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Original Article

Overexpression of rice thaumatin-like protein (*Ostlp*) gene in transgenic cassava results in enhanced tolerance to *Colletotrichum gloeosporioides* f. sp. *manihotis*

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) is the most important staple food for more than 300 million people in Africa, and anthracnose disease caused by *Colletotrichum gloeosporioides* f. sp. *manihotis* is the most destructive fungal disease affecting cassava production in sub-Saharan Africa. The main objective of this study was to improve anthracnose resistance in cassava through genetic engineering. Transgenic cassava plants harbouring rice thaumatin-like protein (*Ostlp*) gene, driven by the constitutive CaMV35S promoter, were generated using *Agrobacterium*-mediated transformation of friable embryogenic calli (FEC) of cultivar TMS 60444. Molecular analysis confirmed the presence, integration, copy number of the transgene all the independent transgenic events. Semi-quantitative RT-PCR confirmed high expression levels of *Ostlp* in six transgenic lines tested. The antifungal activity of the transgene against *Colletotrichum gloeosporioides* pathogen was evaluated using the leaves and stem cuttings bioassay. The results demonstrated significantly delayed disease development and reduced size of necrotic lesions in leaves and stem cuttings of all transgenic lines compared to the leaves and stem cuttingss of non-transgenic control plants. Therefore, constitutive overexpression of rice thaumatin-like protein in transgenic cassava confers enhanced tolerance to the fungal pathogen *C. gloeosporioides* f. sp. *manihotis*. These results can therefore serve as an initial step towards genetic engineering of farmer-preffered cassava cultivars for resistance to anthracnose disease.

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1. Introduction

More than 800 million people worldwide depend on cassava (*Manihot esculenta* Crantz) as a primary source of food [1]. It was initially used as a famine reserve crop but has recently emerged to be a profitable cash crop of industrial importance [2]. However, cassava productivity has been constrained by a number of biotic and abiotic factors that cause significant losses in storage root yield. Cassava anthracnose disease (CAD) caused by *Colletotrichum gloeosporioides* f. sp. *manihotis*, is one of the most destructive fungal diseases in the fields in sub-Saharan Africa and Asia [3,4]. Cassava anthracnose disease can reduce the amount of healthy planting materials and can cause total yield loss [5]. The disease is characterized by shoot tip-die-backs, cankers on stems and branches and leaf spots [6]. The infected stems become weak and break easily during strong winds [7].

The use of chemical fungicides for the control of anthracnose disease is not a viable long-term strategy because of the high cost and environmental impact [8]. Therefore, the development of anthracnose disease-resistance cassava cultivars would be the most economical, safe and effective management strategy to prevent losses caused by CAD. Resistance cassava cultivars can be generated through either conventional breeding or genetic engineering. Transfer of the resistance traits to farmer-preferred cassava cultivars by conventional breeding is hampered by the high heterozygosity, long vegetative growth cycle, low fertility and unsynchronized flowering [9]. Genetic engineering is an alternative and feasible approach to introduce resistance genes through *Agrobacterium*-mediated transformation without altering important agronomic traits of the cultivar [10].

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Plants are usually exposed to a variety of pathogens in the fields throughout their life cycle and they often respond by triggering complex defense mechanisms such as up-regulation of pathogenesis-related (PR) genes to counter pathogen attacks [11]. Thaumatin-like proteins (TLPs) that belong to PR-5 family is one of such genes that have been shown to be induced by pathogen attacks and environmental stress [12]. In addition, TLPs have been shown to exhibit antifungal property which includes inhibition of fungal enzymes (β-glucanase, xylanase, α-amylase and trypsin), ability to lyse fungal cell membrane and spores hence inhibition of fungal growth, reduce viability of germinated spores and induce programmed cell death in fungi [13]. These antifungal activities make TLPs suitable candidate genes for engineering fungal resistance in crop plants. Moreover, constitutive expression of plant TLPs has shown enhanced resistance in tobacco, wheat, rice and banana against various fungal pathogens [11,14–16]. In this study, rice TLP was transformed into cassava plants in order to evaluate the effect of its expression on resistance against the fungal pathogen *C. gloeosporioides* f. sp. *manihotis.*

2. Materials and methods

2.1. Plant material and production of friable embryogenic calli (FEC)

Clean stem cuttings of cassava cultivar TMS 60444 were collected from Kenya Agricultural and Livestock Research Organization (KALRO), Kakamega, Kenya (0° 17′ 1″ North, 34° 44′ 58″ East) and planted onto sterilized soil in plastic pots in the glasshouse at the Department of Biochemistry and Biotechnology, Kenyatta University. Tissue culture and generation of friable embryogenic calli (FEC) were carried out as described by Nyaboga et al. [17].

