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OVEREXPRESSION OF A GENE ENCODING LRR PROTEIN IMPROVES RICE RESISTANCE AGAINST *MAGNAPORTHE ORYZAE*

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Abstract: *Magnaporthe oryzae* is a rice blast fungus and plant pathogen that causes a serious rice disease and, therefore, poses a threat to the world's second most important food security crop. Plant transformation technology has become an adaptable system for cultivar improvement and to functionally analyze genes in plants. The objective of this study was to determine the effects (through over-expressing and using the CaMV 35S promoter) of *Pikh* on MR219 resistance because it is a rice variety that is susceptible to the blast fungus pathotype P7.2. Thus, a full coding DNA sequence (CDS) of the *Pikh* gene, 1206 bp in length, was obtained through amplifying the cDNA template from a PH9-resistant rice variety using a specific primer. *Agrobacterium*-mediated transformation technology was also used to introduce the *Pikh* gene into the MR219 callus. Subsequently, transgenic plants were evaluated from the DNA to protein stages using polymerase chain reaction (PCR), semi-quantitative RT-PCR, real-time quantitative PCR and high-performance liquid chromatography (HPLC). Transgenic plants were also compared with a control using a real-time quantification technique (to quantify the pathogen population), and transgenic and control plants were challenged with the local most virulent *M. oryzae* pathotype, P7.2. The *Pikh* gene was up-regulated in the transgenic plants compared with the control plants. The quantity of the amino acid leucine in the transgenic rice plants increased significantly from 17.131 in the wild-type to 47.865 mg g^{-1} in transgenic plants. The *M. oryzae* population was constant at 31, 48 and 72 h after inoculation in transgenic plants, while it was increased in the inoculated control plants. This study successfully clarified that over-expression of the *Pikh* gene in transgenic plants can improve their blast resistance against the *M. oryzae* pathotype P7.2.

Keywords: plant pathogen, coding DNA sequence, *Agrobacterium*-mediated transformation, real-time quantitative PCR, high performance liquid chromatography

INTRODUCTION

Bio-engineering techniques, such as over-expression and RNA silencing, could efficiently improve rice tolerance to fungal diseases (Kanzaki et al., 2002; Li, Wei, Lin, & Chen, 2012; 2014). Currently, approximately 100 blast resistance genes have been identified and mapped in various rice genotypes, while only 19 of these genes have been cloned and characterized (T. Sharma et al., 2012). Most of these

blast resistance genes belong to the nucleotide-binding site-leucine rich repeat (NBS-LRR) class of genes (T. R. Sharma et al., 2012). These genes can be applied with breeding and genetic engineering programs for introgressing a high degree of tolerance into well-performing commercial cultivars with susceptibility to blast disease (Zhang, 2007). This broad-spectrum blast-resistance gene introgression has been reported from *O. rufipogon* in Indica rice cultivars (Ram, Majumder, Mishra, Ansari, & Padmavathi, 2007). Gene cloning is an essential step in understanding resistance gene structure and functions (Alberts et al., 2002). *Pikh* (*Pi54*) was cloned from an Indica cultivar (Tetep) in India (Sharma, Shanker, et al., 2005). The *Pikh* gene was successfully assayed using functional studies after its identification (Sharma, Madhav, et al., 2005). Transgenic lines containing the *Pikh* were established to confer a high degree of resistance to various *M. oryzae* isolates (Rai et al., 2011). *Pikh* gene has been wrongly reported as a member of NBS-LRR gene family (Sharma et al., 2012), while we clearly identified it as LRR type of R protein. *Agrobacterium*-mediated transformation is a desired method for a wide range of plant modifications (Tzfira & Citovsky, 2006). In this study, a direct strategy for manipulating enhanced normal tolerance using constitutive expression of naturally inducible defenses has been suggested; however, activation of defense gene transcription triggers the expression of various inducible defense mechanisms (Zhu, Maherfl, Sameer Masoud, & Lamb, 1994). The objective of this study was to determine the effects (through over-expression) of *Pikh* on MR219 resistance as a rice variety that is susceptible to blast fungal pathotype P7.2.

METHODS

Isolating full coding DNA sequence (CDS) of *Pikh*, and construction of an over-expression vector

The total RNA was extracted from treated leaves of the *M. oryzae* PH9 variety using the TRIzol method (Simms, Cizdziel, & Chomczynski, 1993). Reverse transcriptase-PCR was carried out to isolate the *Pikh* gene from the PH9 variety. One microgram per microliter of total RNA was transcribed to the first-strand cDNA fragments using Super Script III (Invitrogen, Carlsbad, California, USA). The reactions were incubated at 50˚C for 60 min and heated for inactivation at 70˚C for 15 min. Next, the template cDNA was amplified using the following *Pikh* specific primers designed in accordance with the cDNA sequence homologies (GenBank: GU258499.1) using Primer Premier 5.0 software (Ren et al., 2004). Forward primer: 5´- CTAGTTCAATTGCTTTAAG -3´; Reverse primer: 5´- ATGAGTAAAATGAAGAAGC-3´. The expected band (approximately 1206 bp) was purified using a QIAquick® gel extraction kit (QIAGEN, Hilden, North *Rhine Westphalia,* Germany) and sent for sequencing. The purified PCR product (1206 bp) was directly inserted into the pDriveU/A cloning vector (QIAGEN, Hilden, North Rhine Westphalia, Germany) using a PCR Cloning^{plus} Kit (QIAGEN, Hilden, North Rhine Westphalia, Germany). The ligated pDrive vectors were then transformed into E. coli EZ cells and cultured overnight (16 hrs, 37°C) in LB agar medium containing X-gal, IPTG, and ampicillin.

Constructing an expression clone and transforming competent cells

Gateway® Technology with Clonase™II (Invitrogen, Carlsbad, California, USA) was used to construct the expression clone. In the first step, to construct entry clones, the KAPA HiFi Hot Start DNA polymerase kit was used to generate attB-PCR products using following primers designed based on the structure of the pDONOR/Zeo vector and the manufacturer's Gateway technology. Forward *Pikh*-anchor: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC CTAGTTCAATTGCTTTAAG-3′; Reverse *Pikh*-anchor: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC ATGAGTAAAATGAAGAAGC -3′.

The QIAquick® gel extraction kit (QIAGEN, Hilden, North Rhine Westphalia, Germany) was used to the target gene (1267 bp) from the gel electrophoresis results. In the next step, the attB-PCR product was cloned, and recombinant plasmids were purified using the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, North Rhine Westphalia, Germany) to construct expression clones. In the next phase, to constructing expression clones, the LR and BP recombination reaction procedures were performed in accordance with the manufacturer's procedures for the Gateway® Technology kit (Invitrogen, Carlsbad, California, USA). Fially, the *E. coli* TOP10 strain (QIAGEN, Hilden, North Rhine Westphalia, Germany) was used as competent cells for transformation.

Transformation procedure

Ten sterile MR219 seeds were placed in each Petri dish (100×15 mm) containing 20 mL MS-2,4-D medium (MS salts and vitamins, 300 mg/L casamino acid, 2 mg/L 2,4-D, and 2.8 g/L Gelrite, pH 5.8). Three Petri dishes were measured as replicates, and 9 petri dishes in total (3 replicates) were analyzed. Next, the seed cultures were incubated in dark conditions at 25˚C. After 3 weeks, yellowish white embryogenic calli developed on the scutellar surface. *Agrobacterium*-mediated transformation (Strain LBA4404) was used to transfer the expression clone to the MR219 calli. The *Agrobacterium* strain LBA 4404 containing an expression vector was streaked on an LB solid medium containing both streptomycin (100 µg/mL) (antibiotic appropriate for vector used) and rifampicin (50 µg/mL) (bacterial selective agents). *The cultured plates were incubated in dark conditions for 2 days. Agrobacterium*-containing expression vectors were transformed into the MR219 embryogenic calli. Next, transformed MR219 embryogenic calli were regenerated.

Functional studies of transgenic MR219

In this part of experiment we did functional studies on transgenic plants through monitoring the GFP expressed in the transgenic seeds collected from the T_0 and T_1 generations using a florescence microscope (Leica MZFL111). Then, The 3G plant PCR kit (KAPA, Cape Town, Western Cape, South Africa), was used to analyze the putative transgenic plants. The presence of transgenic plants in the T_1 lines was assessed based on PCR amplification using two sets of primers designed specifically for the *Pikh* gene and *CaMV35S* promoter sequences. In addition, Reverse transcriptase RT-PCR was used to study *Pikh* gene expression in the inoculated transgenic and untreated transgenic rice plants using SuperScript III (Invitrogen, Carlsbad, California, USA). The *18S rRNA* gene was used as an internal control and amplified using a specific primer. Moreover, Real-time qRT-PCR was used to evaluate the expression levels of the *Pikh* gene in the leaf tissue of the transgenic plants in response to *M. oryzae* (pathotype P7.2) inoculation after 31 h compared with the non-inoculated plants. All data were from three independent biological replicates. The *Pikh*, *18S rRNA* and *α-Tubulin* genes were amplified using specific primers. In the next step of experiment, the amino acid analysis was performed using the Waters HPLC system (E2696, USA) and 2475 fluorescence detector ACCQ-Fluor™ Reagent Kit (USA). The column featured a 3.9 inner diameter and was 150 mm long, ACCQ-Tag™ (Waters, USA). The mobile phase was as follows: A) 10% ACCQ-Tag™ Eluent solution in water and B) 60% acetonitrile in water. The flow rate was 1 $mLmin^{-1}$. Furthermore, the real-time quantitative PCR assay was used to measure and compare pathogen population in T_1 transformed and wild type MR219 seedlings inoculated by the *M. oryzae* pathotype P7.2 at 3×10^5 spores/mL 31, 48 and 72 h after inoculation. The T_1 plants were also evaluated in the glasshouse for the blast disease reaction to *M. oryzae* pathotype P7.2. Three components of partial resistance, including blast lesion degree (BLD), blast lesion type (BLT) and percentage disease leaf area (%DLA), were measured and compared between T_1 and control plants 7 days after inoculation.

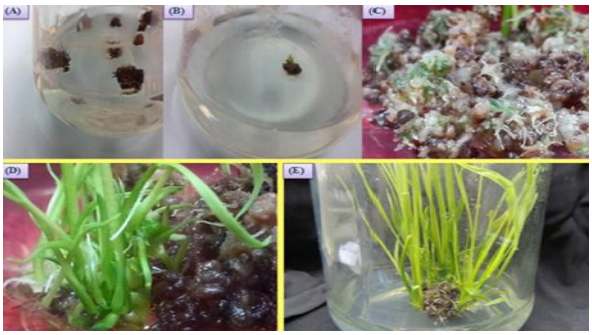

Figure 1: Regeneration of T_0 plantlets from transformed MR219 calli. (A) Transformed calli on the selection media; (B,C) production of green spots on the calli cells, and (D,E) regenerated transgenic shoots of MR219 from calli.

RESULTS AND DISCUSSION

The complete *Pikh* CDS with 1206 bp nucleotides encode a deduced protein of 401 amino acids, which was submitted to NCBI with the accession number (KM501045) and 100% identity with GU258499.1. *Pikh* (*Pi54*) was cloned for the first time from Tetep, which is an Indica cultivar in India. Thereafter, it was thoroughly mapped and molecularly characterized (Sharma, Madhav, et al., 2005; Sharma, Shanker, et al., 2005). The latest study on this gene was carried out to identify a novel allele of *Pikh* using an allele-mining technique. The researchers identified one similar *Pikh* allele from *Oryza sativa* ecotype Bizor-II of 3172 bp as its full length and another allele of 3543 bp as its full length from the *Oryza sativa* Indica Group ecotype Boha Thulasi Joha (Ramkumar et al., 2010). In the next step, the recombinant expression colonies were introduced into the *Agrobacterium* competent cells using a freeze-thaw technique. A PCR colony of the transformed *Agrobacterium* competent cells verified the transformation steps. In fact, transferring foreign genes to the rice plants through *Agrobacterium tumefaciens* has become a routine approach. *Agrobacterium*-mediated transformation showed certain advantages compared with other gene transformation methods and results in a lower copy number for transgene insertion through an easy, simple and low-cost method (Dai, Carcamo, Zhang, Chen, & Beachy, 2001). Four and nine transgenic plants were identified at the end of the selection process as T_0 and T_1 transgenic generations, respectively (Figure 1). The regenerated plants continued to grow and remained green; however, the untransformed calli died after selection. However, the T_0 plants were not strong as MR219 control plants. The results obtained from a PCR analysis of the T_1 transgenic plants yielded two bands at 1206 and 1507 bp. The seeds expressing the *gfp* as observed under the microscope were selected (13.7% and 23% seeds from T_0 and T_1) and used for the next generation and different analysis. The semi-quantitative PCR analysis showed that the *Pikh* allele was originally presented in the susceptible line (MR219); however, the *Pikh* gene expression levels were generally higher in the inoculated transgenic plants than the (untransformed) control MR219 plants. The *Pikh* gene expression levels after 31 h of inoculation in the transgenic plants were higher compared with the treated control plants. Real-time quantitative PCR confirmed the results obtained from semi-quantitative PCR. The *Pikh* gene was up-regulated in the inoculated transgenic rice plants by *M. oryzae* (compared with the inoculated wild-type plants) with a *PH value*=0.000.

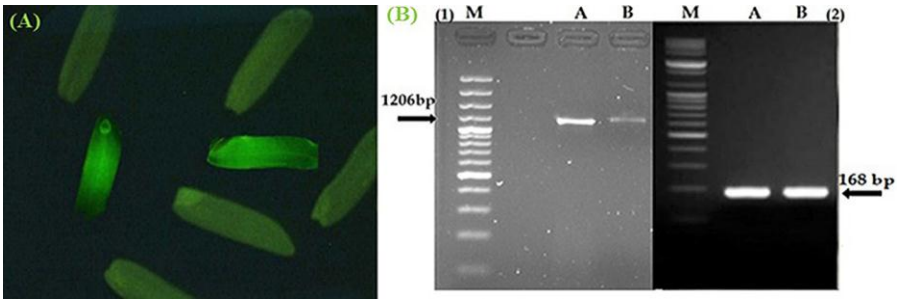

Figure 2: Confirmation of *Pikh* gene expression in the transgenic and control rice plants. (A) The expression of *gfp* flanked our gene of interest in the T1 seeds. (B) Expression pattern of the *Pikh* gene after transgenic rice plant inoculation. (1), RT-PCR analysis was performed using *Pikh*-specific primers using RNA isolated from the inoculated transgenic and wild-type rice plants (A, B, respectively); (2), *18SrRNA* as an internal control.

The peak area from the HPLC chromatogram of certain amino acids significantly increased through *Pikh* gene expression in the transgenic plants. The quantity of leucine amino acid differed significantly between the experimental samples with a probability of (*P<0.05*). The results of this experiment show that the quantity of leucine amino acid differed significantly in the transgenic plants (47.865 mg g^{-1} DW) compared with wild-type rice plants (17.131 mg g^{-1} DW) using Student's t-test. Most of the *R* genes identified contain a leucine rich repeat (LRR) structural motif, which includes a repeating pattern of 20 to 30 leucine amino acids (Azizi, Rafii et al. 2014). This increasing quantity of leucine amino acids in transgenic MR219 plants cannot only be due to the LRR protein encoded by the transformed *Pikh*, but over-expression of the *Pikh* gene may synergistically and/or antagonistically effect the phytohormones, transcription factors, kinase cascades, reactive oxygen species (ROS), etc. The leucine quantity can be affected by certain such activated functions. An important step in plant defense is appropriate perception of stress for a rapid and efficient response. Thereafter, the plants' constitutive defense mechanisms (Andreasson & Ellis, 2010) initiate activation of defense complex signaling cascades (AbuQamar, Luo, Laluk, Mickelbart, & Mengiste, 2009). A Significant variation was detected in the *M. oryzae* content of the inoculated control (untransformed) and transgenic plants in all samples collected 31, 48 and 72 h after inoculation. To be more exact, the real-time quantitative PCR quantification results for the *M. oryzae* population of inoculated control (non-transgenic) and transgenic plants at 31, 48 and 72 h after inoculation. At 48 and 72 h after inoculation, the fungal population in the control plants was significantly (*P*<0.05) higher (4.944 and 5.991 log *28S rDNA* gene copy number/per kg Fresh weight (FW) of plant, respectively) than in the transgenic plants (3.806 and 3.609 log *28S rDNA* gene copy number/per kg Fresh weight (FW) of plant, respectively). However, at 31 h of inoculation, the blast fungus populations in the transgenic plants (3.616 log *28S rDNA* gene copy number/per kg Fresh weight (FW) of plant) did not differ significantly from the control plants (3.628 log *28S rDNA* gene copy number/per kg Fresh weight (FW) of plant). The inheritance of the *Pikh* gene in the selfed T_1 generation was analyzed for the phenotype of blast disease resistance to the *M. oryzae* pathotype P7.2. A larger *M. oryzae* population was previously reported in the leaves of susceptible varieties compared with resistant plants at 48 and 72 h (Azizi et al., 2015) and 4 and 6 days after blast fungal inoculation (Correa-Victoria & Zeigler, 1993). Hence, we can conclude that the genetically transformed plants can provide a unique condition that is not suitable for more fungal growth. The BLT, BLD and percentages of DLA improved amongst the transgenic plants (3, 1 and 5%, respectively) compared with the control plants (4, 7 and 50%, respectively).

CONCLUSIONS

From this research, we generated new information on the expression of *Pikh* and its encoded protein, which are involved in improving the resistance mechanism to the blast fungus pathotype P7.2 in the MR219 rice variety. When the blast fungus enters the plant, the plant's immune system is activated. Effector-triggered immunity (ETI) as a branch of the innate immune system in plants is mediated by intracellular receptor molecules comprising nucleotide binding (NB) and leucine rich repeat (LRR) domains that individually recognize effectors produced by the specific pathogens. In fact, the binding of receptors and effectors proteins results in the initiation of defense systems and often leads to localized cell death (Chen and Ronald 2011). The expression of R genes mediated defense response associates with the hypersensitive response, a type of planned cell death, which occurs at the infection site and restricts growth and distribution of pathogen in the plant (Azizi et al., 2014). Activation and over-expression of *Pikh* gene may control the pathogen in the resistant varieties by producing protein that is involved in this innate immune system. Finally, we emphasize that the results of this study will differ with other isolates due to diverse effectors encoded by different isolates and their specific interactions with the rice blast immunity system. Hence, we conclude that the genetically transformed plants can provide unique conditions that are not suitable for more fungal growth.

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