CHARACTERISATION OF TRANSFORMED SUGARCANE CARRYING ANTI-FUNGAL *β-1,3-GLUCANASE* GENE

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
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MASTER OF SCIENCE
in
BIOTECHNOLOGY
(Minor Subject: Plant Breeding and Genetics)

By

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(L-2014-A-170-M)

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CERTIFICATE-I

This is to certify that the thesis entitled. **"CHARACTERISATION OF TRANSFORMED SUGARCANE CARRYING ANTI-FUNGAL *β-1,3-GLUCANASE* GENE"** submitted for the degree of **Master of Science** in **Biotechnology** (Minor subject: **Plant Breeding and Genetics**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Ms. Shivani Nayyar (L-2014-A-170-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the thesis entitled, **"CHARACTERISATION OF TRANSFORMED SUGARCANE CARRYING ANTI-FUNGAL *β-1,3-GLUCANASE* GENE"** submitted by **Shivani Nayyar (L-2014-A-170-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Biotechnology** (Minor subject: **Plant Breeding and Genetics**) has been approved by the Student's Advisory Committee along with External Examiner after an oral examination on the same.

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ABSTRACT

The present study entitled "Characterisation of transformed sugarcane carrying anti-fungal *β-1,3-glucanase* gene" was carried out to characterize *in planta* transformed sugarcane plants. The objective of this study was to induce red rot resistance caused by *C. falcatum*. The *in planta* transformed T_0 putative transgenic plants (10) were propagated through plant to row progenies leading to formation of 295 axillary bud setts, out of which 127 setts sprouted representing the clonal generation 1 (CG_1). The PCR analysis on T_0 putative transgenic plants of CG_1 (127 clones) revealed the amplification of 1764 bp *β-1,3-glucanase* transgene in six putative transgenic plants. The RT-PCR pointed towards the differential expression of transgene in all the six plants and the relative *β-1,3-glucanase* transgene expression by qRT-PCR confirmed that three plants had 3.197, 4.280 and 4.438 fold increase in transgene expression as compared to non-transformed plant. Bioassay for red rot on six qRT-PCR positive plants with Cf08 pathotype demonstrated that three plants showed resistance against red rot and with Cf09 pathotype demonstrated that two plants showed moderate resistance against red rot. The CG_2 plant analysis through qRT-PCR revealed similar relative transgene expression in the clones suggesting absence of chimerism.

Keywords: Sugarcane, *Agrobacterium* mediated *in planta* transformation, *β-1,3-glucanase*, PCR, RT-PCR, qRT-PCR, Bioassay, Disease Resistance

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ਮੌਜੂਦਾ ਅਧਿਐਨ "ਉੱਲੀ ਵਿਰੋਧੀ *β-1,3-ਗੁਲਾਕਾਨੇਜ਼* ਜੀਨ ਵਾਲੇ ਸਥਾਨਾਂਤਰਨ ਗੰਨੇ ਦਾ ਚਿੱਤਰ-ਚਿਤਰਣ" ਸਿਰਲੇਖ ਅਧੀਨ ਪਲਾਟਾਂ ਸਥਾਨਾਂਤਰਨ ਗੰਨੇ ਦੇ ਬੂਟਿਆਂ ਦਾ ਚਿੱਤਰ-ਚਿਤਰਣ ਕਰਨ ਲਈ ਕੀਤਾ ਗਿਆ। ਇਸ ਅਧਿਐਨ ਦਾ ਮਕਸਦ *C. falcatum* ਦੁਆਰਾ ਹੋਣ ਵਾਲੇ ਤਾਣੇ ਦੁਆਲੇ ਪੱਤੇ ਦੇ ਝੁਲਸ ਰੋਗ ਲਈ ਪ੍ਰਤੀਰੋਧਕਤਾ ਨੂੰ ਉਤਪ੍ਰੇਰਤ ਕਰਨਾ ਸੀ। ਟੀ$_0$ ਪੁਟੇਟਵਿ *ਇੰਨ-ਪਲਾਟਾਂ* ਸਥਾਨਾਂਤਰਤ ਬੂਟਿਆਂ (10) ਨੂੰ ਪੌਦੇ ਤੋਂ ਕਤਾਰ ਵੰਸ਼ਜਾਂ ਜ਼ਰੀਏ ਉਗਾਇਆ ਗਿਆ ਜਿਸ ਨਾਲ 295 ਐਗਜ਼ਲਰੀ ਬੱਡ ਸੈਟ ਤਿਆਰ ਹੋਏ, ਜਿਹਨਾਂ ਵਿੱਚੋਂ 127 ਸੈਟਾਂ ਅੰਕੁਰਿਤ ਹੋਏ ਜਿਸ ਤੋਂ ਕਲੋਨਲ ਜਨਰੇਸ਼ਨ 1 (CG_1) ਦੀ ਪੁਸ਼ਟੀ ਹੋਈ। CG_1 (127 ਕਲੋਨ) ਦੇ ਟੀ$_0$ ਪੁਟੇਟਵਿ ਸਥਾਨਾਂਤਰਤ ਬੂਟਿਆਂ ਦੇ PCR ਮੁਲਾਂਕਣ ਤੋਂ ਛੇ ਪੁਟੇਟਵਿ ਸਥਾਨਾਂਤਰਤ ਬੂਟਿਆਂ ਵਿੱਚ 1764 ਬੀ.ਪੀ. *β-1,3-ਗੁਲਾਕਾਨੇਜ਼* ਟ੍ਰਾਂਸਜੀਨ ਦਾ ਵਿਸਥਾਰ ਹੋਇਆ। RT-PCR ਮੁਲਾਂਕਣ ਤੋਂ ਸਾਰੇ ਦੇ ਸਾਰੇ ਛੇ ਬੂਟਿਆਂ ਵਿੱਚ ਟ੍ਰਾਂਸਜੀਨ ਦੀਆਂ ਵਿਭਿੰਨ ਸਮੀਮਰਨਾਂ ਅਤੇ *β-1,3-ਗੁਲਾਕਾਨੇਜ਼* ਟ੍ਰਾਂਸਜੀਨ ਸਮੀਮਰਨ ਦਾ ਪਤਾ ਚੱਲਿਆ ਜਿਸਦੀ ਪੁਸ਼ਟੀ qRT-PCR ਰਾਹੀਂ ਹੋਈ ਅਤੇ ਗੈਰ-ਸਥਾਨਾਂਤਰਨ ਬੂਟਿਆਂ ਦੇ ਮੁਕਾਬਲੇ ਤਿੰਨ ਬੂਟਿਆਂ ਵਿੱਚ ਫੋਲਡ ਟ੍ਰਾਂਸਜੀਨ ਵਿੱਚ 3.197, 4.280 ਅਤੇ 4.438 ਫੋਲਡ ਦਾ ਵਾਧਾ ਹੋਇਆ। ਛੇ qRT-PCR ਧਨਾਤਮਕ ਬੂਟਿਆਂ ਉਪਰ ਲਾਲ ਰੋਟ ਲਈ Cf08 ਪੈਥੋਟਾਈਪ ਨਾਲ ਕੀਤੇ ਗਏ ਜੈਵਿਕ ਐਸੇ ਤੋਂ ਪਤਾ ਚੱਲਿਆ ਕਿ ਤਿੰਨ ਬੂਟਿਆਂ ਨੇ ਲਾਲ ਰੋਟ ਵਿਰੁੱਧ ਪ੍ਰਤੀਰੋਧਕਤਾ ਵਿਖਾਈ ਅਤੇ Cf09 ਪੈਥੋਟਾਈਪ ਨਾਲ ਨਾਲ ਕੀਤੇ ਗਏ ਜੈਵਿਕ ਐਸੇ ਤੋਂ ਦੋ ਬੂਟਿਆਂ ਨੇ ਲਾਲ ਰੋਟ ਪ੍ਰਤੀ ਦਰਮਿਆਨੇ ਪੱਧਰ ਤੱਕ ਪ੍ਰਤੀਰੋਧਕਤਾ ਵਿਖਾਈ। qRT-PCR ਰਾਹੀਂ ਕੀਤੇ ਗਏ CG_2 ਪੌਦਾ ਮੁਲਾਂਕਣ ਤੋਂ ਕਲੋਨਾਂ ਵਿੱਚ ਇੱਕ ਸਮਾਨ ਵਿਵਹਾਰਕ ਟ੍ਰਾਂਸਜੀਨ ਐਕਸਪ੍ਰੇਸ਼ਨ ਦਾ ਪਤਾ ਚੱਲਿਆ ਜਿਸ ਤੋਂ ਕਾਇਮੇਰੀਜ਼ਮ ਦੀ ਅਣਹੋਂਦ ਦੀ ਪੁਸ਼ਟੀ ਕੀਤੀ