2.2. Isolation of rice thaumatin-like protein (Ostlp) and construction of binary vector

Expanded leaves of rice (*Oryza sativa*) cultivar Japonica were collected from Mwea Rice Irrigation Scheme fields in Kenya and used for isolation of *Ostlp* gene. RNA was extracted from 100 g of leaf tissue using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from DNAse treated RNA using RevertAid First Strand cDNA synthesis Kit (Thermo Scientific, USA). The full-length cDNA of *Ostlp* (Os12g0628600) was polymerase chain reaction (PCR) amplified using forward primer, 5′-CCATGGCGTCTCCGGCCACCTCTTCCGCT-3′ and reverse primer, 5′-CACGTGTTATGGGCAGAAGACGACTTGGTA-3′, containing *Nco*I and *Pml*I sites (underlined) at their respective 5′-ends. The PCR products were cloned into the pJET vector (Fermentas). The vector was digested with *Nco*I and *Pml*I and inserted into the pCAMBIA1305 vector (CAMBIA, Canberra, Australia) replacing the GUS gene. The hygromycin phosphotransferase gene (*hpt*) in pCAMBIA1305 was substituted with neomycin phosphotransferase (*npt*II) gene. The resulting recombinant PCam:*Ostlp* vector contains rice tlp and *npt*II genes driven by the CaMV35S promoter (Fig. 1). The pCam:*Ostlp* plasmid was transferred to *Escherichia coli* DH5α strains by heat shock method. Transformants were selected on LB agar plates containing 50 mg/l kanamycin and confirmed by restriction digestion with *Nco*I and *Pml*I and sequencing. The pCam:*Ostlp* was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Gene pulser ® II, Bio-Rad Laboratories Inc., Richmond, CA). The clones on the LB plate with kanamycin, rifampicin and streptomycin were confirmed by PCR with primers specific to full-length *Ostlp*. The *Agrobacterium* strain LBA4404 harbouring pCam:*Ostlp* was maintained on LB medium (supplemented with 50 mg/l rifampicin, 50 mg/l kanamycin and streptomycin 100 mg/l) and used for transformation experiments.

2.3. Preparation of Agrobacteria suspension culture for cassava transformation

Agrobacterium tumefaciens LBA4404 harbouring pCam:*Ostlp* was streaked on LB media containing 50 mg/l rifampicin, 100 mg/l streptomycin and 50 mg/l kanamycin and grown at 28 °C for 48 h. A colony was picked for inoculation in 3 ml LB liquid media containing 50 mg/l rifampicin, 100 mg/l streptomycin and 50 mg/l kanamycin for 48 h on a shaker, at 200 rpm and 28 °C. Any solids in the bacteria culture was allowed to settle and 0.25 ml of the starter culture was used to inoculate 25 ml of LB liquid medium containing antibiotics in 250 ml flasks and cultured overnight at 28 °C and agitated at 200 rpm. The next day, the optical density OD_{600} of the culture was analyzed using the Nanodrop spectrophotometer and was checked until the readings were between 0.8 and 1.0. The suspended cells were transferred into 50 ml falcon tubes and centrifuged at 5000 rpm for 10 min at 22 °C. The supernatant was decanted and the cells resuspended in 25 ml of liquid Gresshoff and Doy (GD) medium (pH 5.8) [18] using a 25 ml pipette and centrifuged at 5000 rpm for 5 min at 22 °C and the supernatant discarded. *Agrobacterium tumefaciens* LBA4404 was resuspended in GD (pH 5.8) and for the final OD_{600} was set at 0.5. The 50 ml tubes were set horizontally on a shaker at 50 rpm for 30 min. The suspension was used for transformation of cassava FEC.

2.4. Transformation of cassava FEC, selection and regeneration of putative transgenic lines

High quality FEC from 10 petri plates (approximately 50 mg of FEC per petri plate) were transferred into 50 ml falcon tubes containing 15 ml of *Agrobacteria* suspension and the mixture was left to stand for 30 min in laminar flow hood. Using a wide bore 10 ml pipette, the mixture of FEC and *Agrobacterium* suspension was transferred onto a sterile 100 μm plastic mesh on an empty petri dish. Each mesh containing FECs was blotted on a sterile paper towel and placed onto GD medium in petri dish. Co-cultivation of FEC and *Agrobacterium* on the GD media was done under bright light, 22 ± 1 °C for 3 days with alternating 16 h light/8 h dark. After co-cultivation, FECs were washed 4 times with 25 ml GD containing 500 mg/l carbenicillin in a 50 ml falcon tube then spread evenly onto a sterile 100 μm mesh and blotted dry on sterile paper towel. The mesh containing FECs was placed onto fresh GD petri plate supplemented with 250 mg/l carbenicillin and kept under 16 h

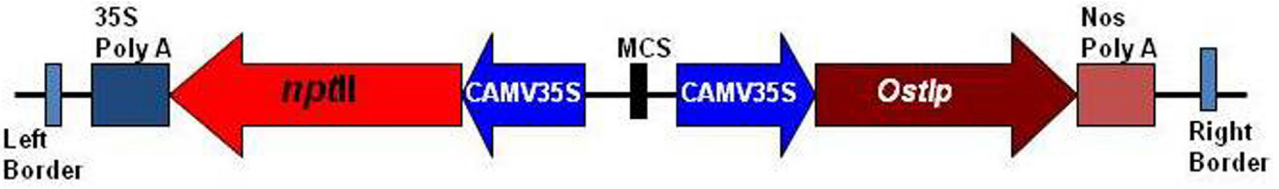


Fig. 1. Schematic representation of PCam:*Ostlp* plasmid used for cassava transformation.

