Expression of C-terminal Prodomain Truncated *Petunia* Floral Defensins Inhibit the Growth of Transgenic Banana Plants

Siddhesh B Ghag, Upendra K Singh Shekhawat and Thumballi R Ganapathi*
Plant Cell Culture Technology Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400 085, India.
*For Correspondence - trgana@barc.gov.in

Abstract

Plant defensins are basic proteins synthesized as part of innate immune response against the attack of fungal pathogens. These defensins show strong antimicrobial activity which is specifically directed towards the invading pathogen. Diverse defensins have been overexpressed in model transgenic plants to show their efficacy in heterologous systems. There are more than 350 plant defensins known today which have occurred due to convergent and divergent evolution over time. Although most of these peptides generally do not react with the host plant cells, some may have deleterious effect on plant organs. As a consequence plants build up advance strategy to nullify the effect by targeting the peptides to vacuoles, preventing its expression in sensitive organs and changing the amino acids composition. The C-terminal propeptide (CTPP) domains probably prevented any lethal effects of plant defensins while simultaneously enabling vacuolar sorting. In order to study the role of CTPP of two *Petunia* floral defensins (*PhDef1-T* and *PhDef2-T*), banana embryogenic cells were transformed with *PhDef1-T* and *PhDef2-T* without the CTPP domain region. The results obtained from this study clearly demonstrated that the absence of CTPP in *Petunia* floral defensins caused significant reduction in the number of embryos and its poor growth. Regenerated shoots were considerably slow in growth signifying the relevance and the importance of the prodomain region in floral defensins.

Key words: Floral defensins, *Petunia*, banana, CTPP domains

Introduction

Plant defensins are low molecular weight antimicrobial peptides having 45-54 amino acid active domains. These cysteine-stabilized \( \alpha\beta \) (CS\( \alpha\beta \)) structures mainly act as effective membrane destabilizers (1) and protein inhibitors (2). Potent activity of these defensins, derived mainly from seeds and floral tissues, against a wide array of fungal pathogens has been described in the past (3). Further, expression of some of these defensins in model transgenic plants has resulted in efficient resistance towards fungal infection. Although their potency against fungal pathogens is more or less established, their interactions with the target and the host organisms are not clear in most cases. Floral defensins from *Petunia* and *Nicotiana* possess strong antifungal activity and a highly stabilized structure having five intramolecular disulphide linkages (4). These defensins are synthesized as inactive proproteins having a 27-33 amino acids C-terminal prodomain which is postulated to be responsible for neutralizing the cationic defensin domain and possibly also for vacuolar targeting. The C-terminal prodomains of these defensins are rich in acidic and hydrophobic amino acids giving them a net negative charge.
at neutral pH. This helps in avoiding any disturbance in the optimum plant cell milieu during synthesis and translocation of these defensins. Once the defensins reach their site of storage (intracellular vacuole), the C-terminal prodomain region is cleaved off by specific vacuolar proteases.

Recent report demonstrated that high level expression of full-length Petunia floral defensins (PhDef1 and PhDef2) in banana cultivar Rasthali provides resistance to pathogenic Foc race 1 (5). Apart from having defined signal peptides and defensin domains, these defensins are unique in having C-terminal prodomain regions which probably also take part in intracellular sorting. Earlier reports demonstrated the role of plant defensins in growth and development of the host plant (6, 7). In the present study we demonstrated that transformed banana cells overexpressing Petunia floral defensins (PhDef1-T and PhDef2-T) showed reduced embryo formation and regeneration pointing towards the function in growth and development of plants. The shoots so obtained showed stunted growth and were unable to form roots. These results comprehend the role of CTPP in plant defensins that are produced in plants during pathogen attack.

Materials and Methods

**Isolation and cloning of defensin genes:** Total RNA was extracted from Petunia hybrida flowers using Concert Plant RNA Reagent (Invitrogen, USA) and was purified using RNeasy Plant Mini Kit (Qiagen, Germany). This RNA (approx. 5 µg) was then used to make first strand cDNA using Oligo (dT)12–18 primer (Invitrogen, USA) and AccuScript Reverse Transcriptase (Stratagene, USA) according to manufacturer’s instructions. The C-terminal truncated versions of the two defensins PhDef1-T and PhDef2-T were amplified from the flower derived cDNA using the following primers (from 5’ to 3’): PhDef1-T Fw: GATCCGCAGAGATGCTGCTCCATCTGTTTC, Rv: ACTGGTACCTAA CACTCTTTAGTGCACAGACATCTTC; PhDef2-T Fw GATCCGTG CAGGATGGCTGCTCCATCTGTTTC, Rv: ACTGGTACCTAA CACTCTTTAGTGCACAGACATCTTC. Thermal cycling conditions set for amplification were 94°C for 5 min followed by 30 cycles each with 94°C for 1min, 55°C for 1min and 72°C for 1min with a final extension of 72°C for 10min. The amplified products so obtained were gel purified and cloned into cloning vector pTZ57R/T (Invitrogen) and sequenced.