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CONTENTS

CHAPTER	TITLE	PAGE NO.
I	INTRODUCTION	1 – 6
II	REVIEW OF LITERATURE	7 – 24
III	MATERIAL AND METHODS	25 – 35
IV	RESULTS AND DISCUSSION	36 – 46
V	SUMMARY	47
	REFERENCES	48-59
	APPENDICES	i – viii
	VITA	

CHAPTER – I

INTRODUCTION

Sugarcane (*Saccharum* spp.) belongs to the major grass family *Poaceae* and sub-family Panicoideae (Watson *et al* 1985). Brazil is the largest producer of sugarcane with 768 million metric tons, followed by India, China and Pakistan (FAOSTAT 2014). In India, it is cultivated with total area coverage of 4.2 million hectare (M ha) and is the second most important agro industrial crop next to cotton (Jayashree *et al* 2010). It is an important crop for food and energy production, emerging as a pivotal biofactory for high-value products such as molasses, alcohol, fuel sugar, biofibres, waxes and bioplastic for domestic and export purposes. India is the largest consumer of sugarcane in the world with annual consumption of about 19 million MT (Metric Tons) (Vishwakarma 2013). Ever increasing population and uprising sugar consumption forced farmers to extend the cultivation beyond fertile agricultural lands. In the future, the production may not be sufficient to meet the elevating demand for sugar and related products but left with the choice of growing sugarcane in marginal soils with all possible production constraints. Sugarcane production is hampered by various biotic and abiotic stresses. Biotic stresses such as fungal diseases i.e. red rot, bacterial diseases i.e. leaf scald and viral diseases adversely affect the sugarcane production. Besides biotic stresses, abiotic factors are also equally harmful and contribute towards reduced productivity of sugarcane.

Red rot caused by *Colletotrichum falcatum* (perfect stage: *Glomerella tucumanensis*) is the major reason for low productivity of sugarcane production in India (Alexander and Viswanathan 2002). The pathogen *C. falcatum* affects the economically important cane stalks and deteriorates the sugarcane quality. The perpetuation of red rot is through infected setts, diseased debris and resting propagules in the soil. Symptoms of red rot vary depending on the susceptibility of the sugarcane cultivar, pathogen virulence and the environment. The most specific symptom of the disease is the drying up of the canes, which when split open will show red coloration of the internal tissue with horizontal white patches and the typical foul smell. The disease-infected canes exhibited 25-75 % reduced sucrose content compared to healthy canes (Viswanathan and Samiyappan 1999). A considerable part of sugarcane crop is lost each year due to this disease (Malik and Munir 1990). In India, the red rot disease was responsible for the elimination of many elite commercial varieties such as Co 312, Co 658, Co 997, Co 1148, Co 6304, CoC 671, CoC 8001, CoC 85061, CoC 86062, CoC 90063, CoC 9206 and CoJ 64. About 100 % yield loss has been reported in the recent red rot epiphytotics in Tamil Nadu (Viswanathan and Samiyappan 2000).