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light/8 h dark at 28 °C for 4 days. The mesh was moved onto a fresh GD plate containing 250 mg/l carbenicillin and 30 mg/l paromomycin and kept at 28 °C under 16 h light/8 h dark for 7 days. The process was repeated two more times with subsequent increase of paromomycin concentration by 10 mg/l each time. The mesh was moved onto fresh MSN plate containing 250 mg/l carbenicillin, 50 mg/l paromomycin and kept at 28 °C under 16 h light/8 h dark for 10 days. The process was repeated twice or until green cotyledons appeared. The somatic embryos with developing green cotyledons were moved to shoot elongation medium (CEM) containing 100 mg/l carbenicillin. CEM medium containing 100 mg/l carbenicillin was refreshed every 10 days. After 3 weeks, the well-established initial shoots were transferred to tissue culture jars containing cassava basic medium (CBM) containing 50 mg/l carbenicillin. Well established shoots were subcultured onto fresh tissue culture jars containing CBM supplemented with 50 mg/l carbenicillin.

2.5. Polymerase chai reaction (PCR) analysis

For PCR analyses, total genomic DNA was isolated from 100 mg of *in vitro* leaves of transgenic and non-transformed control plants using DNeasy® plant mini kit (Qiagen, GmbH, Germany). The presence of transgene was confirmed by PCR amplification using gene-specific forward primer 5′-GGGTGGAGAGGCTATTCGGCTATGA-3′ and reverse primer 5′-ATTCGGCAAGCAGGCATCGC-3′, designed to amplify 542 bp of *npt*II gene. Plasmid DNA was used as a positive control and non-transformed plant genomic DNA as a negative control. PCR reactions were carried out in 25 μl reaction mixture containing 1 × PCR buffer containing 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of each primer, 0.5 U of HotStarTaq DNA polymerase (Qiagen, GmbH, Germany) and 100 ng of template DNA. The PCR conditions were initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 40 s, and extension at 72 °C for 50 s; and a final 10 min extension at 72 °C. The amplified PCR products were resolved by electrophoresis on 1% agarose gel stained with GelRed™ (Biotium) and visualized under UV light.

2.6. Southern blot analysis

Southern blot analysis was done to confirm integration of transgenes into the cassava genome and to determine copy number of transgene according to DIG DNA Labeling and Detection Kit (Roche Applied Sciences, Mannheim, Germany) following manufacturer's instructions. Genomic DNA was extracted from leaves of *in vitro* grown plants using cetyltrimethylammonium bromide (CTAB) method as described by Soni and Murray [19]. A total of 20 μg genomic DNA was digested overnight at 37 °C with *Pml*I (New England Biolabs, USA) for which there is a single recognition site in the T-DNA of pCam:*Ostlp*, separated by electrophoresis on 1.0% (w/v) agarose gel followed by depurination, denaturation and neutralization steps and transferred to a positively charged nylon membrane (Roche Applied Sciences, Mannheim, Germany) by capillary transfer. After cross-linking, the blots were hybridized with DIG-labeled *npt*II-specific probe generated using a PCR DIG Probe Synthesis Kit (Roche Applied Sciences, Mannheim, Germany). Hybridization and detection were performed using a DIG Luminescent Detection Kit for Nucleic Acids (Roche Applied Sciences, Mannheim, Germany), following the instructions of the manufacturer.

2.7. Expression analysis of Ostlp gene by semi-quantitative RT-PCR

Semi-quantitative reverse transcriptase (RT)-PCR was used to detect the presence of *Ostlp* mRNA transcripts in transgenic plants. Total RNA was prepared from approximately 100 g leaf tissues of transgenic and non-transgenic control plants using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Extracted RNA was treated with DNaseI (Promega) prior to RT-PCR. RT-PCR was carried out using one step RT-PCR kit with Hotstar-*Taq* DNA polymerase (Qiagen, Hilden, Germany) and *Ostlp* gene-specific primers (forward primer 5′-CGTCTCCGGCCACCTCTTCCGCT-3′ and reverse primer 5′-TTATGGGCAGAAGACGACTTGGTA-3′, amplifying 534 bp of *Ostlp* gene). The RT-PCR was performed under the following conditions; incubation at 55 °C, 30 min for cDNA synthesis, 2 min at 95 °C for denaturation, followed by PCR amplification with the subsequent conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 40 s; and final extension for 5 min at 72 °C. To confirm the quality of synthesized cDNA, amplification of Protein phosphatase 2A (*PP2A*) was performed as described by Nyaboga et al. [20]. The amplification products were visualized by electrophoresis on a 1% (w/v) agarose gels and photographed by using gel documentation system (Syngene, USA).

