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Expression of β-*defensin* Gene in Potato Confers Enhanced Resistance to *Ralstonia Solanacearum* L

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ABSTRACT

An optimised methodology of *Agrobacterium*-mediated stable genetic transformation of potato (*Solanum tuberosum* L.) using the shoot organogenesis potential of internodal stem segments for increased resistance to bacterial plant pathogen, *Ralstonia solanacearum* L. was developed. Improvised plant regeneration protocol for expression of antimicrobial *β-defensin* transgene and efficient selection of tissues in plant selectable marker, kanamycin sulphate was successfully utilised for transformation of potato. Stable integration and expression of antimicrobial peptide was observed in leaves and tubers. The results were validated by associated molecular analysis by RT PCR, Southern hybridisation, northern hybridisation and western blotting. The bacterial wilt disease progression was monitored in controlled greenhouse and Percent Disease Index (PDI) was measured by analysis of variance (ANOVA) that selected superior resistant plants. These transformed plants were able to contain the disease progression and complete the life cycle stages and developed healthy tubers.

Keywords: Antimicrobial peptide; *Agrobacterium tumefaciens*; β-*defensin;* Kanamycin sulphate; *Solanum tuberosum*; Transformation

NOMENCLATURE

MS	Murashige Skoog
LB	Luria bertani
YEM	Yeast extract mannitol
RM	Regeneration medium
GA	Gibberellic acid
IAA	Indole acetic acid
NAA	α Napthalene acetic acid
CPG	Casamino acid peptone glucose
AMPs	Anti-microbial peptides
npt II	neomycin phospho transferase
PBS	Phosphate buffer saline
HR	Hypersensitive resistance

1. INTRODUCTION

The potato crop loss from *Ralstonia Solanacearum* L. is estimated to be in excess of $ 950 M[1]. *Ralstonia Solanacearum* L. (phylotype II, sequevar I) is the most destructive bacterial pathogen of family solanaceae and is responsible for suboptimal yield of potato in Africa, Asia and Latin America[2-4]. Inheritance studies in solanaceous crops against pytopathogenic bacterial disease outlined the role of root cortical cells and phenolic content as primarily responsible for restricting the multiplication of bacteria[5]. The crop management strategies and use of healthy tubers are preventive measures; but limiting for this endemic disease. Moreover these methods are required to be replenished in the field and address to long-term strategy is not economically sustainable. Breeding for host resistance in potato are being attempted[2] however; adequate resistance in the field is still a concern[6-7]. Preventive methods including crop rotation, soil fumigation are inadequate to control the disease as bacterium persists in the soil for indeterminate period. Application of chemical elicitor on moderately resistant tomato genotypes are reported to induce wilt resistance[8]; chemicals and natural plant extracts/oils (jasmonic acid, coconut milk, mango malformed inflorescence extract, and ethyl salicyclic acid) are reported to reduce wilt severity[9]. Resistant crops developed through breeding have been successful against bacterial wilt in Africa, Latin America and some parts of Asia[10-6]. In potato, bacterial wilt resistance is derived from native *Solanum phureja* which is however unstable in environmental flux suggesting interaction between host plant-pathogen-environment[11]. Host plant resistance to bacterial wilt has been extensively studied[12-13] and screening for host resistance sources has enabled identification of promising clones with moderate resistance[14]. Identification of resistance within potato accessions including tuberosum and andigena clones have been unsuccessful[15].

Recently successful transfer of wilt resistance genes from interspecific symmetric protoplast fusion of somatic hybrids developed from eggplant and cultivated potato have been reported[16] but the stability of derived resistance in field and at different temperatures remain uncertain[17]. Concurrent to breeding strategies, advances in biotechnological approaches for increased resistance against pest and pathogens in cultivated crops offer viable options. Crop fortification to biotic and abiotic stresses through the application of genetic transformation procedures have been successfully demonstrated

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in important food crops[18]. *Agrobacterium*-mediated bacterial transformation system offers several advantages over the direct DNA delivery system such as transfer of fewer transgene copies at higher efficiencies, ability to transfer and integrate large DNA fragments into plant genome components with minimum rearrangements. Moreover, the method offers minimum explant manipulation and coupled with cost-effectiveness of the procedure has resulted in its wider applicability. The potentials of shoot organogenesis culture for efficient transfer of β-*defensin*, antimicrobial transgene through *A. tumefaciens* mediated transformation in potato was the method of choice in the present study.