Available disease management practices are cultural practices, resistance breeding, biological control and chemical control. Cultural and physical control are not much effective as no cultivar completely resistant to fungus has been found, so far. It is difficult to manage red rot through chemotherapy due to the impervious nature of rinds and fibrous nodes at cut ends that do not allow sufficient absorption in setts (Agnihotri 1990). Limited success was achieved under field conditions with various fungicides (Singh and Singh 1989) as it leads to drastic effect on soil biota, pollutes the atmosphere and is environmentally harmful. Thermotherapy is though useful for inactivation of pathogen (Singh 1973) but it is difficult to remove deep-seated infections and has limitation to check the secondary infection. Moreover, most of management of the disease is by the use of disease-free seed canes for planting which is impractical due to the difficulty in diagnosis of the dormant infections in seed canes under field conditions. Several efforts are continued to increase the life of the high sugar cultivars at farmer's field (Kalaimani 2000). Among the different approaches attempted to tackle the disease, cultivation of resistant cultivars failed due to the development of new variants, as *C. falcatum* is a facultative parasite which keeps on mutating and as a result new races of the pathogen are frequently emerging. Adaptation of the pathogen to the newer sugarcane cultivars is considered as a major cause for the disease susceptibility. Therefore, in the long term, the development of crops that possess durable genetic resistance provides the best solution for effective, economical and environmentally safe control of the epidemic spread of notoriously difficult to manage red rot disease.

An effective alternative is biological control by the use of antagonistic micro-organisms against phytopathogenic fungi (Howell 2003). *Trichoderma* has been extensively used as a biocontrol agent for fungal plant pathogens (Papavizas 1985). It is free living fungus common in soil and root ecosystems. It decreases growth, survival, or infections caused by pathogens by different mechanisms like competition for food and space, mycoparasitism, hyphal lysis and production of various secondary metabolites that include mainly antibiotics and cell wall lytic enzymes. The direct mycoparasitic activity of *Trichoderma* spp. is the major mechanisms proposed to explain their antagonistic activity against soil-borne plant-pathogenic fungi (Ridout *et al* 1986). Singh (1994) reported that sett treatment with *T. harzianum* improved germination and were effective against red rot disease development in the field. Mohanraj *et al* (2002) reported that red rot can be managed by the application of *T. harzianum* since it directly acts on *C. falcatum* and induces systemic resistance in plants developed from treated setts. Srivastava *et al* (2006) observed increased germination and yield parameters of sugarcane by the use of *Trichoderma*.

Mycoparasitic *Trichoderma* produces cell wall degrading enzymes which allows to bore holes into other fungi and extract nutrients for their own growth. *β-1,3-glucanases* and chitinases have found to be directly involved in the mycoparasitism interaction between *Trichoderma* and its hosts (Kubicek *et al* 2001). Chitnases hydrolyase chitin and *β-1,3-glucanases* hydrolyse β-1,3-linked glucans belonging to PR-1 and PR-2 family respectively. *β-1,3-glucan* and chitin are the major structural component of fungal cell wall. *β-1,3-glucanases* and chitnases act in concert to degrade the chitin and *β-1,3-glucan* component of cell wall, and hence inhibit the fungal growth at hyphal tips.

Abiotic and biotic environmental parameters such as low temperature, dry soil and antagonistic soil bacteria have negative influence on the biocontrol efficacy of *Trichoderma* strains (Kredics *et al* 2003). Therefore, there are reports on isolation and cloning of genes encoding antifungal enzymes from *Trichoderma* (Sharma *et al* 2011). Lora *et al* (1995) studied heterologous expression of *endo-β-1,6-glucanase* gene from the mycoparasitic fungus *T. harzianum*. Montagu (1998) cloned and studied the expression of *β-1,6 glucanase* from *T. harzianum*. Biochemical characterization showed that enzyme has specificity for β-1,6-linkages. El-Katatny *et al* (2001) purified and biochemically characterized *endo-β-1,3-glucanase* from *T. harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. Recently, antifungal gene encoding for *β-1,3-glucanase* (GenBank accession no. GU300766) was isolated from a local potent strain of *T. viride* at Molecular Biology Laboratory, School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana. The gene shares a sequence homology with the antifungal genes earlier isolated from *Trichoderma* indicating a similarity between the characteristic properties and structures of enzymes with the antifungal genes earlier isolated from *Trichoderma*.

A strategy proposed to increase fungal protection has been the over-expression of PR proteins in genetically engineered crops (Velazhahan and Muthukrishnan 2004). There are several reports where gene cassettes carrying antifungal genes (chitinase and *β-1,3-glucanase*) have been used to genetically engineer plants to enhance their protection against fungal pathogens (Ceasar and Ignacimuthu 2012). Genes encoding PR proteins were overexpressed in tobacco (Zhu *et al* 1994), rice (Nishizawa *et al* 2003) and Indian mustard (Mondal *et al* 2007), which resulted in enhanced tolerance of transgenic plants to fungal pathogens. The transgenic expression of *chit42* and *chit33* genes encoding *T. harzianum* endo-chitinases, increased fungal tolerance in apple (Bolar *et al* 2001), broccoli (Mora and Earle 2001), carrot (Baranski *et al* 2008) and lemon (Distefano *et al* 2008). Another report on cloning of *β-1,3-glucanase* gene (*bgn13.1)* from *T. harzianum* followed by its transfer in strawberry through

Agrobactrium-mediated transformation was given by Mercado *et al* (2015) that revealed the antifungal activity against *Colletotrichum acutatum.*

