J. Trop. Plant Pests Dis.
Vol. 24, No. 2, September 2024
Pages: 274–283

ISSN: 1411-7525
E-ISSN: 2461-0399
DOI : 10.23960/j.hptt.224274-283

RESEARCH PAPER

Unveiling alliinase gene candidates in shallots using resistance gene motif-based degenerate primers

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Manuscript received: 22 April 2024. Revision accepted: 3 July 2024. Available online: 5 September 2024.

ABSTRACT

Shallots are integral to Indonesian daily life, with annual production reaching two million tons. However, the superior varieties are often susceptible to disease, highlighting the need for new resistant varieties. Understanding the genetic basis of disease resistance is crucial for breeding efforts aimed at developing new varieties. Alliinase, an enzyme crucial for garlic defense, is a potential candidate for enhancing shallot resistance. This study aims to identify alliinase gene candidates in shallots using degenerate primers. Genomic DNA from the *Bima Brebes* genotype was isolated, and degenerate primers successfully amplified 600–800 bp fragments. Three sequences were selected for further analysis, with one sequence showing high similarity to known resistance genes. Multiple sequence alignment revealed characteristic resistance gene motifs, supporting their candidacy as resistance genes. Phylogenetic analysis grouped these sequences with known resistance genes, further supporting their potential. These findings provide valuable insights for breeding programs aiming to enhance resistance in shallots through a molecular breeding approach. The identified alliinase gene candidates can be used to develop disease-resistant shallot varieties. Incorporating these genes into breeding programs can enhance resistance, improving yield and stability in shallot production.

Key words: alliinase, degenerate primer, defense mechanism, molecular marker

INTRODUCTION

Yield loss due to plant pathogens is a major limiting factor in shallot production, increasing the need to prioritize disease resistance or tolerance in the development of superior plants. Plant defense mechanisms against disease are crucial and require in-depth understanding. Regardless of how great a plant's performance is in terms of production capacity, if it is not supported by its ability to withstand the onslaught of diseases in the field, the consequences can be severe. Researchers must focus on three key areas to combat the emergence of virulent biotypes/strains of various plant pathogens: (i) developing new plant varieties with enhanced host resistant mechanisms; (ii) reconstituting resistance that is broken over time through the pyramiding of multiple disease resistance genes; and (iii) developing disease-resistant cultivars that are resilient against both current pathogens and emerging new biotypes (Thudi et al., 2021). Points (i) and (iii) are closely related, but they emphasize different aspects of developing disease-resistant plants. The relationship between these two aspects lies in their shared goal of developing disease-resistant plants, but they differ in focus. The first (i) emphasizes creating new plant varieties with enhanced resistance mechanisms by incorporating novel resistance genes, aiming to strengthen the plant's overall defenses. Meanwhile, the second (iii) focuses on developing cultivars that not only resist specific diseases but also maintain their resilience under varying environmental conditions, ensuring durable and multi-faceted resistance over time. Together, these approaches contribute to a comprehensive strategy for improving plant disease resistance.

Shallots play a crucial role in the daily life of Indonesians, with current production reaching 2 million tons per year. However, one of the main challenges in shallot production is the susceptibility of superior varieties to disease. The most relevant solution to this problem is the development of resistant varieties. To achieve this, understanding the source of disease resistance is crucial. Therefore, research focused on identifying genes involved in disease resistance responses is essential. Alliinase, an enzyme encoded by a member of a multigene family, plays a

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significant role in garlic's defense against pests and diseases (Ovesná et al., 2015; Nwachukwu et al., 2012; Viswanathan et al., 2014).

The alliinase gene family has been extensively studied in various *Allium* species, particularly for its role in sulfur metabolism and defense mechanisms (Al- Khayri et al., 2023). Alliinase enzymes are responsible for converting cysteine sulfoxides into allicin, a compound known for its antimicrobial properties and contribution to plant defense (Al-Khayri et al., 2023; Tiku et al., 2018). Previous research has explored the enzymatic functions of alliinase, focusing on its biochemical pathways and the resulting bioactive compounds that enhance plant defense against pathogens (Ovesná et al., 2015; Prajapati et al., 2023). However, there is limited information on the potential role of alliinase as a resistance gene in shallots. Therefore, it is important to address this gap by employing resistance gene motif-based degenerate primers to identify alliinase gene candidates in shallots, thereby providing insights into their potential role in plant resistance.

