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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.

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

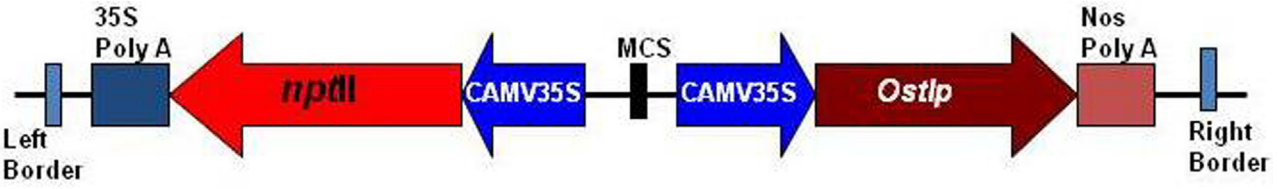

Fig. 1. Schematic representation of PCam:*Ostlp* plasmid used for cassava transformation.

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