Several parameters require optimization at the cultivar level in sugarcane to reach higher transformation efficiencies (Scortecci *et al* 2012). The first protocol developed for genetic transformation of sugarcane was particle bombardment of cell suspension (Bower and Birch 1992), but the efficiency of the method depends on callus formation and plant regeneration, which varies with genotype and culture conditions (Scortecci *et al* 2012). Arencibia *et al* (1998) reported the recovery of morphologically normal transgenic sugarcane plants from cocultivation of calluses with *Agrobacterium tumefaciens.* Sugarcane plants have been transformed either by *Agrobacterium* mediated genetic transformation (Elliott *et al* 1998, Enrı´quez-Obrego´n *et al* 1998, Enrı´quez *et al* 2000, Manickavasagam *et al* 2004, Zhangsun *et al* 2007 and Kalunke *et al* 2009) or the micro-projectile method (Gallo-Meagher and Irvine 1996, Arencibia *et al* 1999, Ingelbrecht *et al* 1999, Zhang *et al* 1999, Ma *et al* 2004, Jain *et al* 2007, Wu and Birch 2007, Molinari *et al* 2008, Arvinth *et al* 2010 and Weng *et al* 2011) followed by *in vitro* regeneration either by direct indirect or somatic embryogenesis (Snyman *et al* 2000, Kalunke *et al* 2009 , Vander Vyver 2010 and Sandhu *et al* 2016). Though these methods are widely used, they have some disadvantages such as requiring highly sterile conditions, time consuming, occurrence of somaclonal variations, genotype specificity and recalcitrance.

Direct gene introduction for preferred traits into vegetatively propagated sugarcane, requires to avoid somaclonal variation becomes superseding in the selection of regeneration systems (Birch 1997). Feldman and Marks (1987) established *in planta* transformation for *Arabidopsis*, it is a tissue culture-independent process which is simple, less time-consuming and cost effective. There are several reports regarding *Agrobacterium*-mediated *in planta* transformation on many plant species such as peanut (Rohini and Rao 2000), strawberry (Spolaore *et al* 2001), radish (Park *et al* 2005), citrus (Ahmad and Mirza 2005), wheat (Supartana *et al* 2006), pigeon pea (Rao *et al* 2008), rice (Lin *et al* 2009), tomato (Yasmeen *et al* 2009), maize (Mamontova *et al* 2010) and cotton (TianZi *et al* 2010). It was reported that transformation efficiencies through *in planta* transformation were higher (five fold) than conventional tissue culture-based transformation in rice and wheat (Supartana *et al* 2006).

Mayavan *et al* (2013) standardized a protocol for *in planta* transformation of sugarcane using seed as explants. Sugarcane seeds were infected with *Agrobacterium tumefaciens* EHA 105 harboring pCAMBIA 1304-bar plasmid and transformed plants were established without undergoing *in vitro* regeneration. The transformed sugarcane plants were selected against BASTA and screened by GUS and GFP visual assay, PCR and Southern

hybridization. Mayavan *et al* (2015) gave an efficient, reproducible, and genotype-independent *in planta* transformation using sugarcane setts as explant. Sugarcane setts were pricked, sonicated and vacuum infiltered in *A. tumefaciens* C58C1 suspension harboring pCAMBIA 1304-bar plasmid. The three-stage selection process completely eliminated the chimeric transgenic sugarcane plants and variety Co 6907 showed the maximum transformation efficiency (32.6 %).

In transgenic lines besides the confirmation of transgene integration by PCR technique, there are a number of reports on quantification of gene expression through semi-quantitative RT-PCR e.g. tobacco (Tang *et al* 2005), banana (Kumar *et al* 2005), citrus (Barbosa-Mendes *et al* 2009) and maize (Nguyen and Sticklen 2013). Khan (2012) reported the production of putative sugarcane transgenic plants against red rot pathogen *C. falcatum* by introducing Har*Chit* and Har*Cho* genes under the control of constitutive *Ubiquitin-1* promoter and *nos* terminators along with selectable marker gene (*bar*). The putative transgenic plants were confirmed and verified at genomic level by PCR analysis, Southern hybridisation and RT-PCR analysis. Khamrit *et al* (2013) transformed sugarcane calli using *Agrobacterium* strain LBA4404 harbouring antifungal chitinase gene under the control of *CaMV35S* promoter. The transgene integration was confirmed by PCR and RT-PCR.

Transgene copy number has a great impact on the expression level and stability of gene in transgenic plants. Real-time PCR technique (qRT-PCR) has been widely accepted as an accurate, time-saving method on determination of copy numbers in transgenic plants. qRT-PCR has been employed to detect transgene copy number in several plant species e.g. potato (Toplak et al 2004), maize (Shou *et al* 2004) and wheat (Gadaleta *et al* 2011). Casu *et al* (2007) used bioinformatic techniques followed by *in vitro* testing, and identified two genes that appear to occur once per base genome of sugarcane. Using these as reference genes, a high-throughput assay employing qRT-PCR was developed and tested using a group of sugarcane plants that contained unknown numbers of copies of the *nptII* gene encoding kanamycin resistance. Using this assay, transgene copy numbers from 3 to more than 50 were identified.

The activation of defense mechanisms in plants is considered to be consequent upon an initial recognition event in which the host plant detects molecular components of the pathogen, known as elicitors (Van't and Knogge 2002). Sundar *et al* (2008) investigated the induction of PR proteins in sugarcane leaves and suspension-cultured cells in response to treatment with a glycoprotein elicitor isolated from *C. falcatum*. Treatment of leaves and cells with the elicitor resulted in a marked increase in the activities of chitinase and *β-1,3-glucanase* in a red rot resistant plants. Khan (2012) performed pathological assay by spraying

non-transgenic and transgenic plants (Har*Chit* and Har*Cho* genes) with *C. falcatum* spores. Pathogenicity index was developed on the basis of leaf tip dryness, dead heart on leaf surface, mid rib redness and red leisions. Data was collected after two weeks and all transgenic lines showed a decrease in the damage caused by *C. falcatum*. Mercado *et al* (2015) reported that the constitutive expression of *β-1,3-glucanase* gene (*bgn13.1*) from *T. harzianum* improved strawberry tolerance to crown rot diseases caused by *Colletotrichum acutatum* in transgenic lines. Disease severity index (DSI) was calculated according to the (0–5) scale described by Denoyes and Baudry (1995) after six weeks period.