Identifying genes and functional markers that are strongly associated with variations in plant phenotypes is a major challenge, especially as a breeding strategy for improving agronomic traits and resistance to biotic and abiotic stress (Salgotra & Stewart, 2020). Advances in molecular biology, including genomics, high-throughput sequencing, and genome editing, are enabling the development of increasingly rapid and precise cultivars. On the other hand, these advances also make it easier to explore new genes, including those that play important roles in plant defense mechanisms. Advances in bioinformatics also open greater opportunities to obtain a variety of functional genes, especially when combined with a degenerate-primer approach for targeted gene sequence amplification (Zuiter et al., 2012; Yu & Yan, 2013; Aminfar et al., 2019). Degenerate primers are often used to amplify gene sequences related to plant defense mechanisms because they are usually designed based on specific motifs that characterize the response function of the plant defense system (Kiselev & Dubrovina, 2009; Pan et al., 2007). Apart from their high specificity, degenerate primers are also highly productive, increasing the oportunity to acquire new genes whose roles in plant defens mechanisms have not yet been confirmed.

This study aims to identify functional genes in shallot plants using a bioinformatics approach with degenerate primers. The research demonstrates that degenerate primers can successfully amplify the alliinase gene sequence, a crucial gene in shallot plants involved in pathogen defense. This finding is highly valuable for plant breeding programs, providing essential gene sequence information for future breeding efforts, particularly in the development of molecular markers for shallots.

MATERIALS AND METHODS

Research Site. This study was conducted in the greenhouse and microbiology laboratory of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) in Bogor (6°34'31.58"S, 106°47'07.37"E; 221 meters above sea level) in January 2020.

Plant Material. The plant material used in this study was the *Bima Brebes* cultivar, which is widely favored by farmers in Indonesia.

Genomic DNA isolation. DNA isolation was performed following the protocol outlined by Doyle & Doyle (1990). The quality and quantity of DNA were assessed using a Nanodrop machine (Thermo Scientific 2000). Shallot genomic DNA extraction began by adding 500 µL of pre-warmed CTAB extraction buffer (100 mM Tris HCl pH 8, 2% CTAB, 50 mM EDTA, 0.7 M NaCl, 0.17% β-mercaptoethanol, and 1% PVP) to ground samples. The samples were then incubated at 65 ºC for 30 min in a water bath, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding two volumes of absolute ethanol and pelleted by centrifugation at 15,000 g for 15 min. The pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). To degrade RNA, the DNA was treated with RNase A (50 mg/mL) for 30 min at 37 ºC. DNA concentration and purity were assessed using the NanoDrop machine. DNA purity was determined by the absorbance ratio at 260 nm to 280 nm, with a good purity falling within the range of 1.8 to 2.0 (Sambrook et al., 1989).

Amplification, Cloning and Sequencing. PCR amplification was conducted using a set of forward and reverse primers detailed in Table 1. These primers were designed based on conserved motifs from the gene encoding the NBS-LRR protein. The PCR reaction was carried out in a 50 µL volume containing 200 ng of genomic DNA, PCR buffer with $(NH_4)_2SO_4$

(Fermentas Inc., USA), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP mix, 1.2 mM of each degenerate forward and reverse primer, and 1 unit of recombinant Taq polymerase (Fermentas Inc., USA). The PCR reaction was performed using a PCR machine (Mastercycler pro, Eppendorf Inc., Germany) with the following cycling conditions: initial denaturation at 95 ºC for 1 min, followed by 36 cycles of denaturation at 94 ºC for 50 s, annealing at 48 ºC for 1 min, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. PCR products were analyzed alongside a 100 bp DNA ladder using agarose gel electrophoresis stained with EtBr (0.5 mg/ml) in 1% (w/v) agarose gel in EDTA buffer (TAE) (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The amplified products were visualized, their sizes estimated, and then purified using a Purification Kit (Qiagen Inc., USA). The purified PCR products were sent to 1st Base (Malaysia) for cloning and sequencing.

