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Transgenic Sugarcane Expressing Rice Chitinase (*G11*) and Rice Basic Chitinase (RCH 10) and Alfalfa β-1, 3-Glucanase (*AGLU*1) Showing Enhanced Resistance to Red Rot Disease

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Abstract

Productivity and sugarcane quality are severely affected by the fungus Colletotrichum falcatum, which causes red rot disease. Genetic engineering of sugarcane with antifungal genes encoding pathogenesis-related (PR) proteins offers an opportunity to develop resistance to red rot. In this study, transgenic sugarcane clones were developed by over expressing either the rice chitinase (G11) gene alone or a combination of the rice basic chitinase (RCH 10) and alfalfa β-1, 3-glucanase (AGLUI) genes. Particle bombardment was performed using the pAHG11 plasmid, which carries the rice chitinase (G11) gene driven by the maize ubiquitin promoter, and the pZ100 plasmid, which carries both the rice basic chitinase (RCH 10) and alfalfa β-1,3-glucanase (AGLUI) genes, both driven by the CaMV35S promoter. Of the 23 sugarcane transgenic events obtained, 9 harboured G11 alone, and 14 harboured both RCH 10 and AGLUI. Southern and Western analyses of T0 transgenic events showed constitutive expression and accumulation of rice chitinase and β-1, 3-glucanase. An assay of T1 transgenic events for their reaction to red rot disease revealed increased resistance to Colletotrichum falcatum.

Keywords: *β-1, 3-glucanase; Chitinase; Red rot resistance; Transgenic sugarcane.*

Introduction

Sugarcane (*Saccharum officinarum*) is exposed to several biotic and abiotic stresses during its growth, and new varieties must be able to withstand a range of stress conditions. In India, the most important sugarcane disease is red rot, caused by the fungus *Colletotrichum falcatum* Went (perfect state: *Glomerella tucumanensis* (Speg.) Arx and Muller) [1]. The disease causes severe losses in yield and quality [2]. Deploying red rot-resistant sugarcane cultivars is the most important strategy for managing the disease [3]. Transferring disease resistance genes to sugarcane plants through conventional breeding methods may take several years, and breeding resistant cultivars is challenging because of long generation times, variable ploidy levels, the sterility of most commercial cultivars, and limited genetic variability [4]. The first major achievement toward developing an integrated molecular/conventional breeding system for sugarcane improvement was the establishment of an efficient transformation system [5]. Subsequently, substantial efforts have been made to genetically engineer agronomic traits in sugarcane varieties [6].

Chitinases, such as poly [1, 4-N-acetyl-D-glucosaminid] glycan hydrolase (EC 3.2.1.14), are low-molecular-weight, pathogenesis-related proteins that are often extracellular, acid-soluble, and protease-resistant [7, 8]. They catalyze the hydrolysis of β-1,4 linkages in the N-acetyl-D-glucosamine polymer chitin in fungal mycelial walls, yielding N-acetyl glucosamine oligomers, which cause in vitro lysis of hyphal tips and inhibition of spore germination [9]. There are reports of developing fungal-resistant plants in various crops, such as transgenic tobacco [10], canola [11], cotton [12], and rice [13]. In many cases, a pronounced synergistic effect was observed when β-1, 3-glucanase and chitinase transgenes were expressed in combination, including in transgenic tobacco [14] against *Fusarium solani*, in frog eye spot against the fungal pathogen *Cercospora nicotianae* [15], and in Brassica napus plants against *Sclerotinia sclerotiorium* [16]. However, evidence shows that chitinase and glucanase enzymatic activities can confer fungal disease resistance through genetic transformation to develop disease-resistant plants.

There are reports of single-gene over expression of Gloxalase III isolated from wild sugarcane enhancing abiotic tolerance [17, 18]. Hence, the main objective of this study was to transform the red rot-susceptible sugarcane variety CoC 671 with a rice chitinase gene (*G11*) alone and in combination with rice basic chitinase (RCH10) and alfalfa glucanase (*AGLU*I) genes using particle bombardment to enhance resistance against *C. falcatum*.

Materials and Methods

Plasmid vector construction:

The binary vector pAH*G11*, containing the bar gene as a selection marker, is digested with *BamHI* and *HindIII* to clone the *G11* gene encoding rice chitinase, which will be driven by the maize *ubiquitin* promoter (Figure 1A). Another binary vector, pZ100, containing the *npt II* gene as a selection marker, is digested with *EcoRI* to clone the RCH10 and *AGLU*I genes, encoding rice basic chitinase and alfalfa glucanase, respectively, each driven by the CaMV35S promoter (Figure 1B). The plasmid constructs were isolated and purified by the alkali lysis method [19].

Plant material and tissue culture:

Young meristematic leaf segments from the red-rot-susceptible sugarcane variety CoC 671 were dissected, surface-sterilized, and placed on modified Murashige and Skoog (MS) I medium (MS medium supplemented with 3 mg/L 2-4-dichlorophenoxyacetic acid, 10% tender coconut water, 100mg/L myoinositol, and 20g/L sucrose) [20]. The explants were incubated at 25°C in the dark and sub cultured on fresh MS I medium every 14 days until embryogenic calli were generated [21]. To assess the minimal inhibitory concentration (MIC) of phosphinothricin and geneticin, the embryogenic calli were cultured in MS I medium containing various concentrations of phosphinothricin (0–10 mg) and geneticin (0–50 mg). Final observations on callus survival were recorded after 15 days.

Transformation and regeneration of transgenic material:

Particle bombardment of sugarcane calli was performed according to [22]. Before bombardment, friable embryogenic calli 2–3 mm in size were placed on osmotic medium (MS I with 0.25M mannitol and 0.25M sorbitol) for 4hrs. Explants were bombarded with gold particles (1µm and 1.5–3µm) coated with pAH*G11* and pZ100 plasmids using a Bio-Rad PDS 1000/He biolistic system at 1100 psi of helium. Bombarded explants were incubated on the same osmotic medium overnight. After 24hrs, calli bombarded with pAH*G11* were transferred to callus induction medium (MS I) with 3mg/L phosphinothricin, and those bombarded with pZ100 were transferred to callus induction medium (MS I) with 30mg/L geneticin. Phosphinothricin-resistant calli that proliferated in MS I were transferred to MS II regeneration medium (MS I + kinetin 1mg/L + NAA 0.5mg/L + 3mg/L phosphinothricin for pAH*G11* and 30mg/L geneticin for pZ100) and incubated at 25°C with a 16h light/8h dark cycle.

When green shoots reached 10–12 cm in height, they were transferred to the rooting medium (Whites medium) with 3mg/L phosphinothricin for pAH*G11* or 30mg/L geneticin for pZ100. Rooted plants were transferred to pots containing a mixture of sterilized sand, soil, and farmyard manure in a 1:1:1 ratio. Pots were covered with polythene bags to maintain humidity. After 15 days, the acclimatized plantlets were transferred to a transgenic glasshouse.

Molecular analysis of transgenic plants:

PCR analysis:

Genomic DNA was isolated from putatively transformed sugarcane plants using the cetyl trimethylammonium bromide (CTAB) method [23]. All putative transgenic plants and untransformed control plants were subjected to PCR using *G11* and *AGLU* gene-specific primers (Table 1). The PCR reaction mixture contained 0.25µM of each primer, 200M dNTPs, 50ng of genomic DNA, 0.5 units of Taq DNA polymerase, 1X buffer, and 2.5mM MgCl2. The reaction mixture was subjected to an initial denaturation at 94°C (3 min), followed by 30 cycles of denaturation at 94°C (45 sec), annealing at 56°C for *G11* and 61°C for *AGLU* (45 sec), and extension at 72°C (1 min). The reaction was terminated by a final extension at 72°C (10 min). The amplified PCR products were electrophoresed on a 2% (w/v) agarose gel at 100 V for 2hrs, stained with ethidium bromide, visualized under UV light, and photographed using a gel documentation system. The transgenic events were further confirmed by PCR using selection marker- and promoter-specific primers (Table 1).

Southern blot analysis:

Southern hybridization was performed [19]. 50µg of genomic DNA extracted from the leaves of V0 transgenic events over expressing *G11* was digested with *BamHI*, which cuts once in the upstream region, 1.1 kb from the 5' end of the maize *ubiquitin* promoter DNA sequence. 1 - 2ng of the pAH*G11* plasmid was digested with *BamHI* and *HindIII*, which served as a positive control. Similarly, genomic DNA extracted from the leaves of V0 transgenic events over expressing RCH10 and *AGLU*I was also digested with *BamHI*. The pZ100 plasmid was digested with *BamHI* and *SacI* and served as a positive control for the gene combination experiment. All samples were electrophoresed on a 1% agarose gel and transferred to a nylon membrane by capillary transfer. The transferred membranes were hybridized with a 32P-dCTP-labeled 1.1Kb chitinase gene cassette (released by digesting the plasmid with *HindIII* and *BamHI*) and a 2.4Kb glucanase gene cassette (released by digesting the plasmid pZ100 with *BamHI* and *SacI*), respectively. The blot was subsequently auto-radiographed.