The sugarcane cultivar CoJ 83 is an early maturing, high sugared genotype that performs well under frequent irrigation and high nutrition conditions. The cultivar is highly susceptible to red rot infection. In the present study characterization of transformed sugarcane plants (CoJ 83) carrying *β-1,3-glucanase* antifungal gene cloned from *T. viride* and mobilized in *Agrobacterium* strain LBA4404 to meet following objectives:

- Confirmation of putative transgenics through PCR, RT-PCR and qRT-PCR.
- Challenging of transgenic plants with disease inoculum to evaluate the level of resistance

CHAPTER II

REVIEW OF LITERATURE

Red rot disease caused by fungus *C. falcatum* Went is the major constraint for production of sugarcane in India. About 100 % yield loss has been reported in the recent red rot epiphytotics in Tamil Nadu (Viswanathan and Samiyappan 2000) and therefore called the "Cancer" of sugarcane (Khan *et al* 2011). Cel wall of all fungi is composed of two major components i.e. chitin and glucans. The production of enzymes such as chitinases and glucanases are capable of degrading the cell walls of pathogenic fungi, became the important component of the defense response of the plants against fungal pathogens (Ceasar and Ignacimuthu 2012). The introduction of gene cassettes carrying antifungal genes (chitinase and *β-1,3-glucanase*) into the plant genome could confer resistance against fungal pathogens.

This chapter reviews literature on red rot of sugarcane, management strategies, mode of action of antifungal genes, their isolation, cloning, genetic transformation, expression and overexpression analysis in different transgenic crops. The literature is divided into the following subheadings:

2.1 Red rot of sugarcane: general background and disease management

2.2 Structure of fungal cell wall

2.3 Mode of action of *Trichoderma* spp.

2.4 Isolation and cloning of antifungal gene (glucanases) from *Trichoderma* species

2.5 Genetic transformation of antifungal gene (glucanases)

2.6 *In planta Agrobacterium* mediated genetic transformation

2.7 Characterization of putative transgenics through RT-PCR and qRT-PCR

2.8 Bioassay for red rot resistance

2.1 Red rot of sugarcane: general background

Red rot caused by fungus *C. falcatum* was first reported in Java (Indonesia) known as "*Sereh*" (Went 1893). Earlier the perfect stage of the fungus was identified as *Physalospora tucumanesis* (Carvajal *et al* 1944) and finally known as *Glomerella tucumanesis* (Arx et al 1954). In India, the disease was first noticed in 1895 in Godavari delta of Andhra Pradesh. It is the major reason for low productivity of sugarcane in India (Alexander and Viswanathan 2002). The disease-infected canes showed 25-75 % less sucrose content as compared to healthy canes (Viswanathan and Samiyappan 1999). A major part of sugarcane crop is lost each year due to this disease (Malik and Munir 1990). In India, the red rot disease was responsible for the extinction of many commercial varieties such as Co 312,

Co 658, Co 997, Co 1148, Co 6304, CoC 671, CoC 8001, CoC 85061, CoC 86062, CoC 90063, CoC 9206 and CoJ 64. Red rot infect economically important stalks and leaf mid ribs of sugarcane which resulted in major losses in crop yield and sugar quality. The pathogen produces sufficient amount of acid invertases which break the sucrose into glucose and fructose, thus consumed by the pathogen. Pathogen infection results in increased levels of total soluble salts and acidity thus, leading to decrease in pH, sucrose and purity of cane juice of affected canes.

Sources of infection

The fungus is sett-borne and the perpetuation of red rot is through infected setts, diseased debris and resting propagules present in the soil (Butler *et al* 1913). The pathogen attacks the cane plant from the very beginning i.e. germination stage (Agnihotri 1996) and causes death of germinated seedlings. Post-germination infection in young emerging shoots is due to dormant mycelia present in the bud scales. Favourable conditions for disease development requires mean temperature (29.4 - 31°C), pH 5-6, high atmospheric humidity (90%) and water-logged conditions of the soil.

Dissemination

Red rot manifestation varies depending on the nature of infection, time of the season and the prevailing environment. Diagnostic symptoms are observed during monsoon or post monsoon period (Agnihotri *et al* 1977). The secondary spread of the fungus during monsoon is mediated through irrigation and rain water which results in the infection of mid-rib, lamina, leaf sheath and stalk, while in winter air currents aid in the spread of the pathogen.

Mode of infection

The pathogen mainly infects canes through nodes and main portal of entry are leaf scar, growth ring and buds. After the fungus invades the tissues of the stalk, the mycelium spread from cell to cell leading to gum formation through the vascular bundles. Infected internode tissues develop a rot with a characteristic red colour that often contains interspersed areas with normal colour called “white spots”.

Disease Management

Management of red rot has been a challenging area of work for the pathologists and sugarcane breeders as the epiphytotic of the disease depends upon weather conditions, genotypes, presence of virulent pathogen and time for disease development. These factors must be studied in depth so as to achieve effective control of the disease. Thus, there is a need for understanding the basic mechanism operating in sugarcane-red rot pathogen interaction and to find out conditions which favor development of new races of *C. falcatum.*

a. **Use of fungicides**: A number of fungitoxicants have been tried but a little success is found in controlling red rot because of impervious nature of rind, presence of fibrous nodes at the cut ends, low solubility of fungicides, lack of broad spectrum fungicides and presence of abundant nutrients in the sett (Satyavir 2003).

b. **Themotherapy:** Thermotherapy (moist hot air or hot water) is though useful for inactivation of red rot pathogen (Singh 1973) but it is difficult to remove deep-seated infections and also limitation to check the secondary infection.

c. **Use of biocontrol agents**: An alternative strategy that is ecologically sound and environmentally safe for the management of soil-borne diseases is the use of biocontrol agents (Harman *et al* 2004). Many fungal and bacterial antagonistic strains suppress the growth of pathogens by competition for food and space, hyperparasitism, hyphal lysis and production of various cell wall lytic enzymes. Fluorescent *pseudomonads* and *Trichoderma* spp. have been exploited for the management of red rot in sugarcane. Abiotic and biotic environmental parameters such as low temperature, dry soil and antagonistic soil bacteria have negative influence on the biocontrol efficacy of *Trichoderma* strains (Kredics *et al* 2003). So it is important to understand the most common contributing factors that underpin the control of red rot. Only in-depth understanding of pathogenecity of disease causing organism and agronomics of the crop will allow to develop effective, integrated disease control.

