDJN01012940.1 named GSh200 (protein encoding with retrieval number EKO05_005870) has been the focus of this study. This was due to its different expression pattern under growth conditions on culture medium and during interaction with host plant (Figure 4). It is also noteworthy that, the GDJN01007218.1 transcript encoding the protein with the retrieval number EKO05_004848 is more similar to the effectors AG1IA_07795 and RsAG8_06778 in terms of the number of amino acids and the structure of the domains. Therefore, it is necessary to investigate the expression pattern of this effector in additional studies in different conditions, as well as to study its presence pattern and also its diversity at the level of the genome and transcriptome of different *A. rabiei* pathotypes.

In conclusion, this study successfully identified the homolog of an effector protein known for triggering programmed cell death during the interaction between *R. solani* and the host plant in *A. rabiei*, GSh200. The gene exhibited a conserved sequence across all three isolates belonging to *A. rabiei* pathotypes PI, PIII, and PVI, indicating a shared ability among these pathotypes to induce plant cell death. Natural mutations in the *RsIA_NP8* effector gene have been observed in 25 isolates of *R. solani*, preventing the programmed cell death process in the tobacco model plant as a non-host after transient expression (Wei, et al, 2020). Although the ortholog of this gene in *A. rabiei* is completely conserved in isolates related to the three studied pathotypes, its inducing role in PCD should be confirmed in additional studies through the cloning and expression analysis in the model plant.

Moreover, to investigate the relationship of this gene with pathogenic power (virulence), it is necessary to investigate its sequence in other pathotypes of *A. rabiei*. However, if its role in pathogenicity is proven and if its sequence is proven to be conserved in other isolates, it will provide the possibility to use this gene in specific tracking of *A. rabiei*.

Acknowledgments

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