View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by UKM Journal Article Repository

Sains Malaysiana 47(10)(2018): 2291–2300
http://dx.doi.org/10.17576/jsm-2018-4710-05

The Effect of Pathogenesis-Related 10 (*Pr-10*) Gene on the Progression of Fusarium Wilt in *Musa acuminata* cv. Berangan

(Kesan Gen Berkaitan Patogenesis 10 (*Pr-10*) pada Perkembangan Fusarium Layu dalam *Musa acuminata* cv. Berangan)

NADIYA AKMAL BAHARUM, ROFINA YASMIN OTHMAN, YUSMIN MOHD-YUSUF, BOON CHIN TAN, KAMILATULHUSNA ZAIDI & NORZULAANI KHALID*

ABSTRACT

PR-10 *is a member of pathogenesis-related (PR) genes elicited by the plant's defense mechanism during pathogen attack. Elevated expression of* PR-10 *upon different pathogen invasions has been observed in many plant species suggesting its role as an anti-bacterial, anti-viral and anti-fungal gene. However, the effect of* PR-10 *in mitigating the infection of* Fusarium oxysporum *f. sp.* cubense *(Foc), the causal agent of* Fusarium *wilt in banana has not been reported. In this study, the coding sequences of* PR-10 *gene isolated from Foc resistant* Musa acuminata *ssp.* malaccensis *(MaPR-10) were integrated into a local Foc susceptible commercial banana cultivar, Berangan via co-cultivation of embryogenic cell suspension and* Agrobacterium tumefaciens. *Out of 17 putative transgenic lines established, 11 of them positively harbored MaPR-10. Among these, Line-19 plantlets showed the most rapid* in-vitro *propagation and successfully over-expressed the transgene. Following a nursery challenge experiment with a virulent Foc race 4 (CI HIR) isolate, about 30% of Line-19 plants showed a one-week delay in disease progression when compared to the untransformed controls. From the final evaluation performed in the 5th week-post-inoculation, the leaf symptoms index (LSI) and rhizome discoloration index (RDI) of Line-19 was 3.4 and 6.1, respectively, indicating the disease had progressed. The findings of this study enrich the current existing knowledge on the roles of* PR-10 *in combating fungal disease in plants.*

Keywords: Fusarium oxysporum *f. sp.* cubense*;* Fusarium *wilt; pathogenesis-related protein;* PR-10*; transgenic banana*

ABSTRAK

PR-10 *merupakan antara gen berkaitan patogenan (PR) yang dihasilkan oleh sistem pertahanan tumbuhan semasa serangan patogen. Peningkatan ekspresi gen* PR-10 *ketika ancaman beberapa jenis patogen yang berbeza telah diperhatikan di dalam banyak spesies tumbuhan menunjukkan peranan gen ini sebagai agen anti-bakteria, anti-virus dan anti-kulat. Namun begitu, peranan* PR-10 *dalam mengurangkan kesan jangkitan* Fusarium oxysporum *f. sp.* cubense *(Foc), patogen penyebab penyakit layu pada pokok pisang masih belum pernah dilaporkan. Dalam kajian ini, jujukan pengekodan* PR-10 *daripada* Musa acuminata *ssp.* malaccensis *(*MaPR-10*) telah diintegrasikan ke dalam genom satu kultivar pisang komersial tempatan, Berangan melalui melalui kaedah penanaman antara* Agrobacterium tumefaciens *dan ampaian sel embriogenik. Daripada 17 jaluran pokok transgenik yang terhasil, 11 daripadanya disahkan positif mengandungi gen MaPR-10. Antara kesemua jaluran yang terhasil, Jaluran 19 menunjukkan kadar propagasi* in-vitro *paling cepat dan menunjukkan ekspresi gen yang lebih tinggi berbanding pokok kawalan yang tidak ditransform. Dalam penilaian tindak balas Jaluran 19 terhadap jangkitan Foc 'race' 4 (C1 HIR), 30% daripada anak pokok menunjukkan penularan penyakit seminggu lebih lambat berbanding pokok kawalan yang tidak ditransform. Melalui penilaian terakhir pada minggu ke-5 selepas inokulasi, skor indeks sistem daun (LSI) dan indeks penyahwarnaan rizom (RDI) bagi Jaluran 19 masing-masing ialah 3.4 dan 6.1, menunjukkan bahawa jangkitan Foc telah merebak. Keputusan kajian uji kaji ini berupaya meningkatkan maklumat sedia ada berkaitan peranan PR-10 dalam menentang penyakit kulat di dalam tumbuhan.*

Kata kunci: Fusarium oxysporum *f. sp.* cubense*; penyakit layu* Fusarium*; pisang transgenik; protein berkaitan patogenan; PR-10*

INTRODUCTION

Global banana production is under continuous attacks by various diseases, including *Fusarium* wilt. *Fusarium* wilt is caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), a type of soil-borne fungus, which initiates the infection through the plant roots. As the fungal hyphae advances into the vessel tissues, distribution of the necessary nutrients to different plant parts is halted causing serious yellowing and wilting symptoms predominantly on the leaves. Foc can be generally classified into three major races (Race 1, 2 & 4), based on their virulence against distinct cultivar groups. Foc race 4 (FocR4) is considered as the most destructive and can be further divided into tropical race 4 (TR4) and subtropical race 4 (SR4) (Ploetz 2015). Recent TR4 reported

in Jordan, Lebanon, Mozambique and Pakistan signifies the unceasing spread of the disease which has now affected the banana plantations outside the common Asian skirt and had caused huge loss to the farmers (Butler 2013; García-Bastidas et al. 2014; Ordonez et al. 2016).

Upon the interaction with the invading pathogens, plant hosts may display different responses. A resistant host would be able to curb the attack and avert the infection while in incompatible host-pathogen interaction, the plant is not able to resist the penetration which eventually leads to plant death. A tolerant host, on the other hand, might able to abate the pathogen from advancing while not necessarily eliminating it (Swarupa et al. 2014). All these responses require the activation of the plants' defense mechanism which may include physical modification of the host's cell wall, production of reactive oxygen species (ROS) as well as the release of defense-associated proteins (Dodds & Rathjen 2010).

Pathogenesis-related (PR) proteins are a part of the defense-related proteins induced in response to various abiotic and biotic stresses including pathogen invasion. PR proteins are classified into 17 subgroups based on similarity in the amino acid sequences, serological relationship and enzymatic activity (Agarwal & Agarwal 2014). Due to their underlying roles in the plants' defense mechanism pathways, PR proteins have been garnering huge interest in the development of transgenic crops with improved resistance against various pathogens. Particularly in combating *Fusarium* wilt, β-1,3-glucanase (*PR-2*), chitinase (*PR-3*) and thaumatin-like (*PR-5*) are among the members of PR families that have been utilized (Mahdavi et al. 2012; Maziah et al. 2007; Sreeramanan et al. 2006) with varying results. Over-expression of native *PR-10* gene for controlling the *Fusarium* wilt infection in banana has not been reported and is presented here.

PR-10 encodes a small, acidic protein with several distinct motifs such as Bet v1 and P-loop. The role of PR-10 in defense-related mechanisms has been associated with its RNase activity, which directly impedes the translational process of pathogen transcripts (Agarwal & Agarwal 2014). Elevated expression of PR-10 during fungal invasion shown in plant species like ginseng (Kim et al. 2014; Lee et al. 2012), pepper (Soh et al. 2012) and maize (Chen et al. 2006) further strengthens its putative role as an anti-fungal protein.

In this study, we aimed to examine the effect of *PR-10* in mitigating the advances of *Fusarium* infection in banana. We had isolated the coding sequences of *PR-10* from FocR4 resistant wild banana, *Musa acuminata* ssp. *malaccensis* (*MaPR-10*) and integrated it into the genome of a FocR4 susceptible commercial cultivar, cv. Berangan via co-cultivation of *Agrobacterium tumefaciens* and embryogenic cell suspension (ECS). The disease progression was then observed for five weeks. Berangan is selected as the target cultivar for this study due to commercial importance, local consumers' preference, good flavor and great storage qualities (Chin et al. 2014).

Materials and Methods

MATERIALS

Embryogenic cell suspension of *M. acuminata* cv. Berangan was provided by the Plant Biotechnology Research Laboratory (PBRL), University of Malaya, Malaysia and was maintained in M2 media at 25±2°C on a rotary shaker at 90 rpm prior to transformation experiments. Foc Race 4 isolate, designated as C1 HIR, was originally a gift from Prof. Dr. Baharuddin Salleh of Plant Pathology and Mycology Laboratory, School of Biological Science, Universiti Sains Malaysia, Malaysia and was maintained in water agar at the Phytomycology Laboratory, University of Malaya. The fungus was isolated from the inner stem of an infected *M. acuminata* cv. 'Cavendish' in Kuala Terengganu, Terengganu, Malaysia. All primer sequences and optimized thermal cycling parameters used are listed in Supplementary File 1.

VECTOR CONSTRUCT

RNA was extracted from young *M. acuminata* ssp. *malaccensis* leaves using a modified CTAB method (Al-Obaidi et al. 2010) and subjected to DNase 1 treatment (Thermo Scientific, U.S.A.). RNA integrity was verified by gel electrophoresis and analysis in a Nanodrop 2000 (Thermo Scientific, U.S.A.). First strand cDNA sample was prepared using random hexamer and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, U.S.A.). Coding sequences of *MaPR-10* (GenBank accession number: KU942376) were amplified using GoTaq® DNA polymerase (Promega, U.S.A.) (Applied Biosystems® Veriti® 96-Well Thermal Cycler platform, U.S.A.). *MaPR-10* was digested by Nco1 and BglII restriction enzymes (New England Biolabs, U.S.A.) and cloned into pCAMBIA1304 resulting in pMaPR-10 construct. The construct was then mobilized into *A. tumefaciens* LBA4404 via a freeze-thaw method (Go 2013). Transformed cells were verified by amplification of the *MaPR-10* insert. Expression of *MaPR-10* was driven by dual constitutive CaMV35S promoters under kanamycin and hygromycin selection in bacterial and plant hosts, respectively (Figure 1).

CO-CULTIVATION OF *A. TUMEFACIENS* AND *M. ACUMINATA* CV. BERANGAN CELL SUSPENSION

A positive *A. tumefaciens* colony harboring *MaPR-10* was cultured in 3 mL Luria-Bertani (LB) broth (Conda, Spain) (supplemented with 100 μg/mL kanamycin and 50 μg/mL rifampicin) and placed on a rotary shaker at 28°C, 220 rpm overnight. The overnight culture was inoculated into 30 mL of fresh LB broth (Conda, Spain) without any supplement. The culture was incubated on a rotary shaker under the same conditions until OD_{600} was ~0.5 followed by centrifugation at 4°C, 2655 × g for 10 min. The pellet was re-suspended in 30 mL of M2 media supplemented with 100 μM of acetosyringone. Depending on the size of banana cells, the suspension was either initially sieved

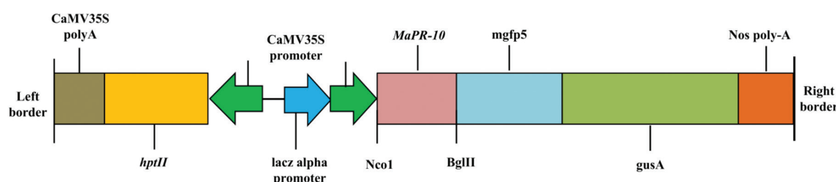


FIGURE 1. Vector construct of pMaPR-10

through 425 μM mesh or settled directly to 1:2 (cell volume:liquid media) in a sterile 50 mL Falcon tube. About 500 μL of the settled cv. Berangan cells were added into 10 mL of fresh M2 media containing 1 mL of transformed *A. tumefaciens* culture. The infected culture was incubated in the dark at 25±2°C, 100 rpm for 30 min before the media was completely removed and replaced with fresh M2 media containing the same supplement. The culture was co-cultivated in the dark on a rotary shaker at 80 rpm. After 4 days, the liquid media was completely removed and replaced with 10 mL of fresh M2 supplemented with 50 μg/mL cefotaxime. The culture was maintained under the same incubation conditions for 48 h before the liquid media was completely removed and replaced with 20 mL of M3 supplemented with 0.2 mg/L hygromycin and 50 μg/mL cefotaxime. The culture was maintained under the same incubation conditions. Untransformed *A. tumefaciens* and *A. tumefaciens* culture harboring pCAMBIA1304 vector were used as negative and positive controls, respectively.

MAINTENANCE AND REGENERATION OF TRANSFORMED CELLS

In M3 media, the cells were sub-cultured every 10-14 days for about 5-9 weeks until immature somatic embryos were formed. The immature somatic embryos were transferred to M4 media containing 8 mg/L BAP (M8B). Potential somatic embryos were maintained in the dark at 25±2°C for 4-8 weeks with a 2 week-subculturing interval. The immature somatic embryos with protruding shoots were transferred to M4 supplemented with 4 mg/L BAP (M4B). They were maintained at 25±2°C in light conditions (light intensity: 1450 lux) until shoot regeneration with a 2 week-subculturing interval. For continuous selection of transformed cells, M8B and M4B were supplemented with 0.2 mg/L hygromycin and 50 μg/mL cefotaxime. Individual shoots were transferred to M4 media supplemented with 2 mg/L BAP (M2B) and maintained under the same conditions for 4 weeks. To enhance the formation of roots, the shoots were cultured in M5 under the same growth conditions until transgene verification. The components of all culture media used were listed in Table 1.

SCREENING OF PUTATIVE TRANSFORMED PLANTLETS

DNA was extracted from the leaves of all regenerated lines using the DNeasy Plant Mini Kit (Qiagen, Germany). Amplification of the *MaPR-10* transgene was performed using Thermo Scientific Phusion® High Fidelity PCR Master Mix (Thermo Scientific, U.S.A.) (Applied Biosystems® Veriti® 96-Well Thermal Cycler platform, U.S.A.). PR10S F/PR10S R primers used in this experiment were designed as such to include a portion of CaMV35S fragment belongs to pCAMBIA1304.

TRANSGENE COPY NUMBER ANALYSIS

Transgene copy number in the selected transgenic Line-19 plants was predicted using qpCR (Sreedharan et al. 2012). *MaPR-10* was amplified from genomic DNA of

TABLE 1. Components of the culture media used for the maintenance and regeneration of *M. acuminata* cv. Berangan cells

Media	Components
M2	MS-macro (1X), MS-micro (1X), FeEDTA (1X), Dhed'a vitamins (1X), ascorbic acid (10 mg/L), 2,4-D (1.1 mg/L), sucrose (2%), myo-inositol (0.01%), zeatin (0.25 mg/L)
M3	MS-macro (1X), MS-micro (1X), FeEDTA (1X), vitamins (1X), sucrose (2%), myo-inositol (0.01%), L-glutamine (400 mg/L) with addition of hygromycin (0.2 mg/L) and cefotaxime (50 μg/mL) for selection of transformed cells
M4 (8 mg/L BAP) or M8B	MS-macro (1X), MS-micro (1X), FeEDTA (1X), vitamins (1X), BAP (8 mg/L), sucrose (3%), myo-inositol (0.01%), gelrite (0.22%) with addition of hygromycin (0.2 mg/L) and cefotaxime (50 μg/mL) for selection of transformed cells
M4 (4 mg/L BAP) or M4B	Refer M8B except for BAP (4 mg/L)
M4 (2 mg/L BAP) or M2B	Refer M8B except for BAP (2 mg/L)
M5	MS-macro (1X), MS-micro (1X), FeEDTA (1X), vitamins (1X), sucrose (3%), charcoal (0.8-1%), gelrite (0.22%)

Line-19 plants as well as from a series of diluted plasmids harboring the transgene using Power SYBR Green (Applied Biosystems, U.S.A.) (QuantStudio™ 12K Flex Real-time PCR platform, Life Technologies, U.S.A.). The copy number of *MaPR-10* was estimated by associating the C_T of *MaPR-10* transgene from Line-19 with the standard curve generated by the serial plasmid dilutions. The calculation was done based on the method described by Bubner and Baldwin (2004) and Sreedharan et al. (2012).

EXPRESSION ANALYSIS OF MAPR-10

Young leaves of one-month-old Line-19 plants as well as untransformed controls grown under the same conditions (26±2°C, 16-8 h photoperiod) were harvested at the same time. RNA extraction and cDNA preparation was performed as described previously. Ribosomal protein S 2 (*RPS2)* was used as a reference gene in which the *MaPR-10* expression was calibrated to the untransformed controls (Chen et al. 2011). qPCR reaction mixtures were run in triplicates using QuantStudio™ 12K Flex Real-time PCR platform (Life Technologies, U.S.A.). Dissociation curves were generated to verify the presence of single peak targets. All data were analyzed using ExpressionSuite Software v1.0.4 (Applied Biosystem, U.S.A.). Non-template and non-RT samples were also included as controls.

SINGLE SPORE ISOLATION AND DNA EXTRACTION

Single spore isolation was performed to prepare pure culture of Foc race 4 C1 HIR isolate (Pérez-Vicente et al. 2014). Pure culture of C1 HIR was incubated on potato dextrose agar (PDA) in the dark at room temperature (26±2°C) for about 3-5 days until vigorous mycelia growth was observed. DNA extraction was performed according to modified method originally described by Lin et al. (2009).

VERIFICATION OF RACE GROUP

Verification of the race group of C1 HIR isolate was done using Foc-1/Foc-2 primer pair (Lin et al. 2009). The amplification was carried out using GoTaq® DNA polymerase (Promega, U.S.A.).

SPORE SUSPENSION PREPARATION

Pure C1 HIR isolate was cultured on PDA in the dark for about 3-4 days at room temperature (26±2°C) until mycelia formation. One loopful of mycelia from the plate was inoculated into 1 L of fresh potato dextrose broth (PDB) (Difco™, BD, France). The culture was incubated at room temperature (26±2°C) and swirled twice a day for one week. The viability test was conducted on the 5th day post-inoculation (dpi) by spreading about 100 µL of diluted spore suspension onto a PDA plate. The plate was incubated at room temperature (26±2°C) in the dark for 36-48 h for spore germination. On the 7th dpi, the concentration of the suspension was estimated by haemocytometer and adjusted to 10^6 spores/mL using sterile distilled water.

FUSARIUM BIOASSAY

Fusarium bioassay was performed using modified 'double tray' technique adopted from Mak et al. (2004). Four biological test groups were prepared: treated transgenic Line-19 Berangan, non-treated transgenic Line-19 Berangan, treated untransformed Berangan and non-treated untransformed Berangan each containing at least 10 plants. All plants were acclimatized in sterile potting mixture (soil:sand (3:1)) for about two months at 29±1°C with a 16-8 h photoperiod.

Prior to inoculation, the plants were carefully uprooted and their roots were rinsed with tap water to remove soil traces. The roots were fully soaked in the prepared spore suspension (10^6 spores/mL) for 2 h before the plants were replanted into the same potting mixture. Simultaneously, negative control plants were treated with sterile distilled water. The plants were watered with tap water every other day and with Hoagland's solution (Sigma-Aldrich, U.S.A.) once a week. Additionally, half tea-spoon of nitrogen:phosphorus:potassium (NPK) (15:15:15) fertilizer was added into each pot once every fortnight.

The contaminated water contained in the bottom tray was treated by adding sodium hypochlorite and left overnight before it was discarded the next morning. All materials used in the experiment including plants and soils were autoclaved at 121°C, 15 psi for 45 minutes prior to disposal. Waste materials were disposed in double-layered biohazard plastic bags. The disease progression on the leaves was observed at least once a week until the final evaluation in the 5th week. The rhizomes were vertically cut to observe the internal discoloration. Disease scoring was performed based on modified leaf symptoms index (LSI) and rhizome discoloration index (RDI) originally described by Mak et al. (2004).