Western analysis:

Proteins were extracted from leaves of putative transgenic events and control plants, and protein concentration was estimated using the Bradford method [24].

Proteins were separated by size using SDS–polyacrylamide gel electrophoresis (PAGE) following [25]. After separation, proteins were electroblotted onto a PVDF membrane, followed by incubation with a primary polyclonal rabbit anti-chitinase antibody for 1hr, then with a secondary IgG antibody for 1hr. Chromogenic detection was performed using the BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) reagent. The reaction was stopped by washing the membrane with distilled water after the formation of the indigo color. The membrane was air-dried and photographed.

Evaluation of the red-rot reaction:

The transgenic, along with the untransformed control plants, were evaluated for red-rot reaction following [26]. Putative transgenic events were vegetatively propagated to raise the V1 generation, which was used for screening. Nine events from each clone (transgenic expressing chitinase alone and transgenic expressing chitinase and glucanase) were multiplied along with the untransformed control. 207 plants, 8 months old, were subjected to red-rot screening. Nodes from the top were selected for inoculation, and leaf sheaths were stripped off without injuring the nodal tissues. The pathogen inoculums was prepared and applied to the freshly exposed nodal regions using cotton strips wetted with a spore suspension (10-6 spores/mL) of the CoC 671 isolate of *C. falcatum*. Inoculated canes were incubated at 90% relative humidity, with the temperature maintained at 30 2°C. After 30 days, the inoculated canes were rated as resistant, moderately resistant, moderately susceptible, susceptible, or highly susceptible on a 0–9 scale, as described [27], based on qualitative symptom characteristics that included drying of tops, lesion width, nodal transgression, and the occurrence and nature of white spots.

Results

Integration and expression of *G11* in transgenic sugarcane

All nine putative transgenic events in the T0 generation showed 746bp amplification when subjected to PCR with *G11*-specific primers (as shown in Figure 2A). This finding was subsequently confirmed by amplifying the resistance marker (bar, 300bp) and promoter (*ubi*, 500bp) with specific primers (as shown in Figure 2B and C). Notably, no amplification was detected when genomic DNA from untransformed control plants was used as a template.

The copy number and integration pattern of the transgenic events were determined by Southern blot hybridization. Genomic DNA was digested, separated, blotted, and probed with a radio labeled *G11* DNA fragment. This approach revealed the integration pattern, showing that several copies of the *G11* gene were integrated into the host genome. Of the nine plants, seven randomly selected plants were analyzed, and all exhibited hybridization with the 1.1 kb *G11* probe (as illustrated in Figure 3). Based on these observations, the V0 plants of *G11*-1, *G11*-2, *G11*-3, *G11*-5, *G11*-4, *G11*-6, and *G11*-7 were found to have two to three hybridization bands.

Transgenic plants were also assessed for chitinase expression. Extracts from V0 plants (*G11*-1 to *G11*-9) displayed protein bands that reacted with a chitinase antibody and were the same size (32kDa) as the positive control from a transgenic rice plant (depicted in Figure 4).

Integration and expression of the *RCH10* and *AGLUI* gene combination in transgenic sugarcane

A total of 14 putative V0 transformants (P1 to P14) were subjected to gene integration analysis by PCR. In all cases, each transformants produced a single DNA fragment of 246bp for *AGLU*I and an 800bp fragment for the *nptII* gene, as depicted in Figure 5. Genomic DNA isolated from leaves of PCR-positive V0 plants was further examined by Southern analysis to provide additional evidence of integration and to determine the copy number of the *AGLU*I gene. The process involved digesting the genomic DNA, resolving it by electrophoresis, blotting it, and probing it with a radio labeled *AGLU*I fragment. This procedure revealed the integration pattern, showing that several copies of the *AGLU*I gene were integrated into the host genome. Of the 14 plants, six were randomly selected for analysis, and DNA from all six plants hybridized with the 2.4kb *AGLU*I probe. These results provide robust evidence confirming the successful integration of the transgene into the plant genome. Importantly, no hybridization signal was detected when untransformed plant DNA was examined, as shown in Figure 6.