The advantages of internodal tissue culture is regeneration of transformed plants directly without intervening callus phase, thus reducing the scope of somaclonal variations and chromosomal aberrations[19]. The stem internodal regeneration protocol has been successfully employed in A. *tumefaciens*-mediated genetic transformation of potato[20-22].

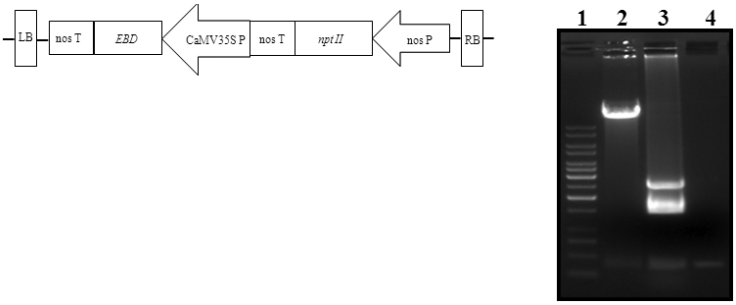

Figure 1. Schematic diagram showing the T-DNA region of the binary vector pBI121-*EBD* used in plant transformation experiments. The *EBD* transgene 194 base pair fragment digested with *Bam* HI- *Xho* I was used as probe to determine the integration and stability of transgene in transformed potato plants. LB: Left Border; nos T: nopaline synthase termination signals; *EBD*: *Enteric* β-*Defensin* transgene; CaMV 35 S P: Cauliflower Mosaic Virus 35S Promoter; *npt II*: *neomycin phosphotransferase* II gene; nos P: nopaline promoter; RB: Right Border.

2. MATERIALS AND METHODS

2.1 Plant Material and Explant Preparation

Virus-free explants of potato cv. Kufri Badshah were surface sterilised and propagated under controlled temperature regime of 25 °C, 16 h photoperiod and light intensity of 30 $\mu Em^{-2}s^{-1}$. The healthy internodal stem segments (2-3 cms) were excised from the *in vitro* grown 21-28 days old potato micro-plants under sterile conditions and were used for plant transformation experiments. The internodal stem segments were placed in 90 mm UV irradiated, petri dishes containing sterile, moistened Whatman1 filter paper and at top of half-strength Murashige and Skoog (MS) medium[23] for 2 days. The above stated temperature and photoperiod regimes were maintained during all stages of *in vitro* regeneration and genetic transformation procedure, unless mentioned otherwise.

2.2 Bacterial Strain, Binary Vector Construct and Culture Conditions

All plant transformation experiments were done using *Agrobacterium tumefaciens* strain EHA 105 harbouring the binary vector pBI121-β-*defensin*. The antimicrobial *β-defensin* gene cloned in phagemid vector pBluescript SK (+) was a gift from Dr. Charles Bevin, Cleveland Clinic Foundation U.S.A. The β-*defensin* transgene was excised at *Bam* HI / *Sac* I and reintroduced in pBI 121, plant transformation vector. T-DNA region of the plant transformation vector contained *neomycin phosopho transferase* (*npt II)* plant selectable marker gene flanked by *nos* termination signals and *nos* promoter and *nos* termination sequences-β-*defensin* transgene-CaMV35S promoter cassette (Fig. 1). The binary vector pBI121-β-*defensin* was introduced into *A. tumefaciens* strain EHA 105 by freeze-thaw technique[24]. *Agrobacterium* cultures were maintained on Yeast Extract Maltose (YEM) medium[25] supplemented with rifampicin 25 mg/L and kanamycin 50 mg/L. A single bacterial colony was transferred to liquid YEM medium containing same amount of antibiotics and grown for 48 h at 28 °C and at 180 rpm in a shaker. The bacterial cells were pelleted by centrifugation at 3000 rpm for 10 min, which was followed by resuspension in a smaller volume of YEM medium supplemented with same quantity of antibiotics to a final optical density of 0.4. The resuspended bacterial culture was incubated at 28 °C for 4 h with continuous shaking at 180 rpm prior to explant inoculation. The 2 days old internodal explants were co-cultured with resuspended bacterial culture for 5 min. with continuous, gentle agitation under aseptic, sterile conditions. The excess bacterial inoculum was blotted dry onto sterile paper and inoculated internodal explants were allowed to re-establish on the same half-strength MS pre-culture medium for additional 2 days under sterile conditions. Post 2 days of cocultivation, the internodal stem explants were transferred to selection medium consisting of MS basal salts, vitamins and supplemented with zeatin riboside (3 mg/L), gibberellic acid (3 mg/L), indole-acetic acid (0.05 mg/L), sucrose (20 g/L), agar (8 g/L) (Sigma), carbenicillin (200 mg/L) and kanamycin sulphate (10 mg/L) for selection of putative transformed shoots.

2.3 Recovery of Transformed Potato Plants

The putative transformed potato plants were monitored for growth in selection medium with stepwise increase in selection pressure of kanamycin sulphate from 10 mg/L to 100 mg/L in duration for 21days for determining stable transformation events and to kill off any residual *Agrobacterium*. Subsequently the putative transformed potato plants were allowed to grow to height of 7 cm - 10 cm and the selection pressure of plant selectable marker; kanamycin was concurrently decreased to 25 mg/L in a phased manner. Transfer of elongated shoots to the rooting medium containing MS salts and vitamins supplemented with kanamycin (25 mg/L) and NAA (2 mg/L) resulted in root development. The healthy independent transformed rooted shoots were transferred to greenhouse, acclimatised and grown till maturity.

2.4 Bacterial Aggressiveness Assay

Aggressiveness of *Ralstonia solanacearum* (phylotype II, sequevar I) was determined on transformed potato plants. To

prepare the inoculum, bacterial strain was grown for 48 h in liquid Casamino acid Peptone Glucose (CPG)[25] at 28 °C with constant shaking at 200 rpm. The bacterial cells were pelleted by centrifugation at 3000 rpm for 10 min. resuspended in 0.9 per cent saline solution and spectrophotometerically adjusted to 10^8 cfu/ml with OD_{600}. Inoculation of bacterium with healthy, independent transformed potato plants with six to eight expanded leaves were performed in triplicates. The 3rd branch of independent transgenic potato plants were inoculated with 10^8 cfu/ml of *R. solanacearum* (phylotype II, sequevar I) by stem stab method [26]. The negative control treatments were done on healthy untransformed potato plant with sterilised water. After inoculation, the plants were incubated in a controlled greenhouse at 28 °C with 12 h photoperiod. Disease manifestation was recorded four days post inoculation. The wilt scoring was done based on the following scale: 1–healthy plant, 2-wilt on 3rd branch,3-wilt on 3rd and 4th branch, 4-infection on 5th and remaining branch and 6-complete wilting ; the disease progression was evaluated for 28 days post-bacterial inoculation.

2.5 Molecular Analysis

2.5.1 Polymerase Chain Reaction

Genomic DNA was isolated and purified from the leaves of greenhouse acclimatised, independent putative transgenic plants using the modified CTAB procedure[27]. The putative plants were screened by polymerase chain reaction for the presence of transgene β-*defensin* and plant selectable marker, *npt II* gene. The 194 base pair (bp) fragment of β-*defensin* gene was amplified using oligonucleotide primer pair, 5'-CGCCATGGCGATGAGGCTCCATCAGC-3'and 5'-CGGGATCCCGTTACCTCCACCTGCAG-3', and the 713 base pair fragment of *npt II* was amplified using the primer pair 5'-CCCCTCGGTATCCAATTAGAG- 3' and 5'-GGGGGTGGGCGAACGAAGAACTCCAG- 3'. The PCR conditions allowed the use of 100ng of transformed plant DNA and the total volume of the PCR reaction was 25 µl. The control, untransformed potato plant DNA used in reaction was 50 ng and control transgene fragment concentration was 25 ng. The PCR reactions were conducted under the following conditions. The samples were carried through 35 cycles by using the following temperature regimes: 94 °C denaturation for 1min, annealing for 1 min at 58 °C (*npt* II) and 61 °C (β-*defensin* gene), 72 °C elongation for 1min and cycles were preceded by an final extension of 10 min at 72 °C. The amplified products were subjected to electrophoresis on a 1 per cent agarose gel, visualised with ethidium bromide in UV light (254 nm) transilluminator. Leaf-tissues of transformed potato plants were harvested and subjected for total RNA isolation through RNeasy Kit (QIAGEN Genetix) following manufacturer's instructions. The concentration of total RNA was measured spectrophotometerically and stored at -80 °C until use. One-step RT PCR conditions were followed using the β-*defensin* primers after DNase I treatment (1Unit/µl). The reverse transcription procedure was performed in a thermal cycler (Applied Biosystem 7600, Perkin Elmer) with following conditions. First strand cDNA synthesis at 50 °C for 30 min, 95 °C for 15 min to activate HotStart Taq DNA polymerase enzyme, followed by gradient amplification of samples through 35 cycles at 94 °C (denaturation) for 1min, 61 °C (annealing) for 1 min, 72 °C (extension) for 1 min followed by final extension of amplified transcripts at 72 °C for 10 min. The samples were sequentially ramped down to 4 °C. The samples were centrifuged briefly for 30 seconds to collect the reaction mix and were visualised through electrophoresis in a 1 per cent agarose gel stained with ethidium bromide (3 µl /100 mL).

2.5.2 Southern Blot Analysis

Total genomic DNA from independent transgenic potato plants was isolated using the modified CTAB procedure as described earlier. Briefly, 500 µg of plant genomic DNA was digested overnight at 37 °C by *Bam* HI (50 Units/µl) and size fractionated on a 0.9 per cent TAE-agarose gel at 30 V. Following denaturation and renaturation washes, the DNA fragments were transferred to Hybond N^+ nylon membrane (Amersham, UK) by capillary transfer in 1X TAE buffer as described [28]. Transferred DNA fragments were cross-linked to charged membrane at 80 °C for 3-4 h. The β-*defensin* fragment (194 bps) obtained by excision by *Bam* HI and *Xho* I was gel purified (QIAquick Gel Extraction Kit, QIAGEN) and labeled with α^{32}P-dCTP using Ready-to-Go labelling kit (Amersham) following manufacturer's instructions and used as probe. Prehybridisation and hybridisation of membranes were performed at 68 °C using BSA and probe respectively. After stringency washes, the membranes were exposed to Kodak films (Eastman Kodak, Rochester, NY) at -80 °C for 24 h.

2.5.3 Northern Hybridisation

Total RNA were isolated from plant tissues using phenol /chloroform procedure [28] and the samples integrity were verified through 1 per cent denaturing formaldehyde gel electrophoresis and quantified spectrophotometerically (BioRad). Equal amount of total RNA was fractionated on a 1 per cent denaturing formaldehyde agarose gel and transferred to positively charged Brightstar (Ambion) membrane as described earlier and were hybridised overnight at 42 °C with α^{32}P dCTP labeled β-*defensin* gene fragment in hybridisation buffers. The membranes were blocked and washed with low and high stringent buffers to remove the background and were exposed to Kodak films with a photo intensifier cassette at -80 °C for 48 h.

2.5.4 Western Blotting

For immunoblotting, the infected leaves of *R.solanacearum* challenged transformed potato plants were grinded in liquid nitrogen and eighty micrograms of protein samples were separated on 14 per cent Sodium dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS PAGE) under a constant electric charge[29]. The fractionated protein bands were transferred to nitrocellulose membranes (HYBOND Millipore, U.S.A.) and blocked with 3 per cent BSA (Bovine Serum Albumin)(Sigma, U.S.A.) in phosphate buffer saline (PBS) containing 0.1 per cent Tween 20 (Sigma, U.S.A.). The membranes were washed and probed with raised β-*defensin* polyclonal antibodies (primary antibody) for 2 h with gentle shaking and washed with secondary antibody anti-goat IgG

HPR conjugate (SantaCruz). The membrane were washed repeatedly and incubated with chemiluminiscent substrate (Sigma, U.S.A.) and bands were developed using X ray film (Kodak UK).

2.5.5 Experimental Design and Statistical Analysis

The experiments on regeneration of transformed potato plants, plant transformation were performed in three replicates. The molecular characterisation studies were performed thrice to confirm the integrity and validity. *R.solanacearum* aggressiveness assay experiments were performed in triplicates with independently generated transformed potato plants. The analysis of variance was computed using PROC GLM in SAS (SAS 9.3, SAS, Institute, NC, USA, version 9.3).

3. RESULTS

3.1 Regeneration from Internodes and Development of Transgenic Plants

The co-cultured internodal segments on regeneration medium were periodically transferred to fresh regeneration medium every 5 days under aseptic conditions. The internodal segments were observed to develop shoots (3-4 cms) with occasional incidence of multiple shooting (3-4 shoots) per explant following 15 days of culture in regeneration medium which consisted of MS salts, vitamins and supplemented with zeatin riboside (3 mg/L) that provided conditions for organogenesis. The excised ends of the co-cultivated explant during the initial stages of regeneration developed undifferentiated callus growth which were restricted by transferring the plant tissue in regeneration medium devoid of IAA and increased concentration of kanamycin sulphate (50 mg/L). Few infrequent multiple shoots per explant were counted as a single independent event and were transferred to glass tubes containing regeneration medium supplemented with kanamycin sulphate (100 mg/L) and carbenicillin (50 mg/L). Putative transgenic shoots in presence of kanamycin sulphate were observed to initiate root development on regeneration medium for additional 10 days (Fig. 2). After rooting, the plants were grown on propagation medium consisting of MS basal salts and vitamins and supplemented with NAA (2 mg/L) to promote root proliferation and in presence of kanamycin sulphate (25 mg/L) for selection pressure. The healthy transformed potato plants were acclimatised for seven days in greenhouse and subsequently transferred to pots. Initial preculturing on half-strength MS salt and vitamins were determined to be crucial for regeneration. Preconditioning enabled explants to maximise its regeneration potentials. Increasing the duration of preculturing of explants from optimised two days were found to decrease regeneration and were associated with increased callus tissues development and inability to develop shoots.

3.2 *Agrobacterium*-mediated Genetic Transformation and Plant Selection

Agrobacterium concentration corresponding to OD_{600} value of 0.4 was utilised for cocultivation experiments. Following cocultivation, the explants were subjected to recovery period of 2 days on half-strength MS salt and vitamins medium, lacking kanamycin sulphate. On completion of recovery period, the cocultivated explants were transferred to regeneration medium following stepwise increment in selection pressure of kanamycin sulphate from 10 mg/L to 100 mg/L for the regeneration of green shoots. The healthy, regenerating shoots (10-12 cms) were excised under sterile conditions and sub-cultured every 10 days to fresh selection medium consisting of MS basal salts, vitamins, plant growth hormone NAA (2 mg/L) and coursed through selection pressure by supplementing the nutrient medium with varying increased concentration of kanamycin sulphate. Out of 580 explants cocultured with *Agrobacterium*, 67 regenerated shoots were obtained following selection in kanamycin sulphate, of which 42 plantlets survived till maturity under greenhouse conditions with mean transformation efficiency of 3.68 per cent. Of the initial 67 regenerated shoots, 7 plantlets turned albino and did not regenerate further than 3-4 leaf-stages. Cessation to develop root system was observed in 13 plantlets in selection medium, 5 regenerated shoots had stunted growth and exhibited necrosis symptoms after 15 days on selection medium. A total of 25 plantlets were discarded.

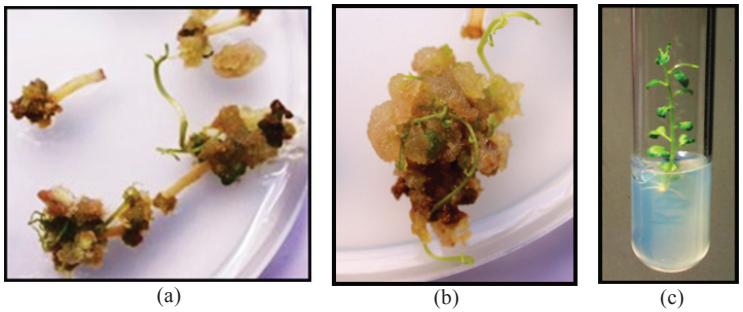

Figure 2. ***In-vitro*** **regeneration and stable genetic transformation of** ***Solanum tuberosum*** **L. (cv. Kufri Badshah). (a) Regeneration of green putative transformed shoots from the excised** ***Agrobacterium*****-cocultivated internodal explants in selection medium consisting of plant selectable marker. (b) Multiple shoot regeneration from the excised** ***Agrobacterium*****- cocultivated internodal stem explants in selection medium. (c) Healthy, normal, transformed shoots with developed root system in regeneration medium.**

3.3 Bacterial Wilt Aggressiveness Assay studies

The bacterial inoculum was prepared and resuspended in 0.9 per cent saline solution with concentration adjusted to 10^8 cfu and was constant in all the treatments. The experiments were conducted in three replicates of each transformed plantlets in a controlled greenhouse. The plantlets (15-20 cms) were inoculated with bacteria inoculum (10^8 cfu) on the third branch of the primary stem following the stem stab procedure[26].

The negative, control treatment were performed on healthy, untransformed potato plant with sterile water. The treated plants were incubated at 28 °C with 12 h photoperiod. The disease progression was recorded 4 day post-inoculation as this timeframe was sufficient for challenged plants to display bacterial infection. The scoring of wilt manifestation on transformed potato plants were done at intervals of 14 days, 21 days and 28 days. The transgenic plants expressing the anti-

microbial peptide, displayed variable degree of resistance to bacterial wilt infection. Of a total of 42 plantlets regenerated, the survival response of nine plantlets (#6,#10,#11, #13, #15, #23, #26, #32, #34) that scored on the scale from 1-4 demonstrated increased resistance to bacterial challenge, while six plantlets (#12, #19, #20, #21, #22, and # 25) had scores between 3-5 scales indicating moderate resistance followed by eight plantlets (#1, #7, #8, #9, #14, #17, #26 and #29) with score ranging between 6-7 scales. Nineteen plants displayed acute wilting symptoms in less than 14 days post-challenge.

3.4 Transgene Characterisation Studies

The molecular analysis was performed on the resistant transformed plantlets with survival response score (1-4) as determined by bacterial challenge assay.

3.4.1 Polymerase Chain Reaction

The transgene expression in plant tissues was established by reverse transcription (RTPCR) procedure (Fig. 3) and molecular screening of plant selection marker (*npt* II) gene in the transformed plants (Fig. 4) confirmed the integration of transgene cassette in plant genome.

3.4.2 Inheritance of Transgene in Transformed Plants

Based on the identification of resistant plants in bacterial challenge assay and presence of anti-microbial β-*defensin* gene in transformed potato plants, Southern hybridisation was performed with 194 bps β-*defensin* gene fragment as probe. The probe DNA effectively hybridised with the transformed plant DNA indicating stable genetic transformation, integration and inheritance of β-*defensin* transgene in potato plants (Fig. 5).

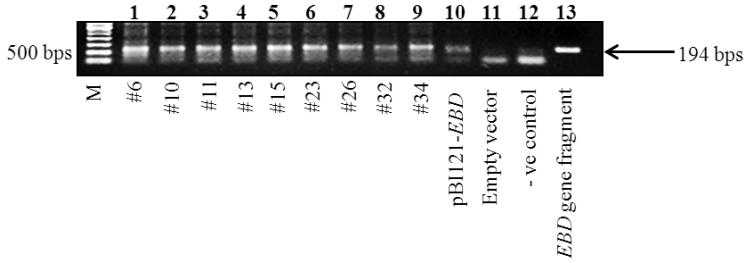

Figure 3. RT PCR analyses of transgenic potato plants. M represents the 100 base pairs (bps) DNA ladder used as molecular weight separator. Lane 1-9 represents the amplification of 194 bps transgene fragment; Lane 10 is positive binary vector pBI 121with cloned transgene; Lane 11 is empty binary vector; Lane 12 represents untransformed plant as negative control; Lane 13 represents purified *EBD* transgene fragment.

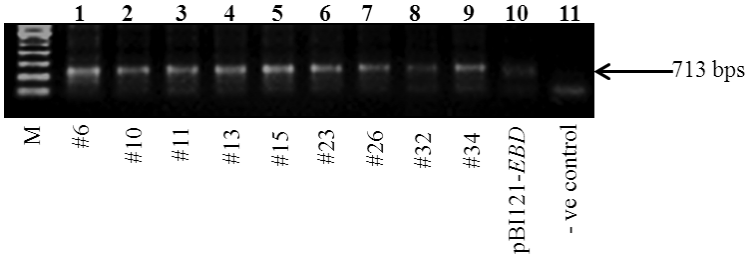

Figure 4. Plant selectable marker gene analyses in transformed potato plants. M represents the 1000 bps DNA ladder used as molecular weight separator. Lane 1-9 represents the amplification of 713 bps *npt* II *neomycin phospho transferase* marker fragment; Lane 10 is binary vector pBI 121with plant selectable marker gene ; Lane 11 is untransformed plant as negative control.

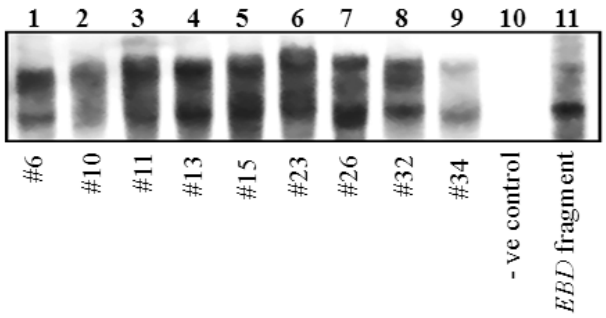

Figure 5. Southern blot analysis of individual transformed potato plants. A total of 500 µg purified genomic DNA was harvested from the transformed tissues and restriction digestion was performed with *Bam* HI (100units/µl) overnight at 37° C. The fractionated DNA fragments were electrophoresed on agarose gel and hybridised with α ^{32}P dCTP labeled *EBD* transgene fragment. Lane 1-9 ; transformed potato plants, Lane 10; untransformed, control potato plant. Lane 11; labeled EBD transgene fragment.

3.4.3 Gene Expression Analysis

The transformed plantlets with survival response score (1-4) for disease challenges were selected for transgene expression analysis study. Total RNA samples were prepared by phenol-chloroform procedure as described by Sambrook et al., 1989 and probed with 194 bps β-*defensin* gene fragment. The positive signals demonstrated stable expression of transgene transcripts in wilt challenged transformed potato plants (Fig. 6). Immunoblotting analysis revealed varying accumulation of β-*defensin* antimicrobial protein in the wilt-challenged transformed leaf-tissues.

4. DISCUSSION

In the present study, we utilised the innate host defence anti-microbial peptide derived from enteric β-*defensin* gene family constitutively expressed in the mucosal epithelial membrane of *Bos tarus* to generate potato plants for enhanced resistance to *Ralstonia solanacearum* infection. Efficient, reproducible *Agrobacterium*-mediated genetic transformation procedures were followed by regeneration of healthy transformed potato plants. The combining effect of *Agrobacterium* and the plant selection marker, kanamycin sulphate resulted in generation of positive transformed potato plants. Pre-conditioning of explants before inoculation and post cocultivation with *Agrobacterium* facilitated potato explants to mobilize essential nutrients from plant growth medium to recover from the excision injuries and increased genetic transformation potential. Enhanced regeneration efficiency was more likely due to transfer of T-DNA strand in the cells that are actively undergoing cell division[30]. The plant binary vector pBI 121-β-*defensin* was introduced into

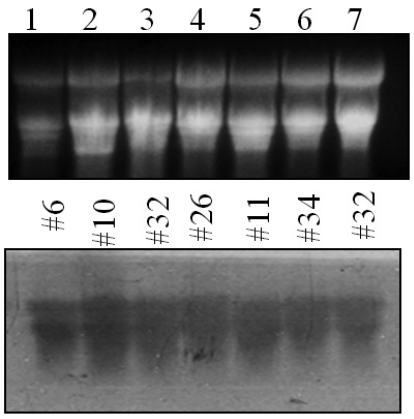

Figure 6. Northern hybridisation of transformed potato plants was performed to determine the stable transgene expression. The transformed leaves tissues were challenged with 10^8 cfu *Ralstonia solanacearum* L. (phylotype II, sequevar I). Total RNA (1µg) from independent transformed tissues were extracted and electrophoresed on 1 per cent denatured formaldehyde agarose gel. The fractionated total RNA was transferred on positively charged membrane and was prehybridised and hybridised with α ^{32}P d CTP labeled *EBD* transgene fragment as probe. (a) Lane 1-7 represents the fractionated total RNA fragments and (b) stable transgene expression.

A. tumefaciens strain EHA 105 with higher transformation efficiencies as compared with other *Agrobacterium* strain, LBA 4404 was in reaffirmation with the studies on evaluating the relative transformation efficiencies between the *Agrobacterium* strains[31]. The plant genetic transformation methodology in the present study using *Agrobacterium tumefaciens* EHA 105 strain, offered high optimum transformation efficiencies and conferred ability to integrate fewer copies of transgene in potato genome.

The initial regeneration of cocultivated explants in selection medium resulted in development of undifferentiated callus tissues around the excised internodal segments, often culminating in explant necrosis. Optimisation of shoot organogenesis protocol by selectively transferring the explants to selection medium with concomitant suppression of IAA and increased zeatin riboside concentration resulted in shoot regeneration. The transformed plants were selected on their ability to develop root system in presence of kanamycin in selection medium was a strong indicator of transgenicity acquired by regenerated potato plants[32]. However, some of the regenerated shoots on kanamycin supplemented selection medium turned brown and did not regenerate any further. The selection of positive transformed tissues is important in genetic transformation studies as all the plants regenerated after cocultivation display normal, healthy phenotypic characters. The conducive shoot regeneration and elongation procedures in parallel with standardisation of doses of selection pressure using plant selectable marker proved to be crucial, as suboptimal conditions of both the treatments resulted in high frequency of escapes. Exceeding the doses concentration of antibiotic (>100 mg/L) for selection of transformed tissues was associated with inhibition of transformed tissues and browning of internodal explants. The optimised concentration of plant selectable marker (*npt* II) gene, was determined to be 10 mg/L post cocultivation with *Agrobacterium* followed by stepwise increment of doses up to 100 mg/L in selection medium. For efficient recovery and to circumvent the continued stress; the internodal explants were passaged for 3-5 days on antibiotic-free regeneration medium prior to transfer in selection medium with step up doses of antibiotics. The bacterial wilt progression is influenced by environment including temperature, humidity, light intensity and age of plant tissues[33-34].

Throughout the study author utilised temperature-controlled greenhouse and the conditions were generated such those that promoted bacterial infection in real time environment. Nine lines viz. EBD 6, EBD 10, EBD 11, EBD 13, EBD 15, EBD 23, EBD 26, EBD 32 and EBD 34 demonstrated disease tolerance and survivability. These were analysed by ANOVA which indicated difference amongst the selected lines and significant higher survivability was obtained in EBD 26, EBD 32, EBD 10 and EBD 6 transformed plants. The bacterial aggressive assay studies provided a strong evidence to support antimicrobial properties of β-*defensin* transgene as potential molecule against bacterial wilt incidences. 4-5 days post bacterial challenge; transformed leaves displayed localised yellow lesions at the base, suggesting possible interaction between *R. solanacearum* and transgene coupled with host resistance repository moieties (Fig. 7 (a)). The lesion signatures gradually progressed in tissues challenged with bacterial infection (Figs. 7 (b) and 7(c)). Interestingly 7-8 days post bacterial infection challenge, the older leaf tissues of transformed plant developed acute necrosis symptoms (Fig. 7(c)) and were detached from the branch indicating the role of selective programmed cell death. The phenomenon may be the result of restricted, localised, independent plant-pathogen interaction mediated defense triggered by plant tissues during an event of acute pathogenesis for sustained growth which has been accelerated in presence of transgene. There are consistent reports on the abilities of cellular components of plant tissues during plant-pathogen interaction to recognise and mediate the resistance through oxidative burst of organic compounds, release chemical and molecular moieties to target tissues and in susceptible event, by inducing autophagy[35-37]. Irrespective of transgene accumulation in transformed potato plants, presence of localised apoptosis in old tissues was similar to *RB*-mediated late blight resistance in transgenic potatoes[38]. In this study, host defence gene expression in conjunction with β-*defensin* transgene during pathogenesis, enabled transformed potato plants to display varying degree of resistance (Fig. 8). However, the antimicrobial peptide transcript accumulation in the transformed plants displaying similar physiological state differed significantly. The correctly incorporated β-*defensin* transgene in transformed plants were obtained by Southern hybridisation. The β-*defensin* gene was presumably incorporated into potato genome at different locations. The transgene resistance in bacterial assay studies and protein expression by western blotting may be attributed to chromatin structure rearrangement or DNA methylation alterations.

Conversely, introduction of transgene into regions

(a) (b) (c)

Figure 7. (a) Hypersensitive response in transformed leaves post *R. solanacearum* L. infiltration, (b) The yellow lesion signatures gradually developed in the infected branches, (c) Programmed cell death of infected plant tissue (as indicated by arrow).

Figure 8. Transformed potato plant and control untransformed plant post-challenge with *R. solanacearum* L.

where transcript levels were not easily altered resulted in consistent defensin gene expression. These results indicate future experiments detailing the interplay of host defence genes, transgene transcript accumulation and impact of transgene positioning in plant genome during pathogenicity. Stacking of resistance genes in potato have been reported for increased resistance to late blight infections[39] however introduction of large foreign DNA fragment is often associated with activation of host plant defence repertories which may result in rearrangement/cleavage of foreign DNA. The optimal expression of enteric β-*defensin* gene in this study was conducive to maintain the biological activity without any toxic effect to host potato plant.

Although with low transformation efficiency obtained, we were able to demonstrate the stable expression of antibacterial β-*defensin* peptide. The studies, on optimisation of β-*defensin* gene for tailored expression in plant system utilising tissue/organ-specific promoters may broaden the spectrum of this antimicrobial peptide in economically important food crops.

5. CONCLUSIONS

In the present study, we successfully introduced β-*defensin*, an antimicrobial peptide into potato and demonstrated the stable integration and expression in transformed potato lines. Challenged transformed lines showed increased tolerance and plants were able to complete their life-cycle. The prospects of introducing novel defensin genes alone or in combination under specific plant promoter will enable strategies in insect-pest management of economically important crops.

Conflict of Interest: None

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He conceptualised, coordinated the planning and provided guidance in the present study.