RESULTS

VECTOR CONSTRUCT AND *AGROBACTERIUM* TRANSFORMATION

Coding sequences of *MaPR-10* (483 bp including the stop codon TGA) were successfully amplified and inserted in between NcoI and BglII restriction sites of a pCambia1304 vector. The amplification of the expected *MaPR-10* band verified the success of *A. tumefaciens* transformation (Figure 2). Selected *A. tumefaciens* LBA4404 culture harboring the pMaPR-10 construct appeared turbid after overnight incubation at 28°C, 220 rpm in LB broth supplemented with 50 µg/mL rifampicin and 100 µg/mL kanamycin, showing the viability of the cells.

PLANT TRANSFORMATION AND REGENERATION

When maintained in M3 media, protruding shoots on the immature somatic embryos started to develop as early as 5 weeks after culture. During plant regeneration on M8B, we observed the appearance of globular-stage embryo on the surface of potentially transformed embryogenic callus

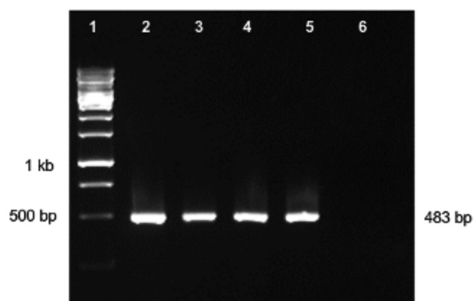
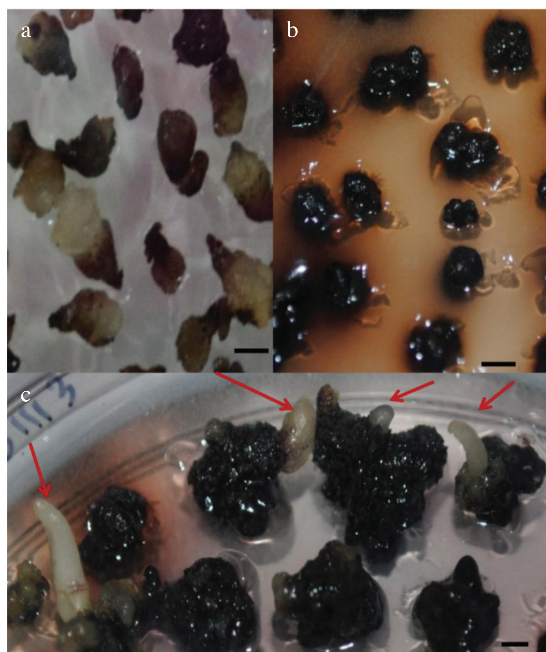


FIGURE 2. Positive *A. tumefaciens* harboring *MaPR-10* were indicated by a 483 bp band (Lane 2-5). Lane 1: 1 kb DNA marker, Lane 6: Negative control

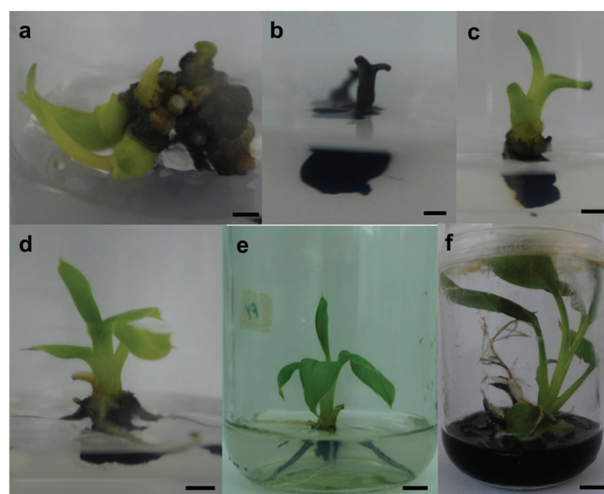
after about 4-8 weeks of culture (Figure 3a). In contrast, the non-viable cells turned black and eventually died (Figure 3b). Out of 3418 pMaPR-10–derived callus cultured on M8B, about 98.4% were non-embryogenic; mostly of watery, compact and yellow nodular types while only about 53 of them or 1.6% were potentially embryogenic callus. Potential embryogenic callus produced protruding shoots as early as 6 weeks after culture on M8B (Figure 3c).



Bar represents 0.25 cm

FIGURE 3. Selection of transformed cells on M4 supplemented with 8 mg/L BAP, 0.2 mg/L hygromycin and 50 µg/mL cefotaxime (M8B), (a) One month-old potential embryogenic cells culture, (b) Somatic embryos turned black and were unable to resist hygromycin selection, and (c) Protruding shoots observed on potential somatic embryos (arrowed) after 6-8 weeks of culture

Further selection in M4B showed that the potential embryogenic callus started to produce multiple shoots as early as one week after culture (Figure 4(a)). Some lines failed to survive the continuous antibiotic selection leading to tissue death (Figure 4(b)). Only 17 lines endured the selection at the M4B stage, which were Lines- 3, 8, 13, 17, 18, 19, 20, 22, 23, 24, 29, 39, 42, 45, 50, 51 and 53. The regeneration rate for this experiment was 0.5%. Potential shoots emerging from the embryogenic callus continued to grow for about three weeks and were sub-cultured onto new M4B to avoid media depletion as well as for propagation (Figure 4c & 4d). Maintenance in M2B and M5 induced individual shoots and vigorous roots formation, respectively (Figure 4e & 4f).



Bar A, B=0.25 cm, C=0.5 cm, D=0.3 cm, E=0.75 cm, F=1.5 cm

FIGURE 4. Regeneration of *MaPR-10*- harboring plantlet on M4B (supplemented with 4 mg/L BAP, 0.2 mg/L hygromycin and 50 µg/mL cefotaxime), M2B (2 mg/L BAP) and M5 media, (a) Mixture of clumps and shoots formed on M4B, (b) Embryogenic cell that was unable to resist hygromycin selection turned dark on M4B, (c) Shoot of *M. acuminata* cv. Berangan harboring *MaPR-10* started to form in M4B, (d) Shoot of putative transgenic Berangan continued to grow in M4B, (e) Shoot of putative transgenic Berangan was cultured in M2B for further shoot regeneration and (f) Regenerated shoot was cultured on M5 for root elongation

TRANSGENE SCREENING

Out of 17 putative transgenic *MaPR-10* lines established, it was possible to amplify a 388 bp gene specific *MaPR-10* band from 11 lines (Supplementary File 2). The positive lines harboring the transgene were Line-18, 19, 20, 22, 23, 29, 39, 42, 45, 51 & 53 whilst Line-3, 8, 13, 17, 24 and 50 were probably escapes. In comparison with other lines, Line-19 plantlets demonstrated the most rapid propagation rate and ability to produce bulk shoots. Due to these reasons, plantlets from Line-19 were chosen for further analysis. Randomly selected Line-19 plantlets also showed the presence of the amplified transgene (Figure 5).

TRANSGENE COPY NUMBER ANALYSIS

C_T values of *MaPR-10* for all tested Line-19 samples fell within the C_T range generated by the serial dilution

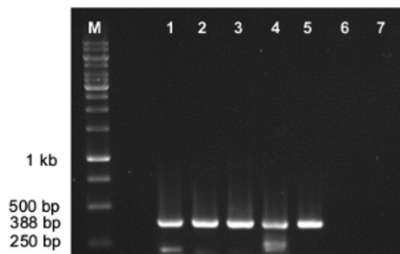


FIGURE 5. Transgene screening of randomly selected Line-19 plants harboring *MaPR-10* using PR10 S F/1304R primer pair. Lane 1-4: Randomly selected Line-19, Lane 5: Positive control (Purified plasmid), Lane 6: Untransformed control (*M. acuminata* cv. Berangan), Lane 7: Negative control PCR, M: 1 kb DNA marker (Thermo Scientific, U.S.A.)

plasmids. Based on the linear equation obtained from the standard curve, the predicted transgene *MaPR-10* copy number in Line-19 was one (Supplementary File 3).

EXPRESSION ANALYSIS OF MAPR-10

Randomly selected Line-19 plantlets (19-1, 19-2 and 19-3) showed variation in the expression of *MaPR-10*. Using $2^{-\Delta\Delta CT}$ formula, the fold-change of *MaPR-10* in 19-1, 19-2 and 19-2 was 62.59, 8.74 and 13.66 respectively when normalized to the untransformed controls (Figure 6).

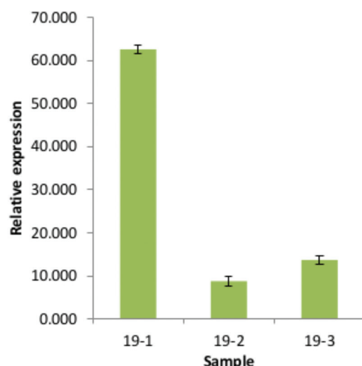


FIGURE 6. Real-time quantitative polymerase chain reaction (qPCR) analysis for over-expression of *MaPR-10* in the leaves of selected Line-19 plants harvested at the same time. All target gene expression values have been normalized against Musa *RPS2* expression levels. The transgene expression in the transgenic samples has been calibrated to of untransformed controls (UTC), which is assumed to be 1. The data represented are means of three technical replicates

VERIFICATION OF FOCR4 (C1 HIR) ISOLATE

After 4-5 days of incubation in the dark at room temperature (26±2°C), extensive white purplish mycelium was observed on PDA. PDB inoculated with the mycelium became turbid and showed significant mycelium growth after 5-7 days of incubation. Germinated spores spotted on PDA confirmed the viability of the pure C1 HIR culture that will be used in the bioassay experiments. C1 HIR was classified as a race 4 isolate based on the presence of a 242 bp-band upon amplification using specific Foc-1/Foc-2 primer pair (Figure 7).

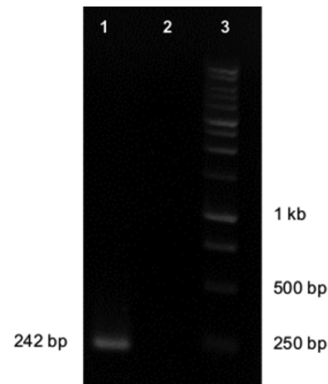


FIGURE 7. Amplification of a 242 bp band indicated the identity of C1 HIR as a race 4 isolate. M: 1 kb DNA marker, N: Negative control PCR

FOC BIOASSAY CHALLENGE OF TRANSGENIC MAPR-10 LINE-19 PLANTS AGAINST C1 HIR ISOLATE

When grown at 29±1°C with 16-8 h photoperiod for two months, about 75% of the transgenic plants depicted slower growth in comparison to the untransformed controls (Figure 8). At 6-dpi, marked yellowing symptoms started to appear on the treated untransformed control plants (Figure 9). In contrast to the untransformed controls, about 30% of the transgenic plants showed delayed disease symptoms that started to appear in the 2nd week post-inoculation. At 20-dpi, the yellowing symptoms on the older leaves of the transgenic plants became more visible initiated at the leaves' edges progressing to the midrib area while some of the leaves completely wilted (Figure 10). At the end of the 5th week, untreated controls remained healthy while infected plants demonstrated different degrees of disease symptoms (Figure 11).

Internal observation on the rhizome of the survived transgenic plants showed incomplete discoloration in the

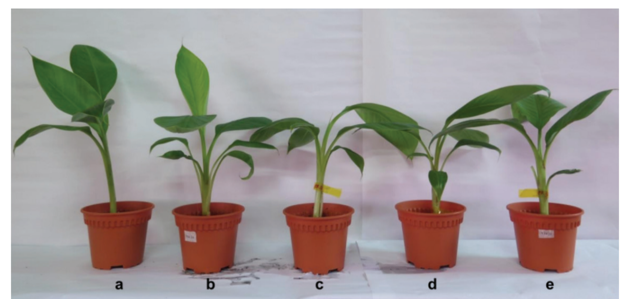


FIGURE 8. At 0 day of post-inoculation. Representatives shown were untransformed control: (a) and Line-19 plants (b-e). Plant (a) and (b) showed similar growth development which were faster than of c-e

FIGURE 9. 6 days post-inoculation. Representatives shown were untreated control (a), treated untransformed control (b) and treated Line-19 plants (c & d). Plant (a) and (c) remained healthy whereas d already exhibited minor yellowing symptoms. Plant (b) started to show marked yellowing symptoms on the leaves. Picture of plant (d) was taken separately

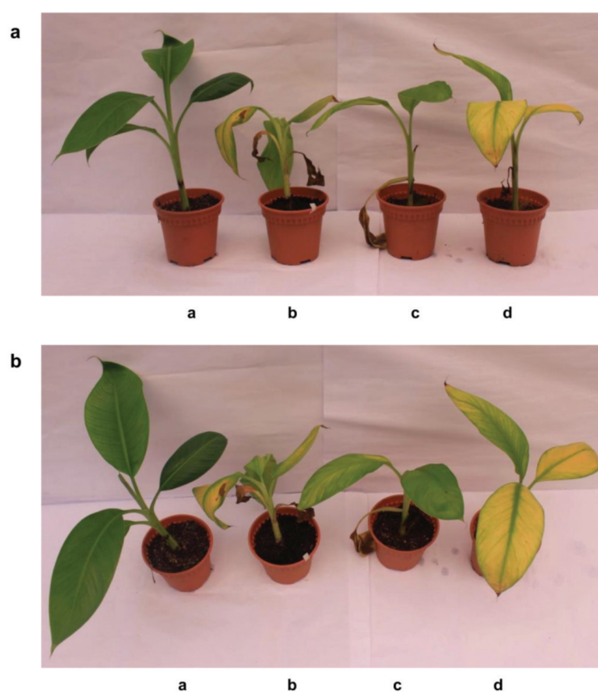


FIGURE 10. 20 days post-inoculation. Representatives shown were untreated control (a), treated untransformed control (b) and treated Line-19 plants (c & d) from front (a) and top (b) view. Plant (a) remained healthy while b, c & d started to show marked yellowing and wilting leaf symptoms. Plant (d) displayed more extensive yellowing symptoms compared to (c)

stele region indicating that the infection did not proceed to the pseudostem. Rhizomes of more severely affected plants exhibited major to complete discoloration (Figure 12). Despite showing delayed infection symptoms, the final disease evaluation performed in the 5th week post-inoculation showed that transgenic Line-19 plants harboring *MaPR-10* was susceptible to FocR4 (C1 HIR) isolate with LSI and RDI scores of 3.4 and 6.1, respectively. The LSI and RDI of treated untransformed controls were 4.5 and 6.3, respectively (Mak et al. 2004).

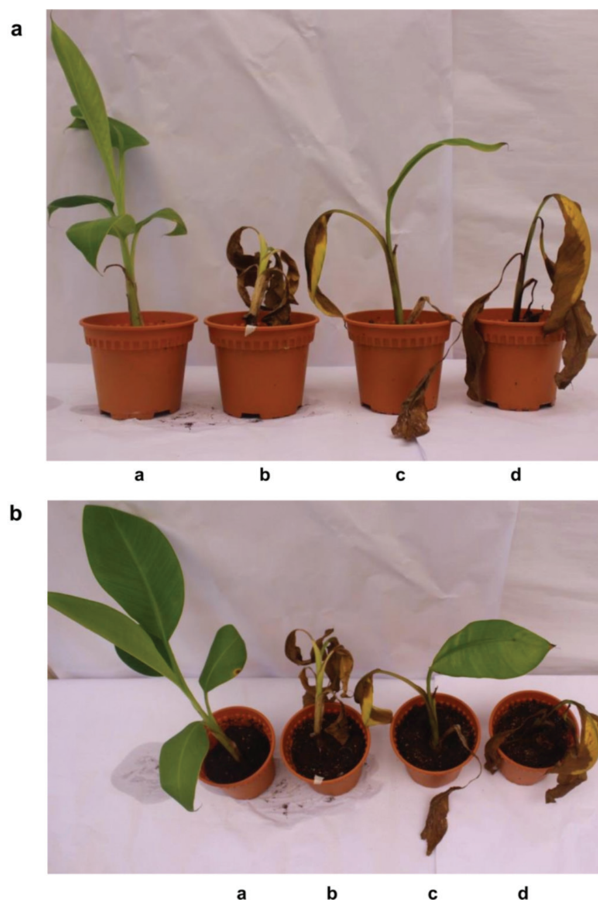


FIGURE 11. 35 days post-inoculation. Representatives shown were untreated control (a), treated untransformed control (b) and treated Line-19 plants (c & d) from front (a) and top (b) view. Untreated control (a) remained healthy and showed steady growth while treated untransformed control (b) almost completely wilted out. The treated transgenic plants (c & d) showed variation in disease severity. Plant (c) showed mixed yellowing and wilting leaf symptoms while plant (d) completely wilted out

DISCUSSION

PR-10 is speculated to possess anti-fungal property which has been demonstrated in many plant species against a number of fungal pathogens such as in *Triticum aestivum* (Zhang et al. 2010), *Zea mays* (Chen et al. 2006) and *Panax ginseng* (Lee et al. 2012). The association between *PR-10* and fungal tolerance was not clearly defined until Moiseyev et al. (1994) showed high sequence similarity between the gene's sequences and ribonuclease's (RNase) derived from phosphate-deprived ginseng cells. Invaded plant cells might benefit from this RNase activity as the nuclease initiates RNA degradation and further restrains the translational process of harmful pathogen proteins thus impeding the incoming attack. The study of *PR-10*'s roles in controlling the disease progression of *Fusarium* wilt affecting banana plants has not been previously reported. In the present study, we have successfully integrated the coding sequences of *PR-10* derived from wild banana (*M. acuminata* ssp. *malaccensis*) (*MaPR-10*) into *M.*

Bar represents 1.0 cm

FIGURE 12. 35 days post-inoculation. Internal observation of the disease symptoms on the rhizomes of the plants. Representatives shown were untreated control (a), treated untransformed control (b) and treated Line-19 plants (c & d). Rhizome of untreated control (a) remained healthy without any discoloration while the treated untransformed control (b) showed extensive rhizome discoloration starting from the junction of root and rhizome as well as in the stele region expanding to the pseudostem. Treated transgenic plants (c & d) showed variation in disease severity. About 50% of the stele region of (c) showed discoloration that did not proceed to the pseudostem whilst more severe symptoms were detected in (d) comprising the stele region as well as the pseudostem

acuminata cv. Berangan embryogenic cell suspension via co-cultivation with *A. tumefaciens*. We isolated *PR-10* from the same plant species to minimize the biosafety concerns and to increase the likelihood of its acceptability to consumers. There is a noticeably growing number of publications in banana transgenic research utilizing banana-derived genes, particularly after the completion of the plant's whole genome sequencing (Negi et al. 2014; Shekhawat & Ganapathi 2014; Sreedharan et al. 2013).

Embryogenic cell suspension (ECS) was chosen as the explant since the plantlets regenerated from ECS were reported to be of single cell origin thus minimizing the chimerism issues which is a general problem in transformation using other explants (Ghosh et al. 2009). The transgene screening has indirectly become easier since the DNA can be extracted from any part of the plants. Despite all the advantages mentioned, the major bottlenecks of using ECS are the long establishment period, low generation rate and reduced embryogenic potential over time (Jain 2008). Throughout the selection process, the concentration of the hygromycin used (0.2 mg/L) was lower than other banana ECS transformation experiments reported (6.25 mg/L-10 mg/L). Effective antibiotic concentrations are influenced by several transformation parameters and thus are usually optimized independently by each study. Several effects of antibiotic toxicity have been exemplified previously that include necrosis on chimeric progenies, growth abnormalities and low survival rate (Maziah et al. 2007; Vishnevetsky et al. 2011). Under continuous hygromycin selection at 0.2 mg/L, the regeneration efficiency obtained in this study was relatively low at around 0.5%. Nevertheless, we were able to select 17 putative transgenic lines.

In general, different plantlets regeneration rate has been observed during tissue culture stage among the putative transgenic lines, which could be linked to transgene 'position effects', or preferential integration of T-DNA region into host genome. Due to positive transgene insertion, vigorous growth and rapid shoot development on hygromycin-supplemented media, Line-19 plantlets were selected for further analysis. When grown under controlled parameters, about 75% of the Line-19 plants depicted slower growth in comparison to the untransformed controls. Similar tardy growth of transgenic plants under greenhouse conditions has been reported in rice seedlings co-expressing chitinase (*RCH10*) and β-1,3-glucanase (*AGLU1*) genes (Mao et al. 2014). This disparity could probably be associated with the effect of somaclonal variation. Somaclonal variation instigated by *in-vitro* micropropagation is commonly known as tissue-induced variation (Kaeppler et al. 2000) and it occurs as a result of strong stress experienced by the *in vitro*-cultured plantlets (Larkin & Scowcroft 1981).

In this study, 30% of Line-19 plants displayed a one-week-delay in yellowing and wilting leaf symptoms, which appeared in the 2nd week post-inoculation when compared to the untransformed controls. Line-19 Berangan plants were eventually found to be susceptible to C1 HIR infection after five weeks of inoculation. Similar cases of delayed disease progression had been reported. In a leaf disk bioassay study against *Mycosphaerella fijiensis* (Kovács et al. 2013), almost half of transgenic 'Gros Michel' lines harboring rice chitinase gene depicted less severe necrosis symptoms at early time points but were later categorized under susceptible group as the disease progressed. Bezirganoglu et al. (2013) also described various level of disease resistance in transgenic melon expressing antifungal protein and chitinase against *Rhizoctonia solani*. Control melon plants underwent leaf necrosis symptoms that led to plants' death early as 5 days post-inoculation. A few transgenic lines survived more than 2 weeks wherein more susceptible lines died at least 3-5 days later than of controls. Bezirganoglu et al. (2013) further elaborated the factors that may have affected the severity of disease development which include developmental stages of the plants, temperature changes or the gene construct. Since this experiment has been optimized under controlled conditions, changes in the test environment may impose more minimal effects on the disease progression rather than the physical condition of the plants. To the best of our knowledge, this is the first report of *MaPR-10* over-expression in *M. acuminata* cv. Berangan banana. Thus, the effectiveness of the gene construct in conferring disease resistance cannot be compared due to lack of information. Nevertheless, this present study built a basis for studying

MaPR-10 and its over-expression effect on the banana's tolerance against *Fusarium* infection.

CONCLUSION

As the first study of *MaPR-10* over-expression in banana against *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4), Line-19 Berangan banana plants harboring *MaPR-10* has shown delayed disease progression following FocR4 C1 HIR infection in comparison to the untransformed controls. More data need to be gathered from the bioassay study of other transgenic lines to further evaluate the effectiveness of *MaPR-10* in improving the plants' tolerance against Foc. The information obtained from this study would be useful for future genetic improvement of banana, particularly in *Fusarium*-host interaction studies.

ACKNOWLEDGEMENTS

This work was supported by grant (53-02-03-1068) financed by the Ministry of Science, Technology and Innovation (MOSTI) Malaysia; a University of Malaya Research Grant (RP005C-13BIO), a University Malaya High Impact Research Grant (UM.C/625/1/HIR/MOHE/SCI/18) and CEBAR Research University Grant (RU006-2017). We also would like to thank Prof. Dr. Baharuddin Salleh and Nurul Farizah Azuddin of Plant Pathology and Mycology Laboratory, School of Biological Science, Universiti Sains Malaysia (USM), Malaysia for providing us the FocR4 (C1 HIR) isolate.

REFERENCES

Agarwal, P. & Agarwal, P.K. 2014. Pathogenesis related-10 proteins are small, structurally similar but with diverse role in stress signaling. *Molecular Biology Reports* 41(2): 599-611.

Al-Obaidi, J.R., Mohd-Yusuf, Y., Chin-Chong, T., Mhd-Noh, N. & Othman, R.Y. 2010. Identification of a partial oil palm polygalacturonase-inhibiting protein (*EgPGIP*) gene and its expression during basal stem rot infection caused by *Ganoderma boninense*. *African Journal of Biotechnology* 9(46): 7788-7797.

Bezirganoglu, I., Hwang, S.Y., Fang, T.J. & Shaw, J.F. 2013. Transgenic lines of melon (*Cucumis melo* L. var. makuwa cv.'Silver Light') expressing antifungal protein and chitinase genes exhibit enhanced resistance to fungal pathogens. *Plant Cell, Tissue and Organ Culture* 112(2): 227-237.

Bubner, B. & Baldwin, I.T. 2004. Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Reports* 23(5): 263-271.

Butler, D. 2013. Fungus threatens top banana. *Nature* 504(7479): 195-196.

Chen, Z.Y., Brown, R., Rajasekaran, K., Damann, K. & Cleveland, T. 2006. Identification of a maize kernel pathogenesis-related protein and evidence for its involvement in resistance to *Aspergillus flavus* infection and aflatoxin production. *Phytopathology* 96(1): 87-95.

Chen, L., Zhong, H.Y., Kuang, J.F., Li, J.G., Lu, W.J. & Chen, J.Y. 2011. Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. *Planta* 234(2): 377-390.

Chin, W.Y.W., Annuar, M.S.M., Tan, B.C. & Khalid, N. 2014. Evaluation of a laboratory scale conventional shake flask and a bioreactor on cell growth and regeneration of banana cell suspension cultures. *Scientia Horticulturae* 172: 39-46.

Dodds, P.N. & Rathjen, J.P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics* 11(8): 539-548.

García-Bastidas, F., Ordóñez, N., Konkol, J., Al-Qasim, M., Naser, Z., Abdelwali, M. & Kema, G.H.J. 2014. First report of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 associated with Panama disease of banana outside Southeast Asia. *Plant Disease* 98(5): 694-694.

Ghosh, A., Ganapathi, T., Nath, P. & Bapat, V. 2009. Establishment of embryogenic cell suspension cultures and *Agrobacterium*-mediated transformation in an important Cavendish banana cv. Robusta (AAA). *Plant Cell, Tissue and Organ Culture* 97(2): 131-139.

Go, P.S. 2013. Expression of anti-toxoplasma scfv antibodies in plants/Go Pei See. Master's dissertation, University of Malaya (Unpublished).

Jain, S. 2008. *In vitro* mutagenesis in banana (*Musa* spp.) improvement. Paper presented at the International Conference on banana and plantain in Africa: Harnessing international partnerships to increase research impact. *ISHS Acta Horticulturae* 879: 605-614.

Kaeppler, S.M., Kaeppler, H.F. & Rhee, Y. 2000. Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology* 43(2-3): 179-188.

Kim, Y.J., Jang, M.G., Lee, H.J., Jang, G.H., Sukweenadhi, J., Kwon, W.S. & Yang, D.C. 2014. Functional characterization of the pathogenesis-related protein family 10 gene, *PgPR10-4*, from *Panax ginseng* in response to environmental stresses. *Plant Cell, Tissue and Organ Culture* 118(3): 531-543.

Kovács, G., Sági, L., Jacon, G., Arinaitwe, G., Busogoro, J.P., Thiry, E. & Remy, S. 2013. Expression of a rice chitinase gene in transgenic banana ('Gros Michel', AAA genome group) confers resistance to black leaf streak disease. *Transgenic Research* 22(1): 117-130.

Larkin, P.J. & Scowcroft, W.R. 1981. Somaclonal variation - A novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* 60(4): 197-214.

Lee, O.R., Kim, Y.J., Balusamy, S.R.D., Khorolragchaa, A., Sathiyaraj, G., Kim, M.K. & Yang, D.C. 2012. Expression of the ginseng *PgPR10-1* in *Arabidopsis* confers resistance against fungal and bacterial infection. *Gene* 506(1): 85-92.

Lin, Y.H., Chang, J.Y., Liu, E.T., Chao, C.P., Huang, J.W. & Chang, P.F.L. 2009. Development of a molecular marker for specific detection of *Fusarium oxysporum* f. sp. *cubense* race 4. *European Journal of Plant Pathology* 123(3): 353-365.

Mahdavi, F., Sariah, M. & Maziah, M. 2012. Expression of rice thaumatin-like protein gene in transgenic banana plants enhances resistance to *Fusarium* wilt. *Applied Biochemistry and Biotechnology* 166(4): 1008-1019.

Mak, C., Mohamed, A., Liew, K., Ho, Y., Jain, S. & Swennen, R. 2004. Early screening technique for Fusarium wilt resistance in banana micropropagated plants. Paper presented at the banana improvement: Cellular, molecular biology, and induced mutations. Proceedings of a meeting held in Leuven, Belgium, 24-28 September 2001.

Mao, B., Liu, X., Hu, D. & Li, D. 2014. Co-expression of RCH10 and AGLU1 confers rice resistance to fungal sheath blight *Rhizoctonia solani* and blast *Magnorpathe oryzae* and reveals

impact on seed germination. *World Journal of Microbiology and Biotechnology* 30(4): 1229-1238.

Maziah, M., Sariah, M. & Sreeramanan, S. 2007. Transgenic banana Rastali (AAB) with β-1, 3-glucanase gene for tolerance to *Fusarium* wilt race 1 disease via *Agrobacterium*-mediated transformation system. *The Plant Pathology Journal* 6: 271-282.

Moiseyev, G.P., Beintema, J.J., Fedoreyeva, L.I. & Yakovlev, G.I. 1994. High sequence similarity between a ribonuclease from ginseng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. *Planta* 193(3): 470-472.

Negi, S., Tak, H. & Ganapathi, T. 2014. Cloning and functional characterization of *MusaVND1* using transgenic banana plants. *Transgenic Research* 24(3): 571-585.

Ordonez, N., García-Bastidas, F., Laghari, H.B., Akkary, M.Y., Harfouche, E.N., Al Awar, B.N. & Kema, G.H.J. 2016. First report of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 causing Panama disease in Cavendish bananas in Pakistan and Lebanon. *Plant Disease* 100(1): 209.

Pérez-Vicente, L., Dita, M.A. & Martínez-de la Parte, E. 2014. Technical manual: Prevention and diagnostic of *Fusarium* wilt (Panama disease of banana caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4). www.fao.org website: http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/caribbeantr4/13ManualFusarium.pdf. Accessed on 26 June 2015.

Ploetz, R.C. 2015. Management of *Fusarium* wilt of banana: A review with special reference to tropical race 4. *Crop Protection* 73: 7-15.

Shekhawat, U.K.S. & Ganapathi, T.R. 2014. Transgenic banana plants overexpressing *MusabZIP53* display severe growth retardation with enhanced sucrose and polyphenol oxidase activity. *Plant Cell, Tissue and Organ Culture* 116(3): 387-402.

Soh, H.C., Park, A.R., Park, S., Back, K., Yoon, J.B., Park, H.G. & Kim, Y.S. 2012. Comparative analysis of pathogenesis-related protein 10 (*PR10*) genes between fungal resistant and susceptible peppers. *European Journal of Plant Pathology* 132(1): 37-48.

Sreedharan, S., Shekhawat, U.K. & Ganapathi, T.R. 2013. Transgenic banana plants overexpressing a native plasma membrane aquaporin *MusaPIP1;2* display high tolerance levels to different abiotic stresses. *Plant Biotechnology Journal* 11(8): 942-952.

Sreedharan, S., Shekhawat, U.K.S. & Ganapathi, T.R. 2012. *MusaSAP1*, a A20/AN1 zinc finger gene from banana functions as a positive regulator in different stress responses. *Plant Molecular Biology* 80(4-5): 503-517.

Sreeramanan, S., Maziah, M., Rosli, N., Sariah, M. & Xavier, R. 2006. Particle bombardment-mediated co-transformation of chitinase and β-1, 3 glucanase genes in banana. *Biotechnology* 5(2): 203-216.

Swarupa, V., Ravishankar, K. & Rekha, A. 2014. Plant defense response against *Fusarium oxysporum* and strategies to develop tolerant genotypes in banana. *Planta* 239(4): 735-751.

Vishnevetsky, J., White, Jr. T.L., Palmateer, A.J., Flaishman, M., Cohen, Y., Elad, Y. & Dgani, O. 2011. Improved tolerance toward fungal diseases in transgenic Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain. *Transgenic Research* 20(1): 61-72.

Zhang, G., Li, Y.M., Zhang, Y., Dong, Y.L., Wang, X.J., Wei, G.R. & Kang, Z.S. 2010. Cloning and characterization of a pathogenesis-related protein gene TaPR10 from wheat induced by stripe rust pathogen. *Agricultural Sciences in China* 9(4): 549-556.

Nadiya Akmal Baharum, Rofina Yasmin Othman, Boon Chin Tan, Kamilatulhusna Zaidi & Norzulaani Khalid*
Institute of Biological Sciences
Faculty of Science
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

Nadiya Akmal Baharum
Department of Cell and Molecular Biology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
43400 UPM Serdang, Selangor Darul Ehsan
Malaysia

Rofina Yasmin Othman, Yusmin Mohd-Yusuf, Boon Chin Tan, Kamilatulhusna Zaidi & Norzulaani Khalid*
Centre for Research in Biotechnology for Agriculture (CEBAR)
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

Yusmin Mohd-Yusuf
Centre for Foundation Studies in Science
University of Malaya
50603 Kuala Lumpur, Federal Territory
Malaysia

*Corresponding author; email: lani@um.edu.my

Received: 15 December 2017
Accepted: 19 June 2018