**Construction of plant expression binary vectors:** PhDef1-T and PhDef2-T coding sequence were digested from pTZ57R/T vector using PstI and KpnI and inserted into the multiple cloning site of pCAMBIA-1301 binary vector (digested with HindIII and KpnI) having nos 3’UTR along with Zea mays polyubiquitin promoter (digested with HindIII and PstI) in a three-way ligation reaction to form pPhDef1-T-1301 and pPhDef2-T-1301 respectively. These binary vectors were subsequently sequenced. pPhDef1-T-1301 and pPhDef2-T-1301 were mobilized into Agrobacterium tumefaciens strain EHA 105 by electroporation.

**Generation of transgenic banana plants:** Banana cv. Rasthali embryogenic cell suspension cultures were transformed by the newly constructed binary vectors as described earlier (8). The transformed cells were cocultured in M2 medium for 3 days and later on banana embryogenic medium for 9 weeks with repeated subculture after every 3 weeks in presence of hygromycin (5mg/L) as selective agent. Putatively transformed embryos were germinated in presence of low concentration of BAP (0.5 mg/L).

**Histochemical GUS assay:** Putatively transformed in vitro leaves were injured with forcep and incubated in GUS buffer overnight at 37°C. The leaves were transferred to 70% ethanol to remove the chlorophyll for easy visualization of GUS staining.

**Detection of transgenic nature by PCR:** Genomic DNA was isolated from in vitro developed banana leaves using GenElute Plant Genomic DNA Miniprep Kit (Sigma, USA). Polymerase chain reaction was carried out using above mentioned primers and amplification...
Current Trends in Biotechnology and Pharmacy
Vol. 7 (1) 505-510 January 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Results

Petunia hybrida defensins namely PhDef1-T and PhDef2-T were successfully amplified from the cDNA of floral tissues. After cloning PhDef1-T and PhDef2-T into pTZ57R/T vector it was sequenced. The sequence of both the amplified products were translated into the protein sequence using online ExPASy translate tool (http://au.expasy.org/tools/dna.html). 24 amino acids signal peptide was identified in both the defensins using online SignalP software (http://www.cbs.dtu.dk/services/SignalP). Active defensin domain in PhDef1-T is of 47 amino acids whereas in PhDef2-T it is 49 amino acids long which undergo characteristic folding to form the CSαβ structure. These PCR amplified products were cloned into pCAMBIA 1301 binary vector as an expression cassette driven by Zea mays polyubiquitin promoter and again sequenced to determine the orientation of the insert between the promoter and the nos 3'UTR. Fresh embryogenic cell suspension cultures were used for Agrobacterium-mediated transformation. Embryogenic cells transformed with pPhDef1-T-1301 and pPhDef2-T-1301 binary vectors (Fig. 1 A) were significantly slow in their growth in the embryo induction and development medium (Fig. 1 B, C). Additionally, the number of putatively transformed embryos which grew on selection medium was less than 20 % of pPhDef1-1301 and pPhDef2-1301 derived embryos (full length defensin constructs described earlier in 5). Owing to their stunted and slower growth, very few transformed shoots could be obtained for these two truncated defensin constructs (Fig. 1D, E). These shoots further failed to produce profuse root system on medium supplemented with NAA. As a result the shoots obtained could not be hardened in the green house. This observation reveals the lethality of overexpressing floral defensins without the CTPP domains. The total number of embryos and shoots which could be recovered after selection in hygromycin medium was less. To confirm the transgenic nature of these shoots, genomic DNA PCR was carried out using primers specific for PhDef1-T and PhDef2-T. Single amplified product derived from PhDef1-T and PhDef2-T coding sequence (~250bp) was obtained in transgenic banana plants overexpressing PhDef1-T and PhDef2-T (Fig.1F). These products were absent in untransformed control plants. The leaf tissue from in vitro shoots was incubated in GUS buffer at 37°C overnight in order to determine the expression of T-DNA in banana leaves. Transformed leaf tissues showed intense blue coloration indicating stable integration and expression of the T-DNA in the transgenic banana shoots (Fig. 1 G, H). Two predicted roles such as vacuolar sorting and transportation of the unique C-terminal prodomains of these defensins can be directly correlated with the observed growth inhibition.

Discussion

Plant floral defensins are stable, cationic peptides produced in flowers to prevent the possibility of fungal attack (9). Plant defensins are secreted during stress conditions such as salt, drought or cold and pathogen attack describing its role in growth, development and defense (10). Several defensin genes have been used to transform economically important crops such as rice (11), banana (5), and potato (12) to provide broad spectrum resistance against phytopathogens. Full length Petunia floral defensins (PhDef1 and PhDef2) when constitutively expressed in banana plants cv. Rasthali it imparted resistance to pathogenic strain of Fusarium oxysporum f. sp. cubense race 1 without affecting the morphology of the transgenic banana plants (5). Interestingly when we constitutively overexpressed the same floral defensins (PhDef1-T and PhDef2-T) without the CTPP in banana cv. Rasthali driven by same Zea mays polyubiquitin promoter there was drastic reduction in number of embryos formed (data not shown) and the ones that were formed showed reduced capability of regeneration. The shoots obtained by germinating the embryo did not undergo profuse multiple shoot development in Inhibition of Growth of Transgenic Banana
Fig. 1. Genetic transformation of banana cv. Rasthali with truncated Petunia defensin constructs and analysis of transformants. (A) T-DNA region of pPhDef1-T-1301/ pPhDef2-T-1301 binary vector wherein C-terminal truncated PhDef1 and PhDef2 coding sequences (without the prodomain) were cloned downstream of Zea mays polyubiquitin promoter and upstream of nos (nopaline synthase) 3' UTR in MCS of pCAMBIA-1301 vector. (B) and (C) Somatic embryos derived from pPhDef1-T-1301/ pPhDef2-T-1301 transformed cells on embryo induction medium 10 weeks post cocultivation. Note the significant browning as well as reduction in number of fresh embryos. (D) and (E) Multiple shoots derived from pPhDef1-T-1301/ pPhDef2-T-1301 transformed cells on shoot induction medium 20 weeks post cocultivation. Growth and multiplication of shoots was significantly slower than those derived from untransformed controls or from pPhDef1-1301 and pPhDef2-1301 transformed cells. (F) Genomic DNA PCR of two selected lines each derived from pPhDef1-T-1301(DT1, DT2), pPhDef2-T-1301(DT1, DT2) and untransformed control (UC) plants (Lane 1 and 5: 1 kb DNA ladder). (G) and (H) histochemical GUS staining of pPhDef1-T-1301/ pPhDef2-T-1301 transformed banana leaves.

Siddhesh et al
medium supplemented with BAP (2 mg/L). In order to know whether these shoots showed root development we transferred them onto the rooting medium. These shoots were unable to form the root system. This study finds that the absence of CTPP domains in both the defensins may have caused severe retardation in growth and development. To further confirm the transgenic nature of the putatively transformed banana shoots genomic DNA PCR was carried out using primers specific to PhDef1-T and PhDef2-T coding sequence. Transformed leaves overexpressing PhDef1-T and PhDef2-T showed positive GUS staining demonstrating that the T-DNA has been successfully integrated in the euchromatin region of banana genome and is expressing the GUS transcript to show the characteristic blue coloration. This clearly indicates that the coding sequence of PhDef1-T and PhDef2-T without the CTPP domain is also expressed in the transgenic banana shoots. Since the transgenic banana shoots overexpressing PhDef1-T and PhDef2-T without CTPP showed abnormal phenotype and poor growth and development explains the importance of CTPP in such potent defense molecules. Many of the plant defensins inhibit fungal hyphal tip growth by disrupting the cytosolic calcium gradients. Root hair development in plants and hyphal tip growth in fungus is thought to be similar phenomenon involving calcium channels; as a result those defensins which target the fungal hyphal tip may attack the growing root hairs of the host plants explaining its absence in *Medicago* roots. *Arabidopsis* seeds when treated with MsDef1, MtDef2, RsAFP2 and KP4 showed root growth inhibition at lower concentrations but later on removal of these peptides resulted in reversal of the phenotype (7). The results presented here clearly demonstrate that plant defensins not only help in antimicrobiosis but also play role in plant growth and signalling. Detailed investigation is needed so as to understand the interactions of these floral defensins with fungal counterparts. There remains much scope to study the mechanism of action, molecular interactions and regulation of these prodomains in plant defensins. Further these prodomains can be tagged to potent broad spectrum antimicrobials to obtain sustainable resistance for wide array of phytopathogens.

**Acknowledgement**

Authors wish to thank Dr. S F D’Souza, Head, Nuclear Agriculture and Biotechnology Division, BARC for his constant support.

Inhibition of Growth of Transgenic Banana
References

Siddhesh et al