Evaluation of the red-rot reaction

At 8 months of age, V1 transgenic events were evaluated for their response to red rot. Disease symptoms became visible 15 days after inoculation in eight transgenic (*G11*-4, *G11*-6, *G11*-9, P-1, P-3, P-5, P-7, and P-13), as well as in the untransformed control. These plants exhibited distinct symptoms, including brownish discoloration on the external surface of internodes near the point of infection, along with yellowing and drying of foliage within 15 days of inoculation. In contrast, no disease symptoms were observed in the remaining transgenic (*G11*-2, *G11*-3, *G11*-5, *G11*-7, *G11*-8, P-6, P-8 to P-12, and P-14), indicating a slower spread of the disease. On the 25th day after inoculation, five transgenic events (*G11*-2, *G11*-3, *G11*-5, *G11*-7, and *G11*-8) exhibited drying of foliage, while seven transgenic (P-6, P-8, P-9, P-10, P-11, P-12, and P-14) showed partial drying of foliage. By the 30th day after inoculation, when the canes were split open, reddish discoloration with alternating white spots, cavity formation, collapse of parenchyma cells, and fungal sporulation were observed. Three remaining transgenic (*G11*-1, P-2, and P-4) with green foliage showed no red discoloration symptoms, as shown in Figure 7. The scoring of red rot symptoms was detailed in Table 2 and categorization of red rot reactions according to [27] was given in Table 3.

Discussion

The particle bombardment approach proved reliable for obtaining sugarcane transformants in our study, with a moderate transformation frequency.

We used phosphinothricin and geneticin in the callus induction medium (MS I), regeneration medium (MS II), and rooting medium to minimize the regeneration of 'escape' plants. Transgenic containing *GII* and *AGLU*I (pZ100) were selected at 30mg/L of geneticin. Only geneticin-resistant cell lines continued to grow under this selection pressure, and the control calli could not develop further at 30mg/L of geneticin. These results are consistent with earlier reports on the co-transformation of wheat with multiple genes [28]. Integration of transgenes into the genome of putative transformants was confirmed by PCR for *chi*, *glu*, *npt-II*, *bar*, and *ubi* transgenes. Stable transformation was confirmed by enzyme assays and Southern analysis.

Multiple bands observed in Southern hybridization of eight transgenic events with *G11* provide evidence of multiple integrations of the transgene. Direct transformation methods are known to produce complex events in which multiple copies of the introduced DNA integrate at one or several loci in the recipient genome [29]. The influence of transgene copy number on gene expression levels is known to be complex. Earlier, it was anticipated that increasing the transgene copy number would enhance expression. However, it is now known that multiple gene copies frequently lead to co-suppression and gene silencing [30]. In contrast, a single copy of the transgene can also be silenced [31]. For example, silencing was observed in transgenic petunia plants with a single copy of the transgene [32].

The expression level also depends on the nature of the promoter used, as demonstrated by [33]. In their studies, they reported that the chitinase gene driven by the CaMV35S promoter was silenced, whereas the bar gene under the control of the maize *ubiquitin* promoter was not silenced in transgenic wheat. Furthermore, the present study confirms that gene silencing is not solely attributable to multiple transgene insertions, as observed in transgenic lines *G11*-4, *G11*-6, and *G11*-7, which over express *G11*. Other studies have also shown that multiple transgene copies do not necessarily lead to silencing [34]. Hence, transgene copy number can have a positive [35, 36], negative [37], or no effect [38] on transgene expression.

Transgenic lines containing the *AGLU*I gene were confirmed by Southern analysis with specific probes. Among the six independent lines tested, two transgenic events (P-1 and P-2) showed two bands; whereas four transgenic events (P-3 to P-6) exhibited three bands (see Figure 6). These results indicate that the *AGLU* gene was stably integrated into the sugarcane genome and was present in multiple copies. Transgene expression was confirmed by Western blotting. Transgenic events (*G11*-1 to *G11*-9) produced chitinase of the expected size (32kDa). The antiserum against chitinase detected a 32kDa protein in all nine transgenic plants and in the positive control. Similar results were observed for chitinase polypeptide expression in Pusa Basmati 1 and IR72 rice plants, which were associated with enhanced resistance to rice sheath blight, as reported [39, 40, 41].

In the present study, the chitinase activity of these transformed plants was higher than that of the untransformed control plant, indicating an increase in chitinase activity due to the introduction and expression of *G11*. Promoters play a significant role in gene expression, and this was studied in monocotyledons using the *ubiquitin* and CaMV35S promoters [42, 43]. Different levels of resistance to the red rot pathogen were observed across transgenic events. Transgenic rice plants that over expressed TLP genes and showed increased resistance to fungal infection [33]. Earlier reports described the deployment of the aprotinin gene for top borer resistance in transgenic sugarcane events [44]. Another report stated that transgenic *Brassica napus* plants that constitutively expressed a chimeric chitinase gene [45]. Transgenic rice plants with chitinase had fewer lesions and delayed the onset of disease caused by *Rhizoctonia solani* [46]. A similar improvement in disease resistance was observed in transgenic sugarcane events in our study.

In event *G11*-4, initial discoloration was observed only at the point of entry in the nodal region. Upon splitting the canes open and examining them, it was observed that the pathogen had entered the internodal region, and the spread of the disease was slower than in untransformed plants, indicating moderate resistance. These results are consistent with those reported by various researchers across multiple plant species. For example, transgenic cucumber plants containing a rice-chitinase gene exhibited enhanced resistance to grey mold disease caused by *Botrytis cinerea* [47]. The transgenic greenhouse experiments reported in this study are similar to studies on the protective synergistic interactions of chitinase/glucanase in transgenic tobacco, tomato, and carrot plants [15, 48]. Seven transgenic events with chitinase and glucanase (P-6, P-8 to P-14) were classified as moderately susceptible, based on partial drying of foliage and slow disease spread. Transgenic events P-2 and P-4, with green foliage and no red discoloration, showed moderate resistance to red rot disease. It can be concluded that co-expression of the two antifungal genes, RCH10 and *AGLU*I, has conferred some resistance to red rot infection. Expression of three antifungal proteins—trichosanthin, tobacco class I chitinase, and tobacco class I glucanase—in *E. coli*, and the recombinant proteins were tested for antifungal activity. All three proteins exhibited inhibitory activity, and their combined activity was enhanced [49].

In general, the incorporated antifungal genes, used alone or in combination for red rot resistance, were successful. However, the level of resistance varied among individual transgenic events, suggesting that the site of integration may have influenced the level of transgene expression. To enhance the protective effects of PR (Pathogenesis-Related) genes, ongoing studies focus on combining antifungal protein genes by hybridizing homozygous parents that express different PR proteins. In our research, both transgenic events over expressing a single gene (*G11*) and combinations (RCH10 and *AGLU*1) show moderate resistance to red rot disease.

This potential resistance could help farmers mitigate yield losses caused by *C. falcatum*. However, further experiments are needed to better understand the underlying mechanism. These additional experiments may include conducting Western blots to quantify the levels of chitinase (RCH10) and glucanase (*AGLUI*) proteins, analyzing the localization of the chitinase protein within sugarcane cells, and assessing the pathogen's susceptibility to the chitinase protein in vitro. Such investigations will contribute to a more comprehensive understanding of the protective mechanisms at play.

Table 1: Primer sequences used for the detection of G11 and AGLU genes in putative transgenic

Gene	Primer Sequence
G11 FP	5'TAAGGGCTTCTAACCTACGA3'
G11 RP	5'CGTCTGCTCGGATCAAATATCAAC3'
AGLU FP	5'CCTTGGCTTCTCCTCCTT3'
AGLU RP	5'GAAGGTCGGAATTAGGCACA3'
Bar FP	5'AACCACTACATCGAG ACAA3'
Bar RP	5'CAACCACGATCTTGAAGCCC3'
Ubi FP	5'TGACAACAGGACTCTACA GT3'
Ubi RP	5'CGTCCTGCCGTCGTGCCGTGAG3'
nptII FP	5'ATGATTGAACAAGAT GGATTGCAC3'
nptII RP	5'TCAGAAGAACTCGTCAAGAAGGCG3'

Table 2: Red rot reaction of transgenic events. Where, * on a 0-9 scale score/symptoms HS: Highly susceptible; MS: Moderately susceptible; MR: Moderately resistant

Genotype	Top	Nodal transgression	Lesion width	White spot	*Reaction
Control	Dry	3	3	3	HS
G11- 4, 6, 9, and P- 1, 3, 5, 7, and 13	Dry	3	3	3	HS
G11- 2, 3, 5, 7, and 8	Dry	3	3	3	HS
P- 6, 8, 9, 10, 11, 12, and 14	Pale green	2	3	1	MS
G11- 1	Green	2	2	0	MR
P- 2	Green	2	2	0	MR
P- 4	Green	2	2	0	MR
G11+M1 and *G11*+M2	Green	2	2	0	MR

Table 3: Categorization of red rot reactions (Srinivasan and Bhat, 1961)

Score on the 0–9 Scale	Reaction category
0.0–2.0	Resistant (R)
2.1–4.0	Moderately resistant (MR)
4.1–6.0	Moderately susceptible (MS)
6.1–8.0	Susceptible (S)
8.1–9.0	Highly susceptible (HS)

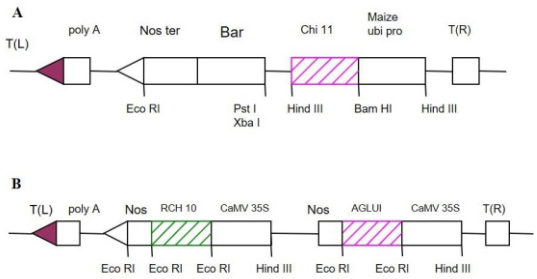

Figure 1: Graphical representation of binary vectors (pAHG11; A and pZ100; B) used for the development of transgenic events

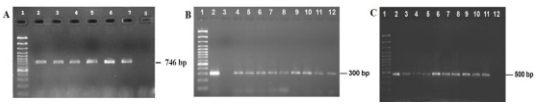

Figure 2: PCR analysis for chitinase (746bp; A), bar (300bp; B), and ubi (500bp; C) gene in putative transgenic events and pAHG11 construct. An empty lane represents untransformed control.

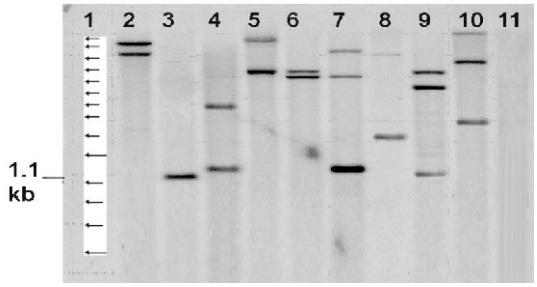

Figure 3: Southern blot analysis for G11 transformed plants (V_0 generation). Plasmid and genomic DNA samples were digested with Hind III and allowed to hybridize with the G11 probe. Lane 1: 1 Kb ladder, Lane 2: DNA from transgenic plant undigested, Lane 3: Plasmid DNA of pAHG11, Lanes 4-10: DNA from transformed plants (G11-1 to G11-7), Lane 11: DNA from untransformed control.

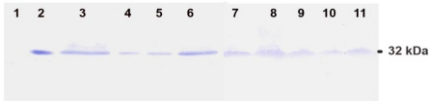

Figure 4: Western blot analysis for chitinases in transgenic expressing the G11 gene. Lane 1: untransformed control, Lane 2: positive control, Lane 3-11: protein extract from transgenic of G11-1 to G11-9

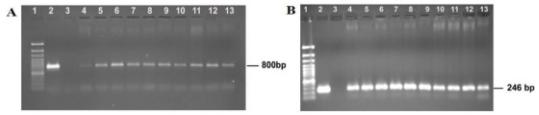

Figure 5: PCR analysis of npt II (800bp; A) and AGLU1 gene (246bp; B), Empty Lane represents untransformed control

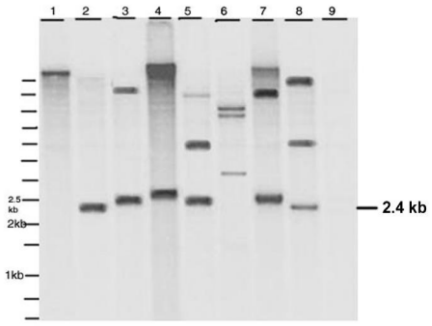

Figure 6: Southern blot analysis for AGLUI transformed plants (V_0 generation). Plasmid and genomic DNA were digested with BamHI and allowed to hybridize with the AGLUI probe. Lane 1: DNA from transgenic plant undigested, Lane 2: Plasmid DNA of pZ100, Lanes 3-8: DNA from transgenic events (P1 to P6), Lane 9: DNA from untransformed control

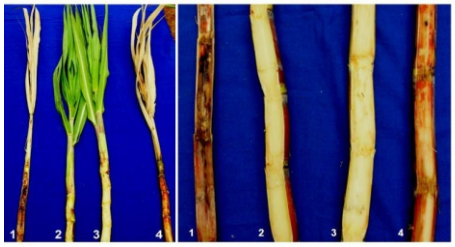

***Figure 7**: Screening of transgenic events expressing G11, RCH10, and AGLUI genes against red-rot pathogen. 1. Susceptible control, 2 and 3 transgenic events showing increased resistance, 4. Susceptible transgenic event*

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