Egypt. J. Bot. **Vol. 64** (Special Issue), pp. - (2024)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Molecular Identification of a Novel *Fusarium fujikuroi* Isolate and Genetic Characterization of Rice (*Oryza sativa* L.) Resistance to Bakanae Disease

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BAKANAE disease, caused by *Fusarium fujikuroi*, is a major threat to rice production globally, especially in Egypt. No rice variety is completely resistant to bakanae disease. Here, we assessed resistance in 11 Egyptian rice genotypes, identified a novel *F. fujikuroi* strain using molecular techniques, and developed molecular markers for resistance breeding. ISSR and InDel PCR were used to determine the genetic distance between the genotypes and develop molecular markers for bakanae disease resistance. QRT-PCR was used to assess the gene expression of four resistance candidate genes. Sakha 103 and Sakha 104 were the most tolerant genotypes, while Sakha 101 and Sakha 108 were the most susceptible to bakanae disease. ISSR and InDel PCR showed high polymorphism values among genotypes and distinctive bands in the most tolerant varieties. Gene expression analysis revealed up-regulation of LOC_Os01g41770, LOC_Os01g41780, and LOC_Os01g41790, which encode leucine-rich repeat receptor-like proteins, in susceptible varieties. Additionally, LOC_Os01g41800, encoding a putative cytochrome P450 monooxygenase, was up-regulated in tolerant genotypes after inoculation.

This study provides tools for screening rice genotypes for bakanae disease resistance and improving resistance breeding programs. The identified molecular markers and candidate genes could potentially be used in future breeding programs to develop new, Bakanae-resistant rice varieties, which is essential for sustainable rice production.

Keywords: Bakanae disease. ISSR. qRT-PCR. Molecular breeding. Marker-assisted selection.

Introduction

Rice (*Oryza sativa* L.) is a vital global food security crop, sustaining over 50% of the world's population (Rathna Priya et al., 2019). To face the demands of a growing population by 2050, rice production must increase by at least 50% (Alexandratos & Bruinsma, 2012).

Rice cultivation faces diverse environmental challenges, including rising biotic stress levels due to rapid climate changes (Jamaloddin et al., 2020). Among these, the Bakanae disease, caused primarily by *Fusarium fujikuroi*, poses a significant threat (Wulff et al., 2010). This disease initially relies on a living host for infection, progressively consuming and destroying host cells (Ma et al., 2013).

Bakanae disease, first identified in Japan in 1828 (Ito, 1931), has since spread to Europe, Asia, Africa, and North America (Ou, 1985). Under field conditions, it can reduce yields by up to 70% and affect grain quality (Fiyaz et al., 2014). The

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Received 20/10/ 2023; Accepted 06/01/ 2024
DOI: 10.21608/ejbo.2024.241138.2523
Edited by: Prof. Dr. Neveen Mahmoud Khalil, Faculty of Science, Cairo University, Giza 12613, Egypt.

severity of symptoms varies depending on the strain, with categories defined by Yamanaka & Honkura (1978) and Bonman (1992). Symptoms of bakanae disease in rice plants are influenced by the levels of gibberellic acid (GA) and fusaric acid produced by the *F. fujikuroi* (Ou, 1985). These acids contribute to distinct symptoms in infected plants, such as abnormal elongation of shoots and leaves, resulting in characteristic stunting and elongation.

Understanding the relationship between pathogen infection and host is essential for effective disease management. *F. fujikuroi* reproduces through sexual and asexual means, with ascospores and conidia playing crucial roles in disease progression (Ou, 1985).

Current management strategies involve seed treatment with hot water or fungicides (Lee et al., 2018). However, these methods have limitations, as hot water treatment may not effectively reach the pericarp layer of severely infected rice seeds (Hayasaka et al., 2001). Furthermore, fungicides may be ineffective due to pathogen resistance (Lee et al., 2011). Developing rice genotypes with resistance genes is a more promising approach, although single resistance genes are vulnerable to evolving fungal races (Lee et al., 2021), posing challenges in achieving higher resistance levels (Fiyaz et al., 2016; Fouad & Hafez, 2019).

The discovery of novel resistance genes from diverse sources plays a pivotal role in rice breeding programs, aiming to establish enduring resistance against bakanae disease. This involves augmenting resistance levels, addressing resistance gene breakdown, or both (Lee et al., 2021). Molecular breeding, particularly marker-assisted selection (MAS), transcends conventional breeding limitations, allowing the rapid and efficient integration of multiple beneficial genes into a single rice variety (Suh et al., 2013).

In recent decades, MAS has shifted plant selection from phenotype-based to DNA-based selection, offering substantial advantages in terms of cost, time efficiency, and selection effectiveness, depending on the trait in question. Various molecular markers, including Amplified Fragment Length Polymorphisms (AFLPs) (Sorkheh et al., 2016), Random Amplified Polymorphic DNA markers (RAPDs) (Bansal et al., 2013), Inter Simple Sequence Repeat (ISSR) markers (Oladosu et al., 2015; Nasser et al., 2023), Single Nucleotide Polymorphisms (SNPs) (Lee et al., 2022a), and Microsatellites or Simple Sequence Repeats (SSRs) (Mattar et al 2016; **Vanlalsanga et al., 2019**), have been employed to characterize the genetic diversity of rice varieties. In this context, ISSR markers were chosen for their high polymorphism and cost-effectiveness, not requiring prior knowledge of the genome sequence, making them valuable tools for plant breeders.

Recently, insertion-deletion (InDel) markers have gained prominence in plant research due to their codominant nature and genome-wide distribution (Hu et al., 2020). InDel markers have found extensive application in assessing genetic diversity and assisting selection in rice (Patel et al., 2014). Therefore, ISSR and InDel markers were employed as reliable tools to assess the genetic diversity of selected rice genotypes concerning bakanae disease resistance.

In the realm of plant stress research, gene expression studies are indispensable for unravelling the biological processes involved in stress responses. Quantitative gene expression analysis using qRT-PCR is a powerful method for deciphering gene function and regulatory networks in biological research. It finds widespread use in the analysis of gene expression in plant tissues across different developmental stages and/or under various biotic or abiotic stresses (Jain, 2009; Xu et al., 2015; Youssef et al., 2021, 2022). Here, qRT-PCR was employed to assess the expression levels of four candidate genes related to bakanae disease resistance in the selected rice genotypes. The connection between the expression of these genes and bakanae resistance had been previously explored by Lee et al. (2019).

In many crops, sources of genetic resistance against *Fusarium* spp. are limited, often providing quantitative resistance. For example, Fhb1 in wheat offers quantitative resistance against *Fusarium* head blight (Hao et al., 2020). Recently, several quantitative trait loci (QTLs) for bakanae disease resistance have been pinpointed within the rice genome (Fiyaz et al., 2016; Ji et al., 2018; Lee et al., 2019; Lee et al., 2022b). These QTLs can be harnessed for

marker-assisted selection in rice breeding and for comprehending resistance mechanisms. Lee et al. (2019) precisely mapped the qBK1 location to a 35 kb interval between InDel 18 and InDel 19-14, identifying six InDel markers for PCR-assisted selection in rice-breeding programs and unveiling four candidate genes within this 35-kb region of qBK1.

The primary objective of this study was to identify the causative agent of bakanae disease in Egypt and provide effective tools for screening rice genotypes for bakanae disease resistance, thereby enhancing resistance breeding initiatives. The identified molecular markers and candidate genes hold promise for future breeding programs, facilitating the development of new rice varieties with resistance to bakanae disease, a critical step in ensuring the sustainability of rice production.

Materials and Methods

Plant materials

Eleven rice (*Oryza sativa* L.) varieties (see Online Resource 1) were generously supplied by the Rice Pathology Department at the Rice Research and Training Centre (RRTC), Sakha, Kafrelsheikh, Egypt.

Fungal isolation and morphological identification

Fusarium fujikuroi was isolated following the procedure outlined by Burgess et al. (1994). Infected rice plants with bakanae disease were gathered from Qallin, Kafrelshikh governorate, Egypt. Plant specimens exhibiting disease symptoms were collected, washed, surface-sterilized, and subsequently incubated on Agar at 25ºC and 20ºC day and night times respectively for 5 days.

Fusarium colonies were sub-cultured on fresh potato dextrose agar media for sporulation. A pure culture derived from a single spore was used for colony morphology examination, employing criteria described by Leslie & Summerell (2006) such as growth rate, phialides, macro conidia, micro conidia, septation, conidial size/shape and length of chains of isolates by using a light microscope.

Molecular identification of Fusarium isolate using Internal Transcribed Spacers 1 and 4 (ITS) molecular markers

Internal Transcribed Spacer 1 and 4 were employed as molecular markers for *Fusarium* isolate identification according to Hussain et al. (2018). Genomic DNA was extracted from the isolate using E.Z.N.A.® Fungal DNA Mini Kit (Omega BIO-TEK, USA) according to the manufacturer protocol., and the ITS region was amplified using specific primers (Online Resource 2). PCR reactions were conducted as follows; initial denaturation at 94°C for 2 min, 40 cycles of 60 sec at 94°C, 90 sec at 52°C and 2 min at 72°C followed by a final extension at 72°C for 7min., and the resulting amplicon was sequenced and analyzed to confirm the isolate's identity. The obtained sequence was aligned and analyzed on the NCBI website (http://www.ncbi.nlm.nih.gov/webscite) using BLAST. Genetic distances and multi-alignments were computed by Pairwise Distance method using ClustalW software analysis (www.ClustalW.com). The nucleotide sequences were also compared with *Fusarium* isolate sequences available in the Gene Bank. Then the sequence was submitted to Gene bank database and alignment for identification, recorded as *Fusarium fujikuroi* isolate MSSR-2 with accession OM283549.

Phenotypic evaluation of bakanae disease resistance

In the present investigation, eleven Egyptian rice varieties have been evaluated against bakanae disease. The virulent *F. fujikuroi* isolate MSSR-2 was used to infect the studied genotypes and to induce the bakanae disease symptoms.

Preparation of pathogen inoculum and seed inoculation

Spores of *F. fujikuroi* isolate MSSR-2 were collected, filtered, and adjusted to a standardized concentration according to Kim et al. (2014). Rice grains were surface sterilized with 70% ethanol for 1 min with shaking, then rinsed 5 times in sterilized distilled water and then soaked in the spore suspension for 48h for infection. Controls were treated with sterilized distilled water.

After inoculation, 90 grains of each genotype were planted in sterilized soil (40% sand, 30% clay, and 30% peat) in 3 trays with 30 grains per tray, and arranged in a completely randomized design. All trays were fertilized one time with urea 46.5 % N at 3 g/tray, watered daily and maintained in the glasshouse at 30–35ºC until the response to bakanae disease was recorded after 3 weeks.

Screening of rice genotypes against bakanae disease under greenhouse conditions

Seedlings were evaluated for bakanae disease symptoms after 21 days of inoculation using a visual index scale modified by Zainudin et al. (2008). Disease severity was graded on a scale of 0 to 4 based on various plant characteristics as follows; 0 = no symptoms; 1 = normal growth but leaves beginning to show yellowish-green, small necrosis localized at the crown level; 2 = abnormal growth, elongated, thin and yellowish-green leaves; seedlings also taller than normal, necrosis on main root and crown; 3 = abnormal growth, elongated, chlorotic, thin and brownish leaves; seedlings also taller than normal, reduced root system with necrosis on secondary roots and basal stem; 4 = dead plants. The disease severity index (DSI) was calculated based on the scoring values of each plant, following a formula provided by Zainudin et al. (2008).

DSI= (∑(number of plants in the specific scale x Disease scale))/(total number of plants)

=(∑(nxo)+(nx1)+(nx2)+(nx3)+(nx4))/(total number of plants)

Extraction and purification of genomic DNA of rice varieties

Genomic DNA extraction was carried out from 100 mg of fresh leaves of each rice genotype. The protocol followed was adapted from Clarke (2009) with modifications by Saad-Allah & Youssef (2018). The DNA concentration and purity were assessed using a Nanodrop ND-1000 spectrophotometer. The 260/280 absorption ratio was maintained within the range of 1.7 to 1.8, indicating DNA purity.

Five ISSR fingerprinting was performed using the specified primers (10pmol) in a reaction mix comprising genomic DNA (100 ng), sterilized water (7µL), and 10µL Dream Taq Green PCR Master Mix #k 1081 (Thermo Fisher Scientific, UK). PCR amplification was carried out following the cycling conditions as described by Abdel-Lateif & Hewedy (2018).

Five InDel markers were amplified with PCR cycling conditions specified by Lee et al. (2019).

Agarose gel electrophoresis

PCR products were analyzed post-amplification through gel electrophoresis using a 1.5% agarose gel (for one hour at 100V). The gel was stained with ethidium bromide and visualized under UV light utilizing a gel documentation system. Molecular size standards included 1kb Thermo Scientific Gene-Ruler and 100bp Mo Bi Tec # P805MBB DNA ladders. Reactions were repeated at least twice, and only reliable products were recorded.

Molecular characterization of resistant and susceptible rice genotypes using qRT-PCR

Based on the study of Lee et al. (2019), four candidate genes for bakanae disease resistance were selected to be measured in the present study. Total RNA was extracted from the leaves of Sakha 101, and Sakha 108 as susceptible genotypes, and Sakha 103 and Sakha 104 as tolerant genotypes, after 12 days of bakanae disease infection with *F. fujikuroi* isolate. The extraction has been carried out using TRizol reagent (15596026, Life Technologies, USA) according to the manufactured protocol. The cDNA libraries were then constructed from total RNA samples (1 µg per sample) using QuantiTects Reverse Transcription Kit (Qiagen, USA). Finally, relative gene expression of LOC_Os01g41770, LOC_Os01g41780, LOC_Os01g41790 and LOC_Os01g41800 was measured using ubiquitin 5 (UBQ5) as the reference gene for the normalization of expression of the target genes. cDNA amplicons were amplified via Maximas SYBR Green/Fluorescein qPCR Master Mix through specific primers (Online Resource 2) with thermal cycling conditions as follows; 10 min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 15s. The fold change in the target gene, relative to the expression of UBQ5, and the control was calculated using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001). Three biological replicates for each sample were used for real-time PCR analysis, and two technical replicates were analyzed for each biological replicate.

Four candidate genes for bakanae disease resistance, following the study of Lee et al. (2019), were assessed. Total RNA was extracted from the leaves of Sakha 101 and Sakha 108 (susceptible genotypes) and Sakha 103 and Sakha 104 (tolerant genotypes) after 12 days of bakanae disease infection with *F. fujikuroi* isolate. RNA extraction was conducted using TRizol reagent (15596026, Life Technologies, USA) following the manufacturer's protocol. cDNA libraries were then constructed from total RNA samples (1µg per sample) using QuantiTect Reverse Transcription Kit (Qiagen, USA). Relative gene expression of

LOC_Os01g41770, LOC_Os01g41780, LOC_Os01g41790, and LOC_Os01g41800 was measured using ubiquitin 5 (UBQ5) as the reference gene for normalization of target gene expression using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001). cDNA amplicons were amplified via Maxima SYBR Green/Fluorescein qPCR Master Mix with specific primers (Online Resource 2) under thermal cycling conditions as follows; 10 min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 15s. Three biological replicates for each sample were used for real-time PCR analysis, and two technical replicates were analyzed for each biological replicate.

Data analysis

ISSR and InDel-PCR amplification products were treated as dominant alleles for a given locus. The presence or absence of an amplified fragment was scored as 1 or 0, respectively. Data analysis was conducted using NTSYS-pc 2.1 software. Dendrograms were constructed from the similarity matrix data of the ISSR and InDel cluster analyses. The significance of differences among the means of disease severity index and relative gene expression was assessed using Tukey's HSD test ($P < 0.05$).

Results

Morphological identification of fungal isolate

The isolate was identified as *F. fujukori* based on morphological characteristics described by Leslie & Summerell (2006). It forms white mycelia that become gray, violet, or magenta with age, and sporulation is abundant. Hyphae are branched and septated, and the fungus has micro and macro conidiophores bearing micro conidia and macro conidia, respectively. The micro conidiophores are single, lateral, and subulate phialides consistent with *F. fujukori* (Fig. 1A, 1).

The fungal isolate exhibited morphological characteristics consistent with *F. fujukori*, as described by Leslie & Summerell (2006). It presented white mycelia that transitioned to gray, violet, or magenta as it aged, with abundant sporulation. The hyphae were branched and septated, and the fungus featured both micro and macro conidiophores bearing micro conidia and macro conidia, respectively. The micro conidiophores were single, lateral, and subulate phialides, confirming the identification as *F. fujukori* (Fig. 1A).

Fig. 1. Colony features of *Fusarium fujukori* on PDA media [(A), micro and macro conidia (B), symptoms of bakanae disease observed in infected rice plants (C)]

Molecular identification of the fungal isolate

PCR amplification of the ITS-rDNA region using universal ITS1-ITS4 primers produced a 561 bp amplicon (Fig. 2A). Sequencing analysis of the PCR product validated the morphological identification, confirming the isolate as *Fusarium fujikuroi*. The sequence obtained has been deposited in the NCBI database with the accession number OM283549. A phylogenetic tree was constructed based on the fungal isolate's Internal Transcribed Spacer 1 and 4 (Fig. 2B).

Evaluation of bakanae disease resistance

In this study, eleven Egyptian rice genotypes were subjected to screening for resistance against bakanae disease. Upon inoculation with the virulent *F. fujikuroi* isolate MSSR-2, various morphological abnormalities were observed; including yellowing and abnormal elongation with slender stems in the inoculated seedlings (Fig. 1C). Adult plants displayed infected stems with elongated internodes covered in dense white mycelium mass and profuse spores.

The results indicated that Sakha 103 and Sakha 104 exhibited the highest level of tolerance to the disease, with notably lower disease severity indices of 1.4 and 1.1, respectively. Conversely, Sakha 101 and Sakha 108 were found to be the most susceptible genotypes, displaying significantly higher disease severity indices, which reached approximately 3.3 (Fig. 3).

Genetic and molecular characterization of rice genotypes using ISSR and InDel PCR

The patterns of amplification products generated by the five ISSR and five InDel primers (Figs. 4 and 5, respectively) were consistently reproducible, establishing their reliability for genotypic analysis and the determination of genetic distances among the studied rice genotypes. A summary of the data analysis from both marker types is provided in Table 1.

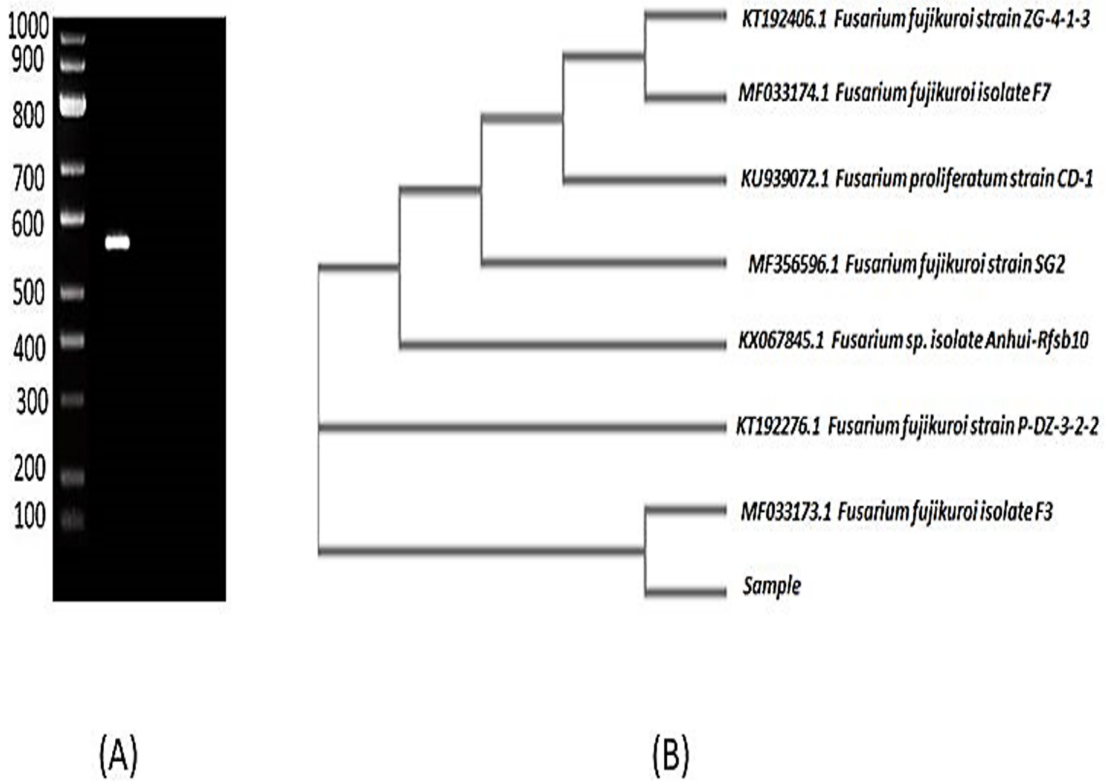

Fig. 2. PCR amplification of ITS-rDNA region (A) and the phylogenetic tree of the sequence of ITS-rDNA region belonging to *Fusarium fujikuroi* (B)

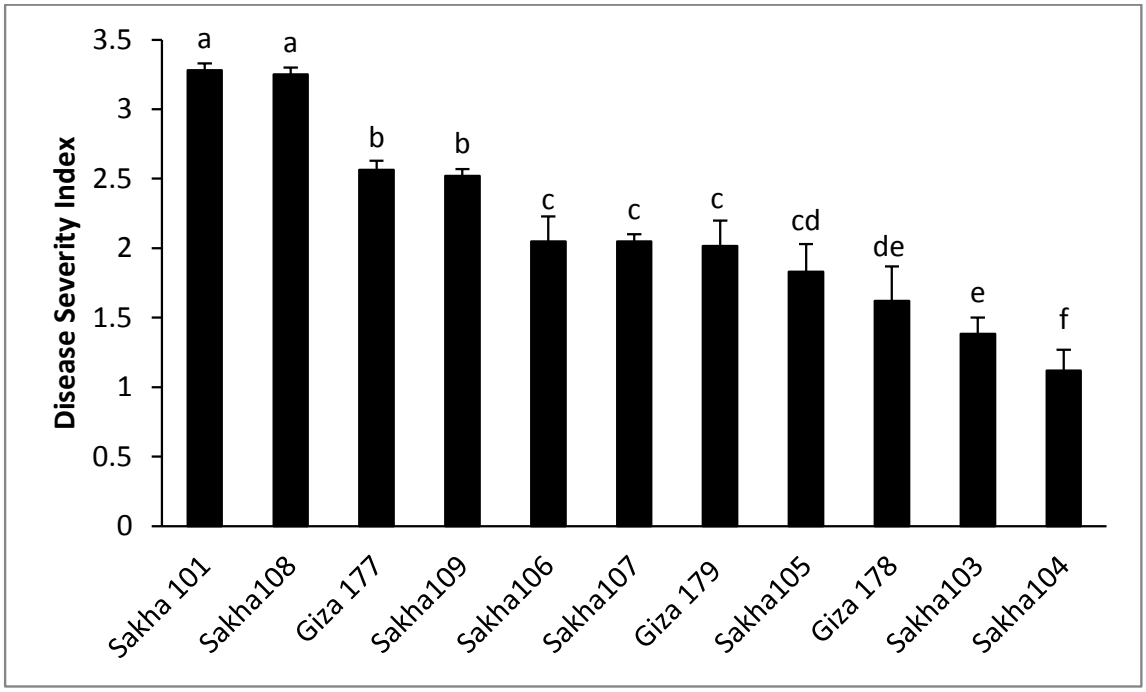

Fig. 3. Histogram representing the bakanae disease severity index of 11 Egyptian rice genotypes. Values + SE are means of three biological replicates each consisting of 30 plants. Different letters indicate statistically significant differences (P<0.05)

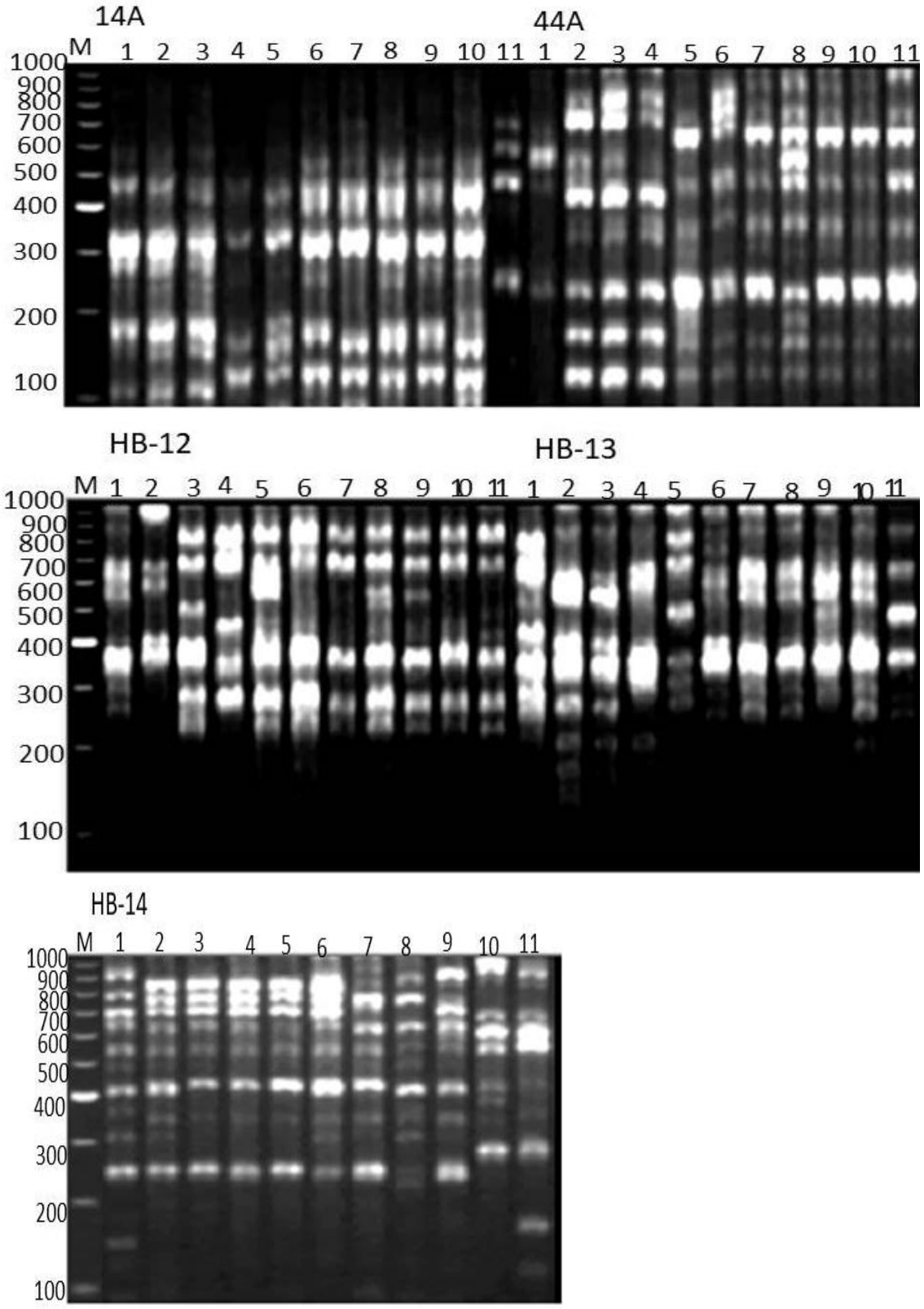

Fig. 4. Amplification products obtained from 11 rice genotypes using five ISSR primers [1= Giza 177, 2= Giza 178, 3 = Giza 179, 4 = Sakha 101, 5 = Sakha 103, = Sakha 104, 7 = Sakha 105, 8 = Sakha 106, 9 = Sakha 107, 10= Sakha 108, 11 = Sakha 109]

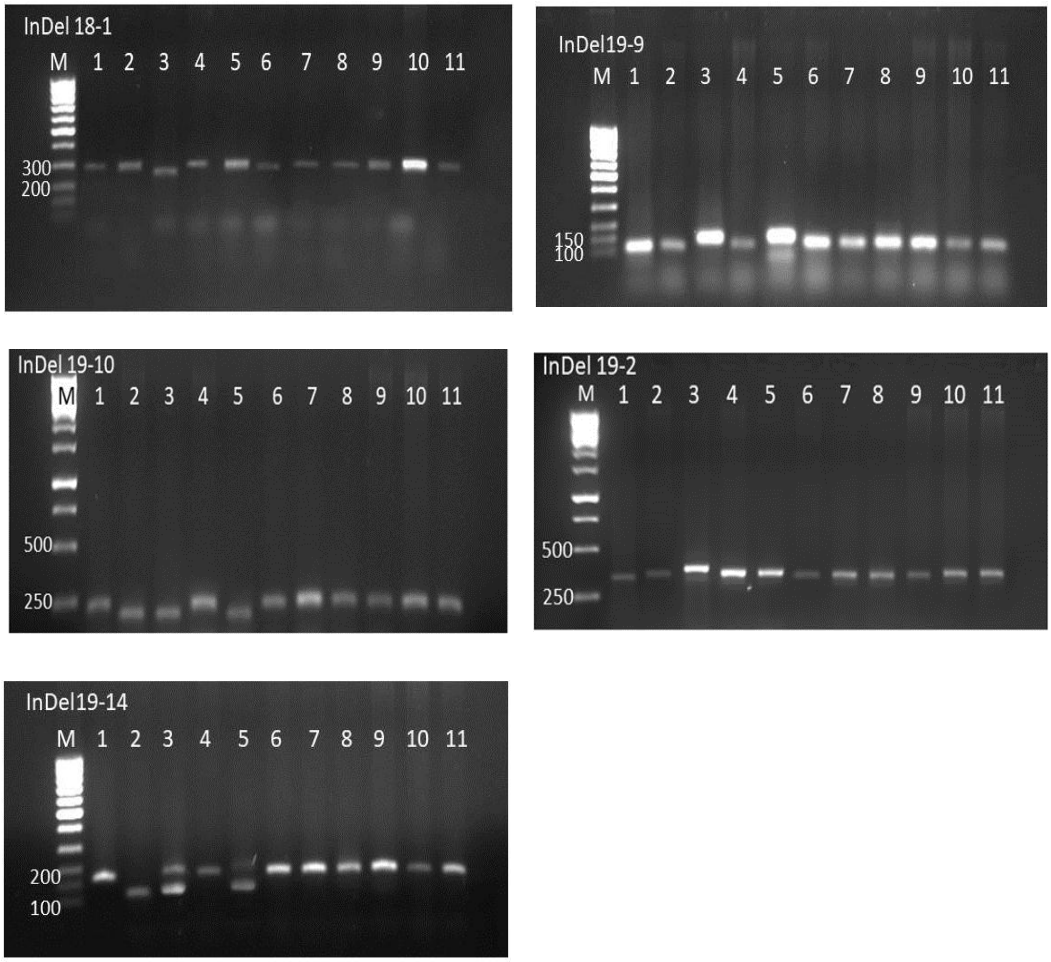

Fig. 5. Amplification products obtained from 11 rice genotypes using five InDel primers [1= Giza 177, 2= Giza 178, 3 = Giza 179, 4 = Sakha 101, 5 = Sakha 103, = Sakha 104, 7 = Sakha 105, 8 = Sakha 106, 9 = Sakha 107, 10= Sakha 108, 11 = Sakha 109]

TABLE 1. Summary of genetic analysis of eleven rice genotypes using ISSR and InDel markers

Primer name	Range of band size	Total bands	Polymorphic bands	% of polymorphism
14A	716-105	12	12	100
44A	1037-115	14	13	92.86
HB-12	932-228	11	11	100
HB-13	940-147	15	14	93.33
HB-14	970-120	13	11	84.61
Total		65	61	
Average		13	12.2	93.85
InDel 18-1	236-310	2	2	100
InDel 19-2	305-410	2	2	100
InDel 19-9	145-176	2	2	100
InDel 19-10	220-258	2	2	100
InDel 19-14	200-142	2	2	100
Total		10	10	
Average		2	2	100

The five ISSR primers collectively revealed 65 total loci across the eleven rice genotypes, with 61 loci showing polymorphism, accounting for 93.85% of the polymorphic loci (Table 1). The number of amplified loci varied from eleven (HB-12) to fifteen (HB-13), with fragment sizes ranging from 105 to 1037 bp and an average of thirteen loci per primer. Notably, two primers (14A and HB-12) exhibited 100% polymorphism, while the lowest level of polymorphism (84.6%) was recorded for the HB-14 primer.

A comparative analysis of banding patterns between the most tolerant genotypes (Sakha 103 and Sakha 104) and the most susceptible ones (Sakha 101 and Sakha 108) revealed potential tolerant-specific bands that could serve as molecular markers for the selection of resistant rice varieties (Online Resource 3a). For instance, the 14A and HB-13 ISSR primers each generated two distinct bands (395bp and 190bp for 14A, and 608bp and 359bp for HB-13, respectively), which were present in the tolerant genotype Sakha 104 but absent in the susceptible varieties Sakha 101 and Sakha 108. Additionally, the Primer HB-13 produced two more bands (490bp and 267bp) present in the tolerant genotype, Sakha 103 and absent in the susceptible genotypes, Sakha 108 and Sakha 101. Moreover, another specific band with a molecular weight of 264bp, found only in the tolerant genotype Sakha 103 and absent in the susceptible genotypes, Sakha 101 and Sakha 108, was amplified using the primer 44A. Furthermore, the HB-12 primer produced two bands with molecular weights of 620bp and 561bp, which were exclusively present in the tolerant genotype Sakha 103 and not found in the susceptible genotypes, Sakha 108 and Sakha 101. These bands represent potential genetic markers for bakanae disease resistance, supporting the use of ISSR primers (14A, HB-13, 44A, HB-12) as reliable molecular markers for marker-assisted selection for bakanae disease resistance.

The UPGMA analysis of the ISSR data was employed to construct a dendrogram based on the genetic dissimilarity distance among the studied rice varieties (**Fig. 6a**). The cluster analysis unveiled substantial genetic diversity among the rice varieties, categorizing them into four main clusters (A, B, C, and D) at a dissimilarity distance of 1.34. Cluster (A) consisted solely of Giza 177, while cluster B branched into two subclusters at a dissimilarity distance of approximately 1.00. The first subcluster contained Giza 178, and the second subcluster encompassed Giza 179, Sakha 101, and Sakha 104. Cluster (C) is further divided into two subclusters at a dissimilarity distance of about 1.15. The first subcluster consisted of Sakha 103, while the second subcluster included Sakha 105 and Sakha 106 together at a dissimilarity distance of 0.85, but Sakha 107 and Sakha 108 were separated. Finally, cluster (D) comprised only Sakha 109.

The five InDel primers used in this study (Online Resource 2) revealed a total of 10 loci across the eleven rice genotypes (Fig. 5). All ten loci were found to be polymorphic, indicating 100% polymorphism among the studied rice genotypes. These loci were distributed across the five InDel primers (InDel 18-1, InDel 19-2, InDel 19-10, InDel 19-14, and InDel 19-9), with fragment sizes ranging from 142 to 410 bp. On average, two loci were identified per primer. Notably, the InDel primers InDel 19-9, InDel 19-10, and InDel 19-14 each produced a characteristic band (176bp, 220bp, and 142bp, respectively) present in the tolerant genotype Sakha 103 but absent in the susceptible genotype, Sakha 101 and Sakha 108 (Online Resource 3b). These specific bands hold potential as genetic markers for marker-assisted selection for bakanae disease resistance.

The UPGMA analysis of the InDel data was used to construct a dendrogram based on the genetic dissimilarity distance among the studied rice genotypes (Fig. 6b). The dendrogram categorized the eleven genotypes into three primary clusters (A, B, and C) at a dissimilarity distance of 1.90. Cluster (A) included Giza 177, Sakha 101, Sakha 104, Sakha 105, Sakha 109, Sakha 108, Sakha 106, and Sakha 107, grouped at a dissimilarity distance of approximately 0.00. Cluster (B) was further divided into two branches, with the first branch containing Giza 178 and the second branch comprising Sakha 103. Finally, cluster (C) included only Giza 179.

Relative gene expression of bakanae-resistant genes

In consideration of the recorded bakanae disease severity index for the studied rice varieties, the expression patterns of the four candidate genes were examined in the two most tolerant genotypes, Sakha 103 and Sakha 104, as well as the two most susceptible genotypes, Sakha 101 and Sakha 108, following 12 days of *F. fujikuroi* inoculation (**Fig. 7**).

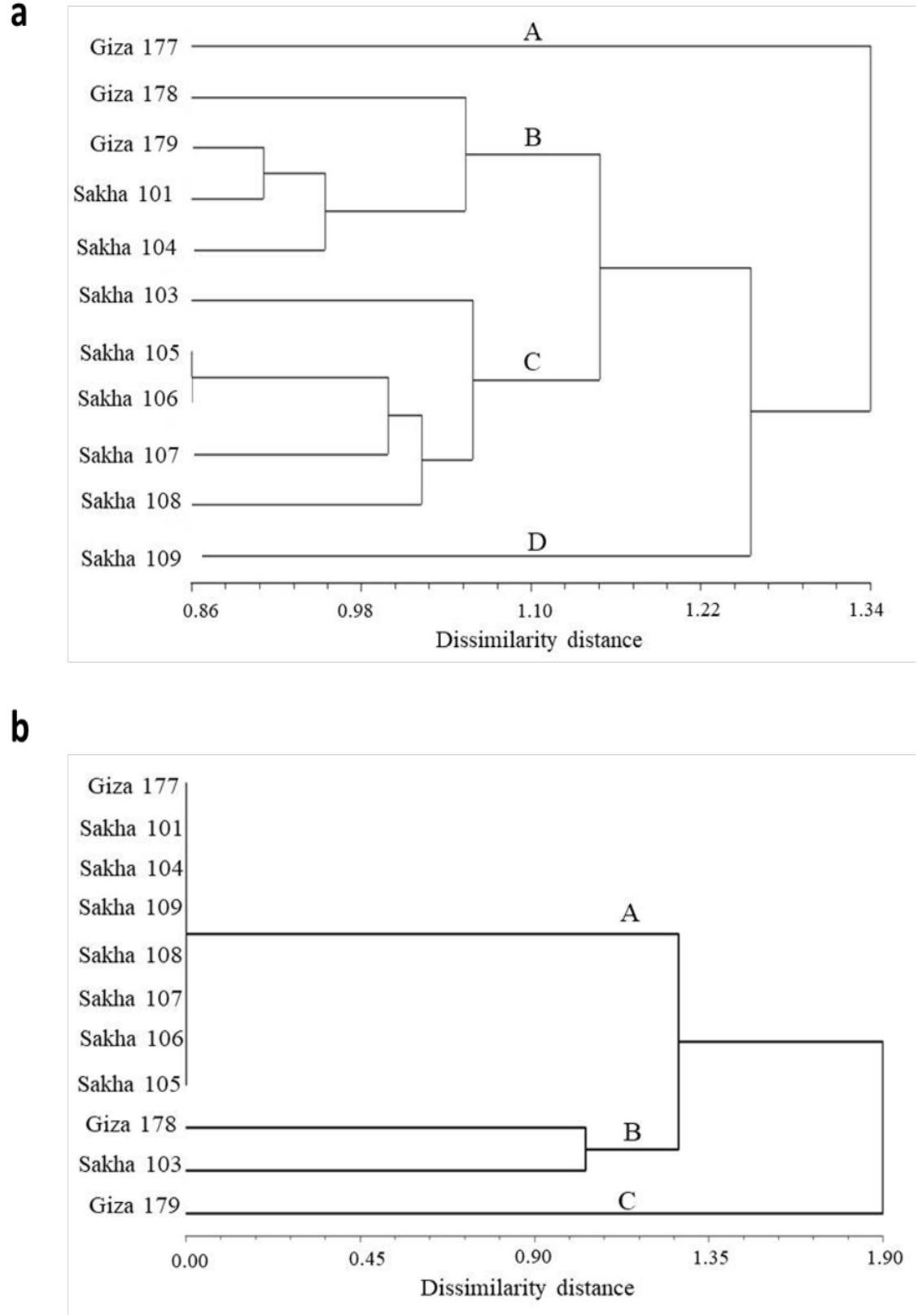

Fig. 6. Dendrograms presenting the genetic relationship among 11 rice genotypes revealed by UPGMA cluster analysis based of ISSR (a) and InDel (b) data analyses

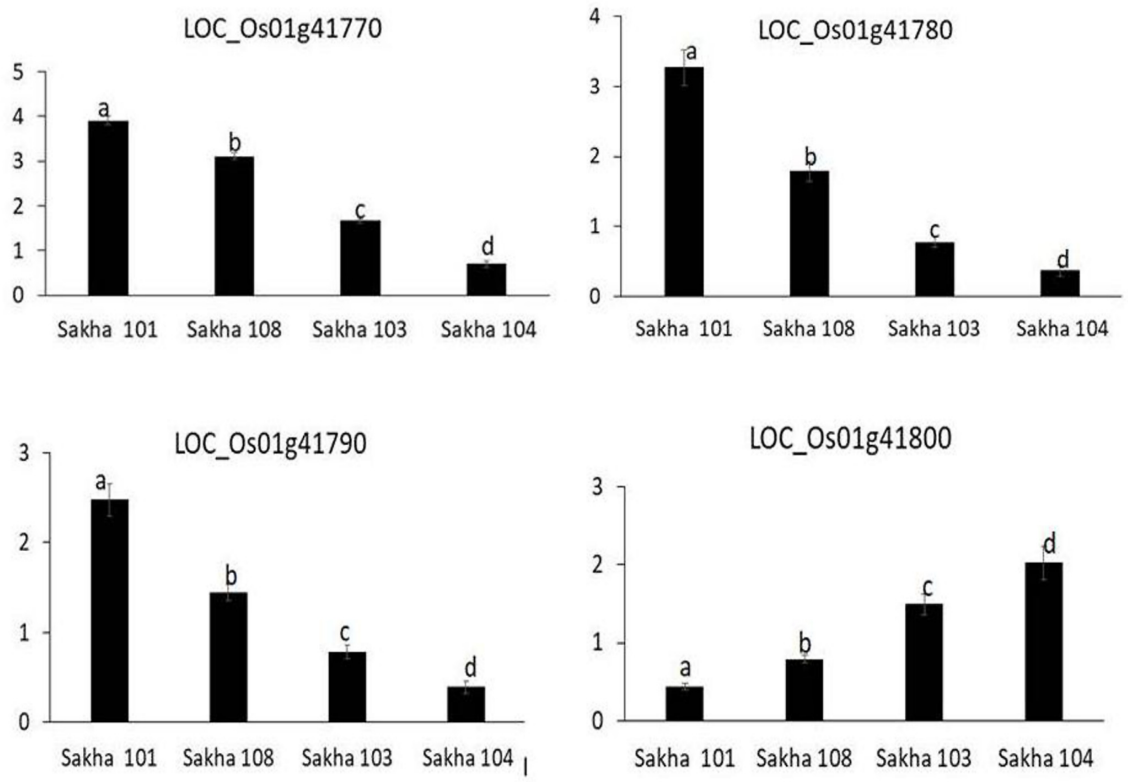

Fig. 7. Relative expression of candidate genes in tolerant and susceptible rice genotypes inoculated with *Fusarium fujikuroi* isolate MSSR-2 at 12 days post inoculation [Values are the means + SE of three biological replicates, each consisting of at least five plants. Different letters indicate statistically significant differences (P< 0.05)]

The relative expression of the four candidate genes exhibited contrasting patterns between the tolerant and susceptible rice genotypes. LOC_Os01g41770, LOC_Os01g41780, and LOC_Os01g41790 showed higher expression levels in the susceptible rice varieties compared to the tolerant varieties (Fig. 7). In contrast, the relative expression of LOC_Os01g41800 was significantly higher in the tolerant genotypes, Sakha 103 and Sakha 104, as opposed to the susceptible genotypes, Sakha 101 and Sakha 108.

Discussion

Bakanae disease, also known as "foolish seedling disease," is a fungal disease that affects rice plants. It is caused by the fungus *F. fujikuroi* and is characterized by the abnormal elongation of the plant's shoots and leaves. The disease can significantly reduce rice yield and, in severe cases, can cause complete crop loss. Niehaus et al. (2017) proved that bakanae disease symptoms in rice such as abnormal elongation or stunting have been associated with gibberellin (GA), fusaric acid, fumonisins and/or novel secondary metabolites produced by *F. fujikuroi*.

To our knowledge, no research has been conducted on the molecular identification of the causing agent of bakanae disease in Egypt. However, *F. fujikuroi* has been identified as the causative agent of bakanae disease in rice plants in various countries worldwide, including Japan, where the disease was first identified (Ito & Kimura, 1931). The use of molecular techniques, such as sequencing the ITS-rDNA region using the universal ITS1-ITS4 primers, is a powerful tool for identifying fungal pathogens like *Fusarium* species (Matsumoto et al., 2003). Accurate identification of the pathogen is essential for developing effective control strategies and understanding the epidemiology of the disease. It is interesting to note that multiple *Fusarium* species including *F. andiyazi, F. fujikuroi, F. proliferatum,* and *F. verticillioides* have been associated with bakanae disease (Wulff et al., 2010), highlighting the complexity of this disease and the need for precise identification. Herein, the identification of

the fungus causing the disease was done at both the morphological and the molecular levels, and the fungal isolate has been identified and recorded in the NCBI database as *Fusarium fujikuroi* isolate MSSR-2 with Accession OM283549.

Screening for and using bakanae-resistant rice genotypes is important for reducing crop losses, minimizing the need for chemical control measures, and protecting local and regional rice-growing industries. In this work, eleven rice genotypes from the most commonly grown rice varieties in Egypt were screened to bakanae disease resistance. Our results showed that Sakha 103 and Sakha 104 were the most tolerant genotypes with significantly lower disease severity indices; 1.4 and 1.1, respectively, compared to Sakha 101 and Sakha 108 the most susceptible genotypes that showed significantly higher disease severity indices ≈ 3.3. These results are congruent with those of Hammoud & Gabr (2014), and Makhlouf & Gabr (2015), who reported that Sakha 101 was the most susceptible genotype to bakanae disease and exhibited the highest infection under non-inoculated and inoculated conditions while, Sakha 104 showed the lowest infection under both conditions, although they applied *Fusarium moniliforme* as the bakanae disease-causing pathogen. While the study of Hassan & Samah (2020) showed Giza 177 as the highly susceptible rice genotype to bakanae disease under greenhouse and field conditions, in our investigation, it was ranked as the third susceptible genotype after Sakha 101 and Sakha 108 with disease severity index 2.57. They also reported Giza 179 and Giza 178 as moderately tolerant genotypes which in somewhat agree with our results. The difference in scoring the disease resistance level of the same varieties among different researchers might be due to a variety of reasons such as, the scoring based on visual rating, testing different isolates and genotypes of the pathogen (Hassan & Samah, 2020), and differences in the plant's growth and development. Other factors, such as soil composition and environmental conditions can also play a role in determining a plant's resistance to bakanae disease.

In the present study, ISSR and InDel markers have been applied to assess genetic diversity among the studied rice genotypes, and the potential use as markers for bakanae disease resistance. ISSR markers are widely used to evaluate genetic diversity within and among different plant species (Saad-Allah & Youssef, 2018; El-Kholy et al., 2015) including rice (Moonsap et al., 2019). ISSR markers are based on PCR amplification of regions between simple sequence repeats (SSRs) sequences, providing highly informative data for comparing genetic relatedness and identifying diversity sources for breeding programs. The five ISSR primers used in the present work showed 93.85% polymorphism and could be used to distinguish between the studied eleven varieties. Furthermore, the UPGMA analysis of the ISSR data separated them into four clusters based on the genetic dissimilarity between the varieties.

On the other hand, the InDel markers resulted in 100% polymorphism among the studied genotypes and are valuable in genotype characterization. InDel markers have been widely employed in evaluating the genetic diversity and in markers-assisted selection for rice genotypes (Patel et al., 2014; Niihama et al., 2015). The UPGMA analysis of the InDel data separated the studied rice genotype into three clusters. The comparison between the ISSR and InDel-derived dendrograms displayed differences in the clustering patterns of rice genotypes within clusters. These variations could be attributed to that each marker targets different sequences throughout the genome. Therefore, both markers are valuable for diversity analysis and genetic characterization in rice.

ISSR and InDel markers were used not only for genetic characterization and diversity evaluation of rice genotypes but also to identify specific genetic variations linked to resistance against various diseases. Prior studies have demonstrated ISSR's ability to identify genetic variations associated with resistance against tungro virus and bacterial blight (Latif et al., 2013; Mazid et al., 2013). In this study, a comparison of ISSR banding patterns between the most tolerant (Sakha 103 and Sakha 104) and susceptible (Sakha 101 and Sakha 108) rice genotypes revealed a potential genetic variation associated with bakanae disease resistance.

On the other hand, InDel markers have been developed by many researchers for the selection of disease-resistant rice genotypes such as rice blast resistance (Hayashi et al., 2006) and bakanae resistance (Lee et al., 2021). In the present study, we analyzed five InDel markers (InDel 18–1, InDel 19–2, InDel 19–9, InDel 19–10 and InDel 19–14) that have been previously used by Lee

et al. (2019) for marker-assisted selection to detect *qBK1*, a major resistant quantitative trait locus (QTLs) for bakanae disease. Three of the studied InDel markers (InDel 19-9, InDel 19-10 and InDel 19-14) showed a characteristic band to the tolerant variety Sakha 103 that was absent in susceptible genotypes, Sakha 101 and Sakha 108. Furthermore, InDel 19-10 and InDel 19-14 showed the same band in the moderate tolerant genotypes, Giza 178 and Giza 179, while InDel 19-9 band appeared in Giza 179. So the above InDels could be used for marker-assisted selection to bakanae disease resistance. None of these bands appeared in the tolerant variety Sakha 104, so the resistance in this variety might be due to another genomic region, this explains the complexity of the genetics of resistance to bakanae disease. To our knowledge there is no study has been conducted before to the present work to analyse the value of InDels or candidate genes as genetic markers for bakanae disease resistance in Egypt.

The expression of LOC_Os01g41770, LOC_Os01g41780, and LOC_Os01g41790 genes showed up-regulation in the susceptible genotypes (Sakha 101 and Sakha 108) while LOC_Os01g41800 was upregulated in the tolerant genotypes (Sakha 103 and Sakha 104). Our findings are consistent with those of Lee et al. (2019), who noted that after inoculation, the expression of leucine-rich repeat receptor-like proteins encoded by LOC Os01g41770 and LOC Os01g41780 was down-regulated in Shingwang, a resistant variety, but significantly up-regulated in Ilpum, a susceptible variety. These two genes might play a negative regulation role in the disease resistance as suggested by Lee et al. (2019). The LOC Os01g41790 transcript, whose functional annotation is unclear, likewise displayed up-regulated and down-regulated expression in the susceptible and tolerant varieties, respectively. However, LOC_Os01g41800, which encodes a putative cytochrome P450 monooxygenase, displayed up-regulated expression in the tolerant variety, and down-regulated expression in the susceptible variety. LOC Os01g41800 appears to be a positive regulator of bakanae disease resistance based on its expression pattern. Plant cytochrome P450 monooxygenases are a class of redox proteins that catalyse a variety of oxidative processes that mediate plant defence, such metabolism of several chemical compounds involved in plant defence against variety of pathogens and herbivores (Isin & Guengerich, 2007).

Conclusion

The genetics of bakanae disease resistance are intricate and not entirely understood. Further research is needed to better understand the genetic basis of this disease's resistance and to develop more effective control strategies for bakanae disease. The identification and introduction of bakanae-resistant rice genotypes are key strategies for managing the disease and minimizing its detrimental effects on rice production. ISSR, InDel and the four genes, LOC_Os01g41770, LOC_Os01g41780, LOC_Os01g41790 and LOC_Os01g41800 could be used to select plants that have the desired resistance trait, enabling more efficient and targeted breeding efforts to develop bakanae disease-resistant rice genotypes.

Acknowledgement: The authors would like to thank all staff of the Department of Botany and Microbiology, Faculty of Science, Kafrelsheikh University, and Rice Pathology Department, Plant Pathology Research Institute, Agricultural Research Center for their technical support in field and laboratory work.

Funding: The present research was funded by the Academy of Scientific Research and Technology (ASRT); Scientists for Next Generation master school cycle6 (SNG 6), Egypt. Grant reference number: ASRT/SNG/AB/2018-11.

Conflict of interest: All authors declare that they have no conflict of interest.

Authors' contributions: The concept and design of the experiments were prepared by all authors. SA conducted all of the procedures and experiments and wrote the first draft. SAH interpreted the results and contributed to the writing of the manuscript. RME helped in manuscript preparation and data analysis. MSY: conceived the idea of the study, performed the data analysis, interpreted the results, and wrote the manuscript. All authors read, revised and approved the paper.

Ethical approval: This research does not involve studies with human participants or with animals.

Data availability: All data generated or analysed during this study are included in this published article [and its supplementary information files].

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تعريف عزله جديده من فطر*Fusarium fujikuroi* وراثيا والتوصيف الجينى لمقاومه نبات الارز لمرض البكانا

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يعتبر مرض البكانا في الأرز المتسبب عن فطر(*Fusarium fujikuroi*) من اكثر الامراض انتشارا في العالم حيث لايوجد صنف مقاوم كليا له .تم تعريف عزله جديده من فطر fusarium fujikori باستخدام Internal Transcribed Spacer. وكذلك تم دراسة التنوع الوراثي الناتج عن الإصابة بالفطر باستخدام تقنيه التكرارات البينية البسيطة (ISSR) وبعض الدلائل الجزيئية المرتبطة بمقاومه البكانا (InDels) لإحدى عشر من الاصناف المصرية. تمت ايضا دراسه التعبير الجينى للاصناف (سخا103 –سخا104- سخا101- سخا 108) باستخدام تقنيه تفاعل البلمره المتسلسل الكمى بعد العدوى بالفطر.

وقد اظهرت النتائج ان الاصناف سخا 103وسخا 104 ذات قدره تحمل عالية علي مقاومة الاصابة بينما كانت الاصناف سخا 101 وسخا 108 الاكثر حساسيه للإصابة. كما اظهرت نتائج التعبير الجيني لبادرات الأرز ان الإصابة بالفطر تسببت في زياده التعبير الجيني للجينات LOC_Os01g41770 وLOC_Os01g4178 LOC_Os01g41790 , والتي تُشفر لبروتينات شبيهة المستقبلات الغنية بتكرارات الوسين، في الأصناف الحساسة وزياده التعبير اللجيني للجين LOC_Os01g41800 المشفر لسيتوكروم P450 مونواكسجينيز في الاصناف الاكثر تحملا للفطر.