2.2 Structure of fungal cell wall

The fungal cell wall accounts for 20–30 % of the dry weight of fungal cells (Klis 1994). It is composed of chitin, glucans and glycoproteins that are covalently cross-linked together (Figure 1). Chitin and *β-glucans* are main components of fungal cell wall, chitin is a linear polysaccharide composed of β-1,4 linked N-acetylglucosamine units existing as α and β-chitin in nature. *β-glucans* are composed of glucose units, *β-1,3-glucan* also known as laminarian, which is the primary glucan composed of polymer of D-glucose in a β-1,3 configuration arranged in a helical coil, several other linkages such as α-1,3, β-1,4 and β-1,6 have also been detected. There are two classes of glycosylated cell wall proteins (CWPs) present, which are linked via either through a glycosylphosphatidylinositol (GPI) anchor or a Pir (protein with internal repeats) sequence to the cell wall (Kapteyn *et al* 1999). The *β-1,3-glucan* molecules are held together by hydrogen bridges which form a three-dimensional network that covers the whole cell. Chitin is attached at the interior side of the cell wall, whereas *β-1,6-glucan* and Pir-CWPs are attached on the exterior side. Infrequent linkage of chitin to *β-1,6-glucan* has also been observed.

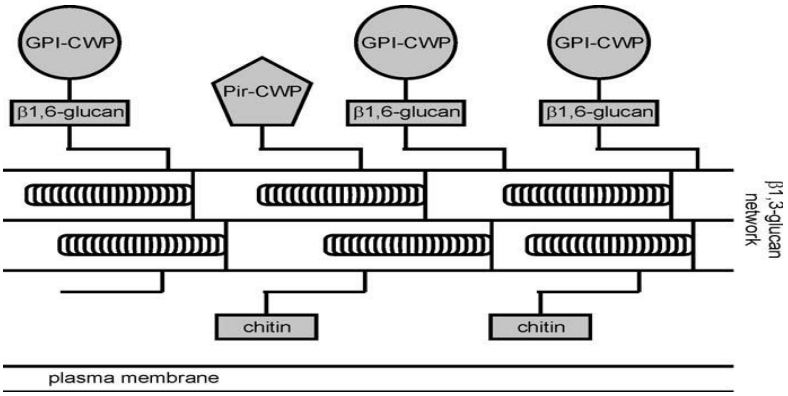


Fig.1: Schematic of fungal cell wall

(GPI: glycophosphatidylinositol, Pir: proteins with internal repeats, CWP: cell wall proteins)

2.3 Mode of action of *Trichoderma* spp.

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. *Trichoderma* strains have been recognized as biological agents for their ability to increase crop productivity, resistance to biotic and abiotic stresses, and uptake of nutrients. Antagonism is one of the major mechanisms involved in mycoparasitism of the *Trichoderma* against phytopathogenic fungi and the process of parasitizing phytopathogenic fungi includes several stages: (1) host-directed growth of *Trichoderma*; (2) recognition of the host and attachment; (3) synthesis and secretion of antibiotics and extracellular enzymes hydrolyzing cell wall components; (4) penetration of hyphae; and (5) lysis of the host. Ultrastructural and histochemical studies reported that enzymes of *Trichoderma* causes localized lysis of cell walls of phytopathogenic fungi at points of contact between the antagonist and the host (Benhamou and Chet 1993). When a *Trichoderma* species is grown in a medium supplemented with fungal cell walls, a significant increase in the production of extracellular chitinases, glucanases and proteinases is found, which forms the basis for the suggestion that lytic enzymes produced by some *Trichoderma* species play an important role in the degradation of fungal pathogens (Chet *et al* 1981).

Chitinases and glucanases are the major hydrolytic enzymes of fungal cell wall, which degrades fungal cell wall by hydrolyzing polymers of glucose with various glycosidic linkages. These enzymes are usually extracellular, low molecular weight, highly stable and are produced in multiple forms or isozymes that differ in charge, size, regulation and stability (Koga *et al* 1991). Glucanases, that hydrolyse the different glucans, are potent antifungal proteins. The most abundant glucanases in the context of host defence are the *β-1,3-glucanases* (EC 3.2.1.39). Further, there are two types of glucanase: exo *β-1,3-glucanase* and endo-*β-glucanase*. Exo *β-1,3-glucanase* hydrolyse the laminarin by sequentially cleaving

glucose residues from the non-reducing end of the polymers or oligomers. Endo *β-1,3-glucanases* act by cleaving the β-1,3-linkages at random sites along the polysaccharide chain releasing smaller oligosaccharides (Cohen-Kupiec *et al* 1999). The oligosaccharides released have been shown to be perceived by the plant cell as elicitors and induce active defence responses (Ryan and Farmer 1991) or the enhanced production of plant protection proteins (Ebel and Scheel 1992). The antifungal mechanism of glucanases can be divided into direct and indirect activities. The direct antifungal activity was due to the digestion of the *β-1,3-glucans* in fungal cell walls, which leads to a weakened cell wall and cell lysis. The indirect effect of glucanases, which has also been observed with chitinases, is due to partial digestion of glucans and chitin.

2.4 Isolation and cloning of antifungal gene (glucanases) from *Trichoderma* species

Among fungus, *Trichoderma* spp. is a major source of antifungal genes that have been cloned and introduced in plant to induce resistance against fungal diseases. Glucanase genes have been cloned from many fungal strains.