Sequence Alignment and Phylogeny analysis. The DNA sequences were first refined using BioEdit software to ensure accuracy. To eliminate any microbial sequence contamination, the VecScreen program from NCBI was employed. Subsequently, the sequences underwent BLASTn analysis through the BLAST program (www.ncbi.nlm.nih.gov/BLAST) to identify the closest matches. Sequences were selected based on high similarity (e.g., e-value < 1e-5) to known resistance and alliinase genes identified via the BLAST tool, ensuring functional relevance. Selected sequences needed to contain key conserved motifs characteristic of defense-related genes, such as P-loop, GLPL, kinase, and LRR motifs. Sequences with uninterrupted open reading frames (ORFs) longer than 300 base pairs were prioritized to ensure potential protein functionality. Sequences with ambiguous bases, low-quality scores, or identified as contaminants were excluded.

The selected sequences were then translated into amino acids using the Transeq tool from the EMBOSS suite software (Rice et al., 2000). Only peptide sequences with uninterrupted open reading frames (ORFs) were retained. For alignment, a total of 25 sequences were used, including 17 alliinase gene sequences and 8 resistance gene sequences obtained from the NCBI database, translated into amino acids. The parameters employed for the alignment included color-based highlights using Clustal dimensions, consensus-based annotations, and a threshold set below 50% (Colour --> Clustal --> By annotation --> Consensus --> Use original color --> Threshold --> Below 50).

The decision to use resistance genes as reference sequences for alignment was motivated by the aim to understand the relationship between the target sequences (alliinase genes) and common resistance genes or resistance gene analogs based on their motifs. This choice was also influenced by the use of degenerate primers with resistance gene-based motifs for gene amplification.

The alignment of the sequences and the construction of the phylogenetic tree were performed using the MAFFT WS alignment method with annotation in Jalview 2.11.13 (Waterhouse et al., 2009). The phylogenetic tree was constructed using the Neighbour-Joining method with the BLOSUM62 substitution matrix (Waterhouse et al., 2009).

RESULTS AND DISCUSSION

Isolation of DNA Genome and Sequence Identity. In this initial study, the *Bima Brebes* variety were used to establish a proof of concept for identifying alliinase gene candidates using degenerate primers. Although the sample size was limited, it allowed us to successfully demonstrate the potential of this methodology. Future research will include a larger number of samples and diverse shallot varieties to validate and expand upon these findings. Increasing the sample size will enhance the robustness and generalizability of the results, providing a more comprehensive understanding of the genetic basis of disease resistance in shallots (Collard & Mackill, 2008; Xu et al., 2017). The genomic DNA from the *Bima Brebes* genotype was successfully isolated and amplified using degenerate primers, resulting in amplicons of 600–800 bp. Subsequently, the amplicons were cloned and sequenced. From the amplification, fragment selection, cloning, and

Table 1. List of degenerate primers used in this study

No	Primer combinations	Sequences (5'- 3')	Motifs (amino acids)
1	Ploop-F/GLPL-R	GGIGGIRTIGGIAAIACIAC	GG(V/I)GKT
		IAGIGYIARIGGIAGICC	GLPL(T/A)L
2	Ploop2-F/GLPL2-R	GGWATGGGWGGWRTHGGWAARACHAC	GGVGKTT
		ARNWYYTTVARDGCVARWGGVARWCC	GLPLAL

sequencing, three sequences were selected for further analysis.

Blast Result Analysis of Sequence Using Conserved Domain Approach. Based on the results of amplification, fragment selection, cloning, and sequencing, we obtained two DNA sequences that qualified for further analysis using Bioedit. Subsequently, both sequences were used as templates for BLASTx analysis on NCBI, resulting in the identification of protein sequences, as represented in Table 2.