2.8. Acclimatization of transgenic plants in the glasshouse

Regenerated plants were multiplied and established in CBM medium and after 5 weeks the rooted plantlets were removed from the tissue culture jars and washed gently with sterilized distilled water to remove any adherent media. The plants were transferred to plastic pots (10 cm diameter) containing sterile forest soil. Pots were covered with transparent polythene bags and placed in a glasshouse at 28 °C. Seven days later, the polythene bags were cut open at the corners to prevent fungal growth. After 2 weeks the polythene bags were removed completely to acclimatize the plants. The plants were used for evaluation of tolerance to *C. Gloeosporioides*.

2.9. Fungal collection, isolation and identification of C. Gloeosporioides

Stem cuttings of cassava cultivar TMS 60,444 showing anthracnose symptoms (sunken lesions or cankers) were collected from the field at Kenya Agricultural and Livestock Research organization (KALRO) Kakamega, Kenya. The infected materials were washed in running water to remove soil particles and other debris. Small sections (3–5 mm in size) were cut from the infection sites, surface-disinfected in 2% sodium hypochlorite solution for 3 min and rinsed in three successive changes of sterile distilled water, blot dried on sterile paper towel in a laminar air flow. The dried stem sections were placed on Potato Dextrose Agar (PDA) supplemented with 100 mg/l sodium novobiocin to inhibit bacterial growth. The inoculated dishes were incubated at 25 °C under alternate 12 h light and 12 h dark regimes daily for 7 days and observed for fungal growth. Identification of the *C. gloeosporioides* was performed by colony characteristics and microscopic observation of spores and confirmed by the procedure described by Barnett and Hunter [21]. The fungal samples identified as *C. gloeosporioides* f. sp. *manihotis* were maintained on PDA plates and used for inoculation of transgenic plants.

2.10. Preparation of spore suspension of C. Gloeosporioides

Spore suspensions were prepared from 8-day-old *C. gloeosporioides* f. sp. *manihotis*. Each Petri plate of the isolate was flooded with 5 ml of sterile distilled water and the acerruli dislodged with a small brush in order to release conidia into the water. The suspension was centrifuged at 2000 rpm for 3 min and the supernatant filtered through sterile muslin cheesecloth to remove the remaining mycelia fragments. The spore concentration was measured with a haemocytometer as described by Duncan and

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Torrance [22] and adjusted the concentration to 3.5×10^6 spores/ml of distilled water.

2.11. Inoculation of Ostlp transgenic cassava lines with C. Gloeosporioides

Two months-old cassava stems and fully expanded leaves of transgenic and non-transgenic control plants established in the greenhouse were used. Five leaves and 5 stem cuttings from each transgenic line and non-transgenic TMS 60444 were placed in sterile Petri dishes containing two layers of sterile Whatman filter papers soaked in 2.5 ml sterile distilled water. Four areas of 1.5 mm^2 of the upper part of each leaf and stem cuttings were selected for inoculation. Each selected area was wounded using insect pin needle and single drops of 10 μl of inoculum were placed on wounded spots. Both wounding and inoculation were carried out under a magnifying lens for good visibility. The plates containing inoculated stem cuttings and leaves were incubated at 25 ± 2 °C and 85% relative humidity in a 12:12 h, light-dark cycle under fluorescent light for 10 days. Both transgenic and non-transgenic plants were inoculated with distilled water as controls. This experiment was repeated three times.

The sizes of necrotic lesions of infected areas were measured after 10 days of incubation and the level of tolerance was based on a lesion diameter size as described by Goodie [23] and Sudi and Podhardizky [24], where <7.0 mm = highly resistant, 7.1–11 mm = resistant, 11.1–15.0 mm = moderately susceptible, >15.1 m m = susceptible. The area of necrosis was measured using Scion Image for Windows (version beta 4.0.2, Scion Corporation). The average necrotic size was calculated and the mean necrotic diameter of the lesion per transgenic line was found by t-test.

3. Results

3.1. Transformation and regeneration of transgenic cassava plants

Cassava cultivar TMS 60444 was used for generation of transgenic plant lines. *Agrobacterium*-mediated transformation of pCam:*Ostlp* plasmid into 60 friable embryogenic calli (FEC) resulted in proliferation of small clusters of pale yellow coloured calli on GD medium (Fig. 2A) and started forming somatic and cotyledonary-stage embryos on MSN media containing paromomycin for selection (Fig. 2B). A total of 31 putatively transformed cotyledons (Fig. 2C) were obtained and 16 developed shoots upon transfer to shoot elongation medium. Out of the 16 formed shoots, 14 putative transgenic cassava lines were selected based on their ability to grow and root on paromomycin supplemented CBM medium (Fig. 2D). Non-transformed control plants failed to grow on the same medium. Transgenic cassava lines were referred as *Ostlp*, and each number represented an independent transgenic line. The rooted plants were transferred to the glasshouse and no phenotypic change was observed during growth of plants.

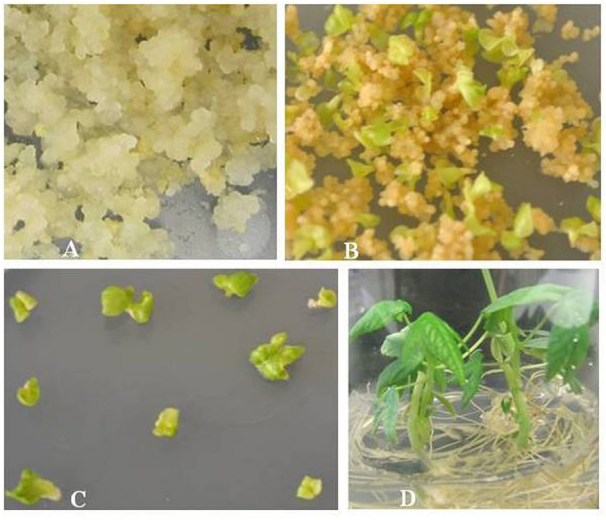


Fig. 2. Production of transgenic cassava plants expressing rice tlp gene. (A) *Agrobacterium*-infected FEC proliferating on GD medium supplemented with paromomycin, (B) Developing somatic and cotyledonary-stage embryos on embryo induction medium containing paromomycin, (C) Maturing and green cotyledonary-stage embryos on shoot induction medium, (D) Regenerated transgenic plants on CBM selective medium.

3.2. Molecular characterization of transgenic lines

Genomic DNA was isolated from all the 14 transformants for PCR analysis to confirm the true transgeni lines using primers specific for *npt*II gene. PCR analysis showed amplification of the expected 542 bp fragment of *npt*II gene in all the 14 putative transgenic lines, similar to the pCam:*Ostlp* plasmid control (Fig. 3A). No amplified PCR product was detected in non-transgenic control plants. Southern blot analysis was performed to confirm the integration and copy number of the transgene in transgenic plants. Southern blot analysis revealed that one to three copies of the transgene were integrated into the genome of all 14 transgenic cassava lines. Eight out of the 14 transgenic lines showed single copy number of the transgene (Fig. 3B). No transgene insertion was detected in non-transformed control plants. RT-PCR analysis showed the expression of rice tlp transcripts in leaves of all tested transgenic lines (Fig. 3C) and no amplification was observed from the cDNA of non-transgenic control plants. Uniform expression of PP2A rRNA was recorded in non-transgenic control and all the rice tlp transgenic lines (Fig. 3C, lower panel). This indicates that *Ostlp* (transgene) was expressed in transgenic lines.

3.3. Identification of C. Gloeosporioides f. sp. Manihotis causing anthracnose disease

Stem cuttings of cassava samples from field grown plants showing symptoms of anthracnose disease (Fig. 4A) produced different types of colonies of fungi upon culture on PDA. Morphological characterization of the colonies cultured on PDA plates identified a colony showing white cotton-like mycelia with a greyish white to dark-grey colour (Fig. 4B). Microscopic observations identified spores with rounded ends and hyphal morphology (Fig. 4C), a characteristic of *C. gloeosporioides* f. sp. *manihotis*. The isolate was maintained on PDA agar and used for screening of *Ostlp* transgenic lines for resistance to anthracnose disease.

3.4. Protection of Ostlp transgenic lines against anthracnose disease

Transgenic cassava lines (*Ostlp*-5, *Ostlp*-9 and *Ostlp*-12) with a single copy number of the transgene as confirmed by southern analysis and different levels of *Ostlp* transcripts by semi-quantitative RT-PCR were selected for evaluation of resistance to anthracnose disease. Leaf and stem bioassays revealed severe necrotic disease symptoms on the non-transgenic plants than in the *Ostlp* transgenic lines at 10 days of inoculation (Fig. 5). The non-transgenic plants showed necrotic symptoms after 2 days of inoculation, while the symptoms were observed in the transgenic lines after 6 days of inoculation. No infection was observed in any of the non-inoculated and mock inoculated leaves and stem cuttings of non-transgenic plants and *Ostlp* transgenic lines as shown in Fig. 5. The non-transgenic leaves and stem cuttings had

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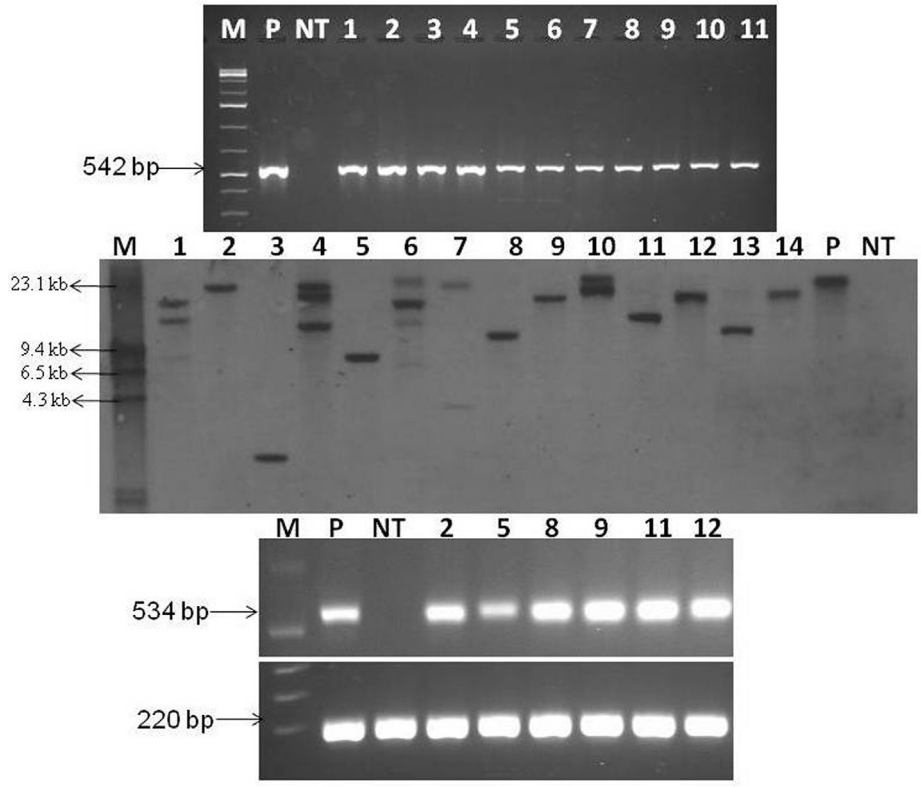


Fig. 3. Confirmation of putative transgenic plants by PCR, southern hybridization and semi-quantitative RT-PCR. (A) PCR analysis of putative transgenic lines using *npt*II gene-specific primers. (B) Southern blot analysis of *Ostlp* gene in transgenic and untransformed control cassava plants. Genomic DNA was digested with *Pml*I, and hybridized with a DIG-labeled fragment containing the *npt*II gene as the probe. (C) Semi-quantitative RT-PCR analysis of *Ostlp* mRNA transcript in transgenic plants leaves. Lane M: DNA molecular marker; Lane P: pCam:*Ostlp* plasmid DNA; Lanes 1–11: *Ostlp*-1 to *Ostlp*-14 transgenic lines; Lane NT: non-transgenic control plant.

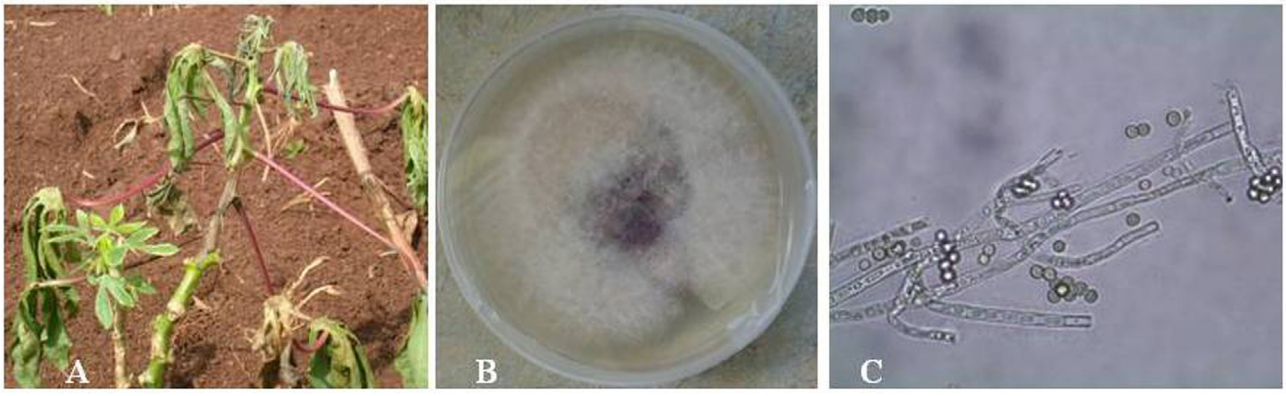


Fig. 4. (A) Cassava plant infected with anthracnose disease in the field; (B) A colony of *C. gloeosporioides* f. sp. *manihotis* isolated from infected anthracnose infected plant on PDA; and (C) Spore and hyphae morphology as observed under the light microscope (40×).

15.56 ± 0.72 and 16.16 ± 0.94 diameter of necrotic lesions, respectively, while the transgenic lines had between 8.88 ± 0.76–10.36 ± 0.67 and 8.64 ± 0.73 – 10.3 ± 0.82 necrotic lesion sizes for the leaves and stem cuttings, respectively (Fig. 6). As compared to the non-transgenic plants, the transgenic lines (*Ostlp*-5, *Ostlp*-8 and *Ostlp*-12) showed delayed disease development and slow spreading of the necrotic lesions around the infection site (Fig. 6).

4. Discussion

In this study, we generated transgenic cassava plants containing and expressing the *Ostlp* gene from rice. This is one of the most important pathogenesis-related (PR) proteins that are induced in plants in response to pathogens, stress, developmental signals and can be utilized to protect plants against fungi infections [25]. Accumulations of PR proteins in plants have been shown to induce systemic-acquired resistance that enables plants to survive against a broad range of pathogens [26]. In the present study, overexpression of rice thaumatin-like protein in cassava provides further evidence of its role in conferring tolerance against fungal pathogens.

Molecular analysis involving PCR and southern hybridization indicated the presence and integration of the T-DNA into cassava genome. The variable hybridization pattern among transgenic lines observed in southern blot indicates that the lines originated from

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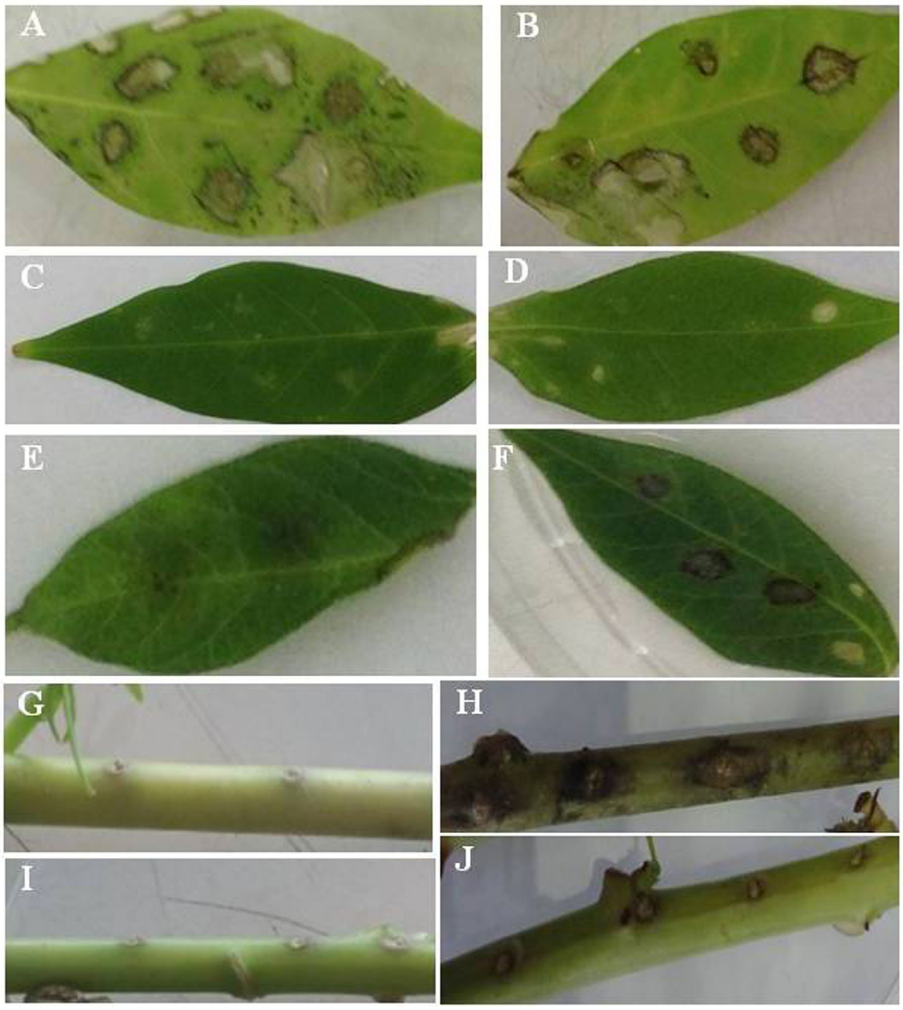


Fig. 5. Representative necrotic lesions caused by *Colletotrichum gloeosporioides* f. sp. *manihotis* inoculation on leaves and stem cuttings of transgenic and non-transgenic control plants. Non-transgenic (A, B and H) and *Ostlp*-5 (E, J) and *Ostlp*-8 (F) transgenic leaves and stem cuttings after 10 dpi with *C. gloeosporioides*; Transgenic (C, I) and non-transgenic (D, G) leaves and stem cuttings after 10 days of inoculation with sterile distilled water.

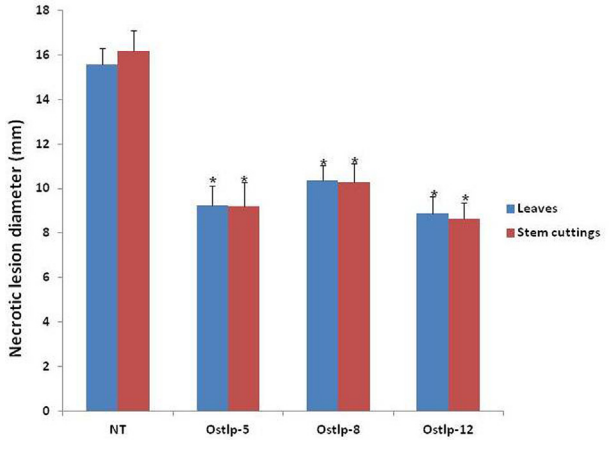


Fig. 6. Size of necrotic lesions of the *C. gloeosporioides*–infected leaf and stem area at 10 days post-inoculation in the transgenic lines (*Ostlp*-5, *Ostlp*-8 and *Ostlp*-12) and non-transgenic plants (NT). Bars represent standard deviation calculated from 5 biological replicates. Asterisks indicate that in comparison with non-transgenic (NT) plants, the infected areas of transgenic lines were significantly different ($P < 0.05$).

independent transformation events. In addition, Southern blot analysis of the six independent lines obtained in this study indicated that nine out of fourteen lines (64.29%) had integrated a single copy of the transgene, and the other four lines had low copy numbers (2, 3 or 4 copies) of the transgene. It has been shown that transgene expression is influenced by the integration site of the transgene [27]. Even if inactivation of transgene expression can occur in plants with a single copy [28], this phenomenon is less frequent than with multiple transgene copies [29]. The expression of *Ostlp* gene in 6 transgenic lines containing a single copy of transgene showed constitutively high levels of expression and varying degrees of expression among the lines possibly due to positional effect. No phenotypic or physiologic abnormality was observed in the transgenic lines. The level of tolerance of transgenic lines following *C. gloeosporioides* infection was tested using 3 lines which showed varying levels of *Ostlp* transcripts.

All the three transgenic lines (*Ostlp*-5, *Ostlp*-8 and *Ostlp*-12) expressing rice tlp gene exhibited increased tolerance to the fungal pathogen *C. gloeosporioides* of cassava. In comparison with the non-transgenic control plants, the leaves and stem cuttings of transgenic lines showed significantly reduced size of necrotic lesions and limited spread of the symptoms, indicating the pathogen

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was inhibited and ceased to develop to other healthy leaf and stem areas due to *Ostlp* accumulation. Delayed development of necrotic symptoms was also observed in all the three transgenic lines expressing rice tlp, compared to the non-transgenic plants. Results from both leaves and stem cuttings bioassay established the anti-fungal role of *Ostlp* toward hemibiotrophic fungus *C. gloeosporioides* that cause Anthracnose in cassava. The protein transcribed by the *Ostlp* gene probably hindered fungal growth due to the degradation of structural components of fungi by thaumatin-like proteins [30]. Thaumati-like proteins have also been reported to have a role in strengthening plant cell wall [31] and probably resist entrance and penetration of fungus into the cell.

This study has illustrated that constitutive expression of rice tlp gene in cassava results to enhanced resistance to Anthracnose disease. Our results are supported by previous studies, where other transgenic crops with a rice tlp gene were reported to have improved resistance to fungal diseases. Transgenic rice with rice tlp gene showed increased resistance to *Rhizoctonia solani*, the rice sheath blight pathogen [11]. Also, transgenic tobacco with rice tlp showed enhanced resistance against *Alternaria alternata* [32]. In addition, Mahdavi et al. [16] developed transgenic banana with *Oryza sativum* thaumatin-like protein gene and demonstrated increased disease resistance to *Fusarium* wilt caused by the fungal pathogen *Fusarium oxysporum* sp. *cubense* (race 4). Therefore, rice thaumatin-like protein gene is important in imparting enhanced resistance against a broad range of fungal pathogens in transgenic crops.

5. Conclusion

Our data suggest that rice tlp is a promising candidate gene for the engineering of cassava plants to confer resistance against Anthracnose disease. Transgenic cassava plants expressing rice tlp gene have a significant potential to control Anthracnose disease. Future plans involve testing of all transgenic cassava cultivar TMS 60444 lines in the glasshouse for confirmation of enhanced disease tolerance. These transgenic lines may be used as additional sources of disease resistance for cassava breeding programs.

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