Gonzalez *et al* (1992) cloned a *β-1,4-endoglucanase* encoding gene *egl1* from *Trichoderma longibrachiatum*. After digestion with *Bam*HI and *HindIII*, cDNA was cloned in pUC18 vector and the expression analysis of clone was done in yeast under the control of *cyc-gal* inducible promoter and reported that the yeast clones were able to secrete the active enzyme. Further, sequencing of gene revealed that it is homologous to the *T. reesei egl1* gene. De La Cruz *et al* (1995) reported the purification, molecular cloning, and characterization of an *endo-β-1,3-glucanase* encoding gene, *BGN13.1* from the mycoparasitic fungus *T. harzianum*, which was specific for β-1,3 linkages and has an endolytic mode of action. For cloning of cDNA corresponding to *BGN13*.1, a synthetic oligonucleotide primer was designed based on the sequence of an internal peptide. For PCR analysis, DNA from a unidirectional cDNA library in λgt11-*Sfi-Not* vector was used as the template and a degenerate oligonucleotide corresponding to the internal sequence and the λgt11 reverse primers were designed. The amplified DNA obtained was the C-terminal region of the *β-1,3-glucanase* protein, corresponding mostly to the 3' untranslated region of the gene. This PCR-amplified band was used as a probe for screening the cDNA library and upon screening, several positive clones were selected and isolated and the one containing the longest insert was chosen for further characterization. The cDNA insert of this phage (named *bgn13.1*) was cloned in the *EcoRI-NotI* sites of the pBluescript SKII vector and then was sequenced. Finally, experimental evidence showed that the enzyme hydrolyzes yeast and fungal cell walls.

Soledad *et al* (1998) analysed the β-1,3-glucanolytic system of the biocontrol agent *T. harzianum* and reported that *T. harzianum* (IM 1206040) secretes *β-1,3-glucanase* in the

presence of different glucose polymers and fungal cell walls. The fungus produces at least seven extracellular *β-1,3-glucanases* upon induction with laminarin, a soluble *β-1,3-glucan*. Thus, properties of this complex group of enzymes suggest that the enzyme might play different roles in host cell wall lysis during mycoparasitism. De la Cruz and Llobell (1999) revealed that treatment with *β-1,6-glucanase* derived from *Trichoderma* together with chitinases or *β-1,3-glucanases* resulted in growth inhibition of filamentous fungi.

Donzelli *et al* (2001) cloned a gene (*gluc78*) encoding an antifungal glucan *1,3-β-glucosidase* from strain P1 of the biocontrol fungus *T. atroviride* (formerly *T. harzianum*). The gene encodes for a protein of 770 amino acids, including a 40 amino acids signal peptide and the encoded protein has similarity to a small group of sequences from filamentous fungi and no significant similarity to *β-1.3-glucanases* or glucosidases from other organisms. Symmetry between the first and second halves of the mature protein was found reporting that *gluc78* may have arisen from a duplicated ancestral gene. The chromosomal copy of *gluc78* coding region was subsequently cloned into the pGEMt vector. Wei *et al* (2001) reported that α-1, 3-Glucanases (EC 3.2.1.59) also named mutanases are extracellular enzymes which are able to degrade polymers of glucose bound by α-1, 3-glycosidic links, that can hydrolyse mutan. It is accumulated during vegetative growth as a cell wall component which is the main reserve material within fungi.

Sanz *et al* (2005) reported the purification, characterization and cloning of an *exo-α-1, 3-glucanase* (AGN13.2) encoding gene *agn13.2* from the antagonistic fungus *T. asperellum* T32. Through biochemical characterization of protein encoded by *agn13.2* revealed the presence of glucose monomers, suggesting an exolytic mode of action of protein. To obtain the cDNA sequence encoding *AGN13.2*, specific primers were designed to clone the 5' end of *agn13.2* mRNA using SMART-PCR system and RACE-PCR system was used to clone the 3' end of *agn13.2* mRNA. Expression at the transcription level in confrontation assays against the strawberry pathogen *Botrytis cinerea* strongly demonstrates the role of AGN13.2 during the antagonistic action of *T. asperellum*.

Djonovic *et al* (2006) revealed the involvement of *β-1,6-glucanase* in mycoparasitism and its relevance in the biocontrol activity of *T. virens.* Transformant strains in which *tv-bgn3*, (*β-1,6-glucanase*) from *T. virens* was disrupted or constitutively over-expressed were compared. Gene expression analysis revealed induction of *tv-bgn3* in the presence of host fungal cell walls and deletion of the gene resulted in significantly reduced levels of disease protection compared to the wild type while constitutively over-expressed strains showed a significantly increased biocontrol capability.

Montero *et al* (2007) cloned and characterized the gene coding for *BGN16.3*, a *β-1, 6-glucanase* putatively involved in mycoparasitism by *T. harzianum*. They cloned the cDNA encoding *BGN16.3*. It showed a significant sequence identity (50 %) to *bgn16.1*. *Bgn16.3* is a single-copy gene and highly homologous sequences are present in all tested *Trichoderma* species. The expression pattern of *bgn16.3* analysed by Northern blot revealed that it expressed during the interaction of *T. harzianum* CECT 2413 with *Botrytis cinerea*, supporting the implication of the enzyme in the mycoparasitic process.

Ganiger *et al* (2008) isolated a gene (1.3 kb) encoding *endoglucanase* from *T. harzianum* and its clone was sequenced and analyzed for the homology at the nucleotide and at the protein level for domain analysis. The reported gene showed 97 % homology with the gene encoding *β-1,6-endoglucanase* both at the nucleotide and at protein level and was identified as *β-1,6-endoglucanase* and was submitted to NCBI database having accession number EU747838. Ganiger *et al* (2009) cloned three genes of *Trichoderma* in the yeast expression vector pYES2/CT namely, *β-1, 6-endoglucanase* from *T. harzianum* (pYESh2) and *β-1.3-glucanases* from *T. virens* (pYESV1) and *β-1,4-endoglucanase* from *T. reesi* (pYESR1) in order to study the expression of *endoglucanase* genes cloned from different species. These were screened for glucanase activity in *Saccharomyces cerevisiea* (INVSc 1 strain) and out of all these, *T. reesi* secreted highest activities. The clones of *T. reesi* showed three times more whereas those from *T. harzianum* and *T. virens* showed twice the activity when compared to control.