Using EMBOSS Transeq with the parameters "all frames", allowing for trimming and reversal, we obtained six protein sequences in each run. We then selected only one sequence from each run for further analysis. The protein sequence derived from Acepa2_Seq001_2 had fewer results compared to Acepa1_Seq001_2a (Table 2). The selected sequences were the longest and did not contain any stop codons. Acepa1_Seq001_2a comprised 279 amino acids, whereas Acepa1_Seq001_2 contained only 84 amino acids. Based on these findings, Acepa1_Seq001_2 is expected to provide a correct gene sequence. However, if a gene similar to the Acepa1_Seq001_2a sequence is retrieved, the accuracy of gene similarity is likely to be closer to the actual gene identity.

Multiple Sequence Alignment. Based on the analysis results, the two tested sequences (Acepa1_Seq001_2a and Acepa2_Seq001_2) exhibited a higher degree of sequence similarity with reference sequences derived from resistance genes. Further phylogenetic analysis confirmed that these two protein sequences clustered with resistance gene sequences. Additionally, ClustalW analysis conducted using Jalview software revealed characteristic patterns of R gene in the cloned sequences. Specifically, sequences located at 10-130 bp (Figure 1a) exhibited the P-loop, GLPL, Kinase, and LRR motifs, while sequences located at 131-210 bp (Figure 1b) contained the RNBS and GLPL motifs.

The analysis revealed that the majority of the analyzed sequences fell below the set consensus value threshold of 50%. In Figure 2, the reference sequences originating from alliinase genes predominantly cluster together. However, three reference sequences—S73324.1 (alliinase from *Allium sativum*), MG742369.1 (alliinase from *A. macrostemon*), and MG742366.1 (alliinase from *A. ampeloprasum*)—stand out by forming a distinct group separate from the other alliinase genes. Specifically, S73324.1 and MG742369.1 are grouped together, while MG742366.1 is positioned separately.

Phylogenic Tree Analysis. The phylogenetic trees were constructed using the average distance method with Percentage Identity (PID) (Table 3). This measure calculates the similarity between each pair of sequences in the alignment, representing the number of identical bases (or residues) per 100 base pairs (or residues). In this analysis, a PID value threshold of 30 was employed. The results indicate that at a > 60% similarity level (marked by the red vertical line), eight clades were identified (Figure 2). This dendrogram visually represents the genetic relationships among the analyzed sequences. The tree divides the sequences into distinct clades based on their genetic distances. Each clade groups together sequences that share a higher degree of similarity, indicating closer evolutionary relationships.

Clade 1 includes sequences S77324.1 and MG742369.1, which are closely related based on their high PID values. Clade 2 contains a more diverse group of sequences (e.g., Prf_U65391, Acepa2_Seq001_2, X87851, and others), indicating a broader range of genetic variability within this clade. Clades 3 to 8 each contain one or more sequences, highlighting distinct genetic groupings. For instance, Clade 5 includes several sequences (M98267.1, L48614.1, MG742367.1, etc.), showing their closer genetic

Table 2. List of protein sequence after blast analysis

Name	Sequence
Acepa1_Seq001_ 2a	YRIPNTGMGGVGKTTIPVPADLKDPNKVLN
	VGSQLNVSVQNALISFLKANLDVFAWTHADMCGISPEVAVHALNIDPKFTPVKQKRRIQG
	LERSTALKEEVDRLMENNFIRESTYPNWVSNPVLVKKANGKWRVCIDFSDLNKACPKDWF
	PLPRIDQLVDGTAGHELLSFMDAYSGYNQIMMYEPDQEDTSFITDVGLYCYRVMPFGLKN
	AGATYQRLVNAMFKPQLGKTMEVYVDGMLVKSKKAVDHIEHLAEMFAILRRYGMKLNPVL
	GIPAAHPLV
Acepa2_Seq001_2	RPLYRIEVWIWKTMARQNSRRLRAE
	RSQQEGSQQASSGGEEVPASIYITRNEMEAITNSLQERLLKSQREMMQTFLEQMRAAGT

relationship.

The dendrogram in Figure 2 complements the data presented in Table 3 by providing a visual representation of the genetic distances and relationships among the sequences. It helps in understanding the evolutionary lineage and potential functional similarities among the sequences. This phylogenetic analysis is crucial for identifying and categorizing gene variants, which can further aid in studying their roles in disease resistance and other genetic traits.

The alliinase gene family can exhibit significant sequence variability among different *Allium* species and even among different cultivars within a species. Degenerate primers are designed to amplify a broader range of related sequences by accommodating this variability, thereby increasing the likelihood of identifying novel alliinase gene variants in shallots (Peška et al., 2019). Using degenerate primers allows for the capture of previously unknown or uncharacterized sequences that might not be detected with specific primers (Nishimura et al., 2024). This approach is particularly valuable in discovering new gene variants that contribute to disease resistance, which may not be represented in existing databases.

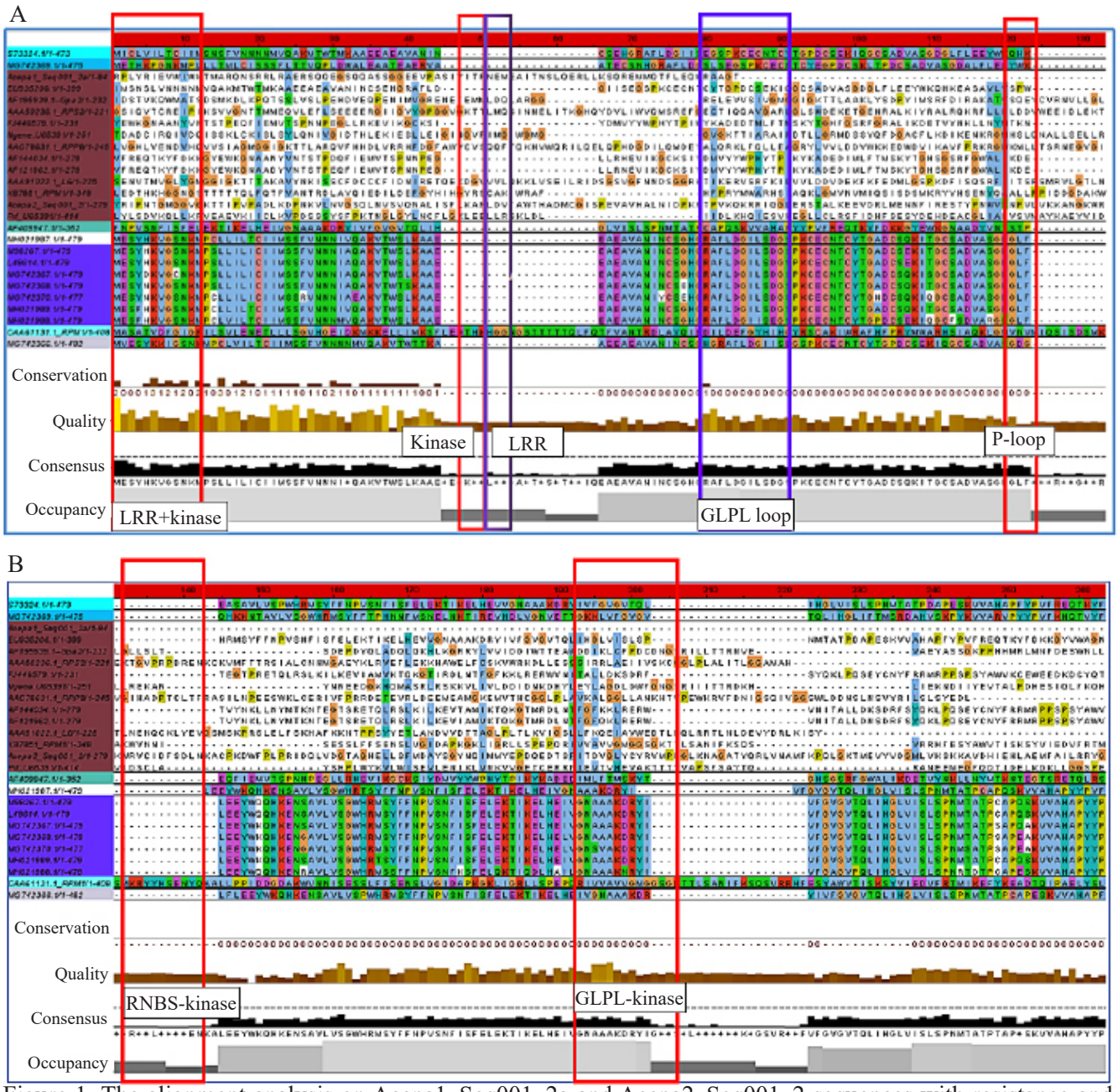

Figure 1. The alignment analysis on Acepa1_Seq001_2a and Acepa2_Seq001_2 sequences with resistance and allinase genes sequences using the Jalview ver. 2.7. A. P-loop, GLPL-loop, Kinase and LRR motif; B. RNBS and GLPL kinase motif.

Table 3. Clades based on average distance method with Percentage Identity (PID)*

Clade	Member of clade
1	S773241; MG742369.1
2	Prf_U65391; Acepa2_Seq001_2; X87851; AAA91022.1; AF121962.1; AF144034.1; Acepa1_Seq001_2a; EU935206.1; AF195939.1; AAA50236.1; FJ446579.1; NgeneU65391; AAC78631.1;
3	AF409947.1
4	MH021987.1
5	M98267.1; L48614.1; MG742367.1; MG742368.1; MG742370.1; MH021989.1; MH021988.1
6	CAA61131.1
7	MG742366.1
8	AB004269.1

*PID value threshold is 30.

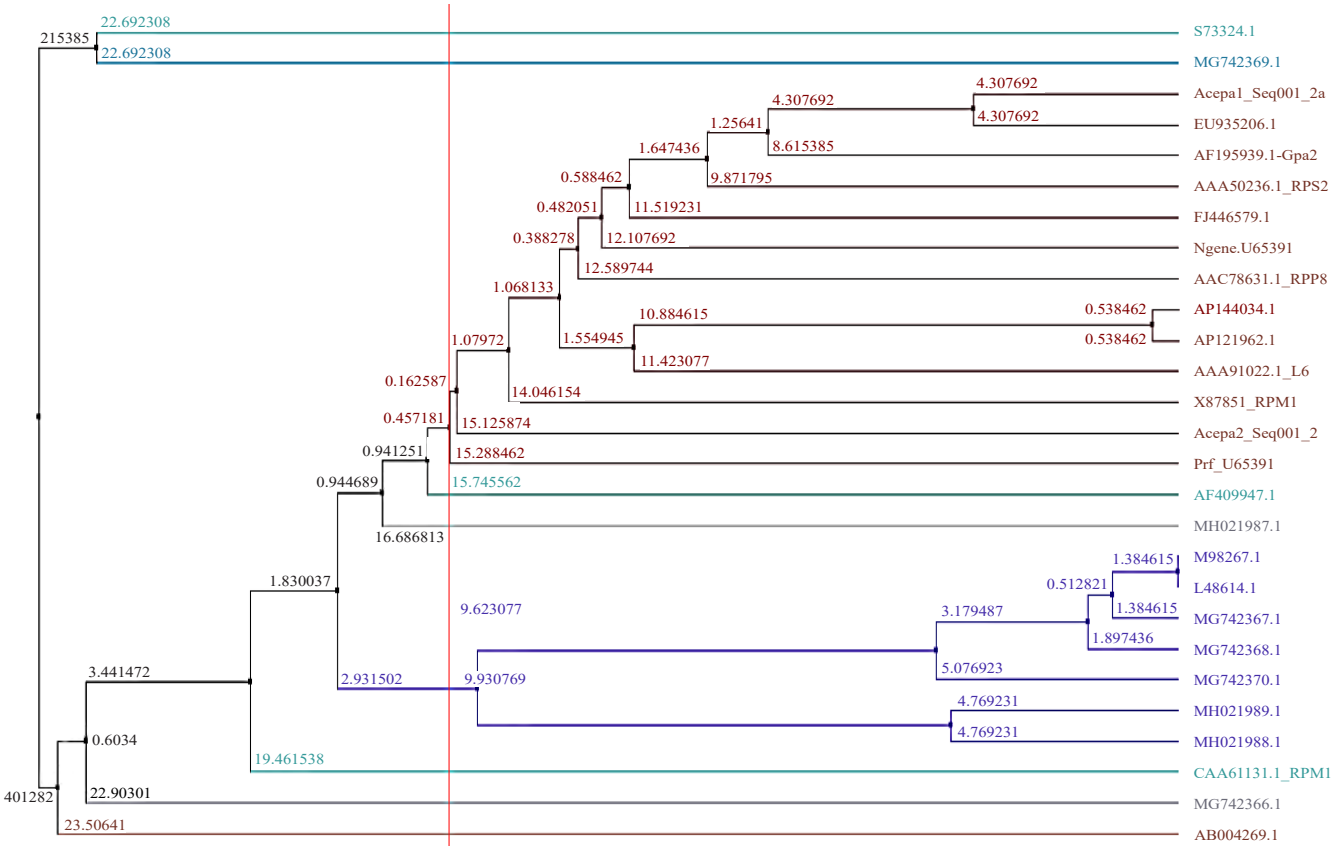

Figure 2. Phyllogenic tree based on average distance using PID.

Degenerate primers are useful for cross-species amplification when the target gene is conserved but not identical across different species. This strategy enables the identification of conserved motifs and functional domains within the alliinase gene that are critical for its role in plant defense (Zhang et al., 2019). For an initial screening aimed at identifying potential resistance genes, degenerate primers provide a more comprehensive approach. Once a range of alliinase gene sequences is identified, specific primers can then be designed for more targeted studies and applications (Chukwuemeka et al., 2020).

Motif-based approaches offer a systematic and efficient way to identify resistance genes in plants, which is crucial for understanding plant-pathogen interactions and developing strategies to enhance disease resistance in crops. These methods involve searching for conserved sequences or motifs associated with known resistance genes within the plant genome. By identifying these motifs, researchers can pinpoint candidate genes that may confer resistance to pathogens. Once candidate genes are identified, they undergo rigorous validation to confirm their role in resistance. This validation includes molecular techniques such

as PCR, sequencing, and gene expression analysis. Validated genes can then be used to develop molecular markers for breeding programs, enabling breeders to select for plants with enhanced disease resistance traits. Overall, motif-based approaches provide valuable insights into plant defense mechanisms, leading to the development of more resilient and productive crop varieties.

The P-loop, GLPL, and Kinase domains are protein components of the NBS-gene (McHale et al., 2006). Their presence indicates a role in nucleic acid binding, crucial for disease-resistant signal transduction and plants' response to pathogen invasion (Wang et al., 2023). The LRR domain consists of a repeating leucine-rich amino acid sequence, typically 20-29 residues long, significant for hormone receptor interaction, enzyme inhibition, cell adhesion, and cell transport. It provides a structural framework for protein-protein interactions and is the primary determinant of NBS-LRR gene-specific resistance (Wang et al., 2023).

RNBS (Resistance Nucleotide-Binding Site) motifs, which are part of NBS-LRR proteins, are crucial for their proper functioning in disease resistance. They participate in nucleotide binding and activate downstream signaling pathways leading to disease resistance responses in plants (Reddy et al., 2015; He et al., 2022; Bouktila et al., 2014; Seshadri et al., 2015; Arya et al., 2014; Ali et al., 2018; Chen et al., 2016; Yu et al., 2018). The conserved nature of RNBS motifs allows for the identification and isolation of disease resistance gene analogs (RGAs) from various plant species. These RGAs are essential for understanding the molecular mechanisms of plant immunity and for devising strategies to enhance disease resistance in agricultural crops (Chandran et al., 2020; Naresh et al., 2017; Nishmitha et al., 2022). The presence of these motifs suggests that the Acepa1_Seq001_2a and Acepa2_Seq001_2 sequences could be potential candidate for resistance genes.

Among the eight groups identified, Clade-2 stands out as the most noteworthy, containing the highest number of members (13) compared to other clades. Notably, Clade-2 is predominantly composed of genes associated with resistance, such as the *Prf* gene, *RPM1* gene, *L2* gene, *Gpa2* gene, *L6* gene, and *RPS2* gene. Interestingly, Clade-2 also includes an alliinase gene from *A. cepa* (FJ446579.1) and *A. wakegi* (AF144034.1). Another intriguing feature is observed in Clade-5, where all members are alliinase genes from seven different *Allium* species.

Our findings on the identification and analysis of alliinase gene candidates in shallots align well with related studies on alliinase genes in other *Allium* species. In this study, the amplified and sequenced alliinase genes from shallots showed high similarity to known resistance genes, suggesting their potential role in defense mechanisms. Based on the comprehensive analysis results and the similarities of Acepa1_Seq001_2a and Acepa2_Seq001_2 to both alliinase and resistance genes, there is a strong indication that these sequences belong to the alliinase gene group. This suggests a potential role in the plant's defense mechanism against diseases in shallots, presenting a highly intriguing outcome.

Research on garlic (*Allium sativum*) has shown significant polymorphism within the alliinase gene family, reflecting various functional adaptations. For instance, different alleles of alliinase genes exhibit tissue-specific expression and are regulated in response to environmental conditions and developmental stages (Ovesná et al., 2015). Similarly, our study found that the alliinase sequences in shallots contain motifs characteristic of resistance genes, supporting their role in plant defense.

Studies on the alliinase gene in garlic have highlighted its crucial role in sulfur metabolism and the biosynthesis of allicin, a compound with antimicrobial properties that enhances plant defense (Soorni et al., 2021, Al-Khairy et al., 2023). The alliinase genes identified in shallots in our study likely perform a similar function, contributing to the plant's resistance to pathogens through the production of sulfur-containing defense compounds. The regulatory networks involving alliinase genes in Allium species, such as garlic, involve complex interactions with other sulfur metabolism-related genes like sulfite reductase (SiR) and superoxide dismutase (SOD), which are crucial for managing the plant's response to stress (Al-Khairy et al., 2023). The presence of conserved resistance motifs in the alliinase genes of shallots suggests that these genes may also be part of a broader regulatory network that enhances disease resistance.

Phylogenetic studies have shown that alliinase genes from different *Allium* species, including onion, garlic, and leek, are closely related and share conserved domains essential for their enzymatic function (Ovesná et al., 2015; Abdelrahman et al., 2020). Our phylogenetic analysis grouped the shallot alliinase sequences with known resistance genes, reinforcing the hypothesis that these genes play a significant role in the plant's defense mechanisms. The alliinase gene, traditionally associated with onions, is crucial for producing the alliinase enzyme, which in turn is

essential for allicin formation. Allicin is known for its role in plant defense against harmful microorganisms and herbivores. Alliinase is a protein present in all garlic tissues (Prajapathi et al., 2023). The identification of the putative alliinase gene using degenerate primers that target resistance genes, including those with nucleic-binding motif component, supports the hypothesis that alliinase may be part of the defense gene repertoire. It is likely involved as a receptor in signal transduction pathways associated with pathogen detection, enabling plants to respond effectively to pathogen invasion.

CONCLUSION

The research successfully isolated genomic DNA from the *Bima Brebes* genotype, yielding 600-800 bp amplicons. Cloning and sequencing produced three sequences, with Acepa1_Seq001_2a generating 279 proteins compared to 84 from Acepa1_Seq001_2. These sequences show promising similarity to known resistance genes and have potential for enhancing pathogen resistance in breeding programs. Further validation studies are warranted.

ACKNOWLEDGMENTS

The author(s) received no financial support for the research, authorship, or publication of this article.

FUNDING

This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

AUTHORS' CONTRIBUTIONS

LH designed the study, conducted the laboratory work, analyzed the data, and wrote the manuscript. BI analyzed the data and contributed to the paper's revision. All authors read and approved the final version of the manuscript.

COMPETING INTEREST

We declare that we have no known competing financial interests or personal relationships that could have to influenced the work reported in this paper.

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