Wiater *et al* (2011) isolated a *α-1,3-glucanase* encoding gene *mutAW*. They designed degenerate primer TrihN1 based on the N-terminal amino acid sequence of the enzyme produced by the isolate. The RT-PCR reaction includes total RNA of *T. harzianum* to obtain cDNA of the *mutAW* gene without introns with the corresponding 5' end of the mutanase gene primer and oligo $(dT)_{15}$ primer. The PCR product obtained was cloned into pJET1.2 vector resulting in pJET1.2T1 plasmid and for sequence analysis, additional reverse Trih2 and forward Trih22 primers were designed. As a result, a DNA fragment containing the leader sequence of the gene was obtained and a full length gene termed *mutAW* was obtained by combining sequences of these two PCR fragments, which is 2000-bp long that encodes a protein of 634 amino acids. The sequence obtained for the *T. harzianum* CCM F-340 mutanase gene *mutAW* has been deposited in the GenBank database (accession no HQ871941).

Kheiri *et al* (2014) isolated *β-1,3-glucanase* encoding gene *bgn*13.1 from the biocontrol fungus *T. virens*-10 and cloned it in pUC19 cloning vector. For the isolation, the oligonucleotide primers were designed based on the sequence similarity of the *T. harzianum*

bgn gene present in the database (X84085). PCR amplification was performed on the genomic DNA, revealed the specific band of 2.4 Kb that was cloned into a cloning vector and again re-confirmed by PCR. Based on *T. harzianum* (*β-glucanase*) gene sequence, the cloned gene was confirmed by using *Pst*I and *Bgl*I enzymes restriction digestion pattern analysis and sequencing. The sequence obtained was submitted to the NCBI under accession number EF426721 and the cloned fragment obtained was confirmed by molecular analysis and revealed two short introns, 52 bp and 57 bp, and an open reading frame coding 761 amino acids. Under the CaMV35S promoter the *bgn*13.1 gene was over-expressed in *B. napus*, R line Hyola 308 by the transformation of cotyledonary petioles via *A. tumefaciens* LBA4404 through pBIKH1 containing *bgn*13.1 gene.

2.5 Genetic transformation of antifungal gene (glucanases)

Fungal diseases cause considerable loss of yield in many plants species, however natural defence response system in plants produces several enzymes which are capable of degrading the cell walls of invading phytopathogenic fungi. Several conventional plant breeding techniques have been employed to improve disease-resistance genetically, but these techniques are applicable only within sexually compatible species and moreover it can take up to 15–20 years (Rommens and Kishore 2000). A strategy proposed is the incorporation of disease resistant genes through genetic engineering which could produce resistance proteins for improving disease resistance (Van der Biezen 2001). The most widely used approach of developing fungus resistant plants has been over-expression of glucanases in transgenic plants by incorporating an antifungal gene under the control of a constitutive promoter. The identification of the genes that are involved in several disease resistance pathways has opened the way to produce fungal-resistant transgenic plants (Grover and Gowthaman 2003).

Wang *et al* (2003) reported co-transformation of a rice chitinase gene and an alfalfa *glucanase* gene into creeping bentgrass using a *bar* gene as a selectable marker. The selectable marker gene *bar* and the non-selectable chitinase and *glucanase* genes were co-introduced into embryogenic calli using microprojectile bombardment and the putative transgenics were analysed for transgene integration. PCR analysis and Southern blot analyses were carried out to evaluate further the co-transfer and insertion of the chitinase, *glucanase*, and *bar* genes in the genomes of the transformed plants. Moreover, northern analyses were used to determine the expression levels of mRNA products from transcription of the transgenes. The glufosinate-resistant transgenic lines revealed resistance to fungal pathogens, *Sclerotinia homoeocarpa* and *Rhizoctonia solani*.

Anand *et al* (2003) transformed wheat calli using plasmid constructs containing chitinases and *β-1,3-glucanase* genes by particle bombardment method. Putative transgenics

were analysed by PCR for transgene integration and only four transgenic wheat lines showed stable inheritance and expression of either a single transgene or transgene combinations up to four generations. The transgenic lines were evaluated for resistance to scab under greenhouse conditions and a line coexpressing a chitinase and *β-1,3-glucanase* gene in combination, showed a delay in the spread of the infection.

Nishizawa *et al* (2003) reported transformation of rice plants with the stress inducible *β-glucanase* gene (*Gns1)* under the control of CaMV 35S promoter through *Agrobacterium*-mediated transformation. The presence of transgene in transgenic plants was confirmed through PCR using the gene specific primers and revealed that 13 lines out of the 15 primary transformants (R_0 generation) were confirmed to carry the transgene. Through PCR, inheritance of *Gns1* gene in the next generation (R_1) was also confirmed. The expression of *Gns1* gene in leaves was analyzed by RNA gel blotting and protein gel blotting and demonstrated that high-level expression of the *Gns1* gene was inherited by the next generations (R_1 and R_2) in most lines. Further, bioassay for leaf blast on transgenic R_0 and R_1 plants was evaluated by inoculation with *Magnaporthe grisea* which revealed that the transformants with over-expression of *Gns1* exhibited significantly higher resistance to rice blast than that of non-transformants. Wro´bel-Kwiatkowska *et al* (2004) transformed the cotyledons and hypocotyls of flax plants with potato *β-1,3-glucanase* gene through *A. tumefaciens*-mediated transformation. The neomycin phosphotransferase gene (*nptII)* was used as a selection marker for transformed plants. Integration of the transgene was analyzed through PCR using gene specific primers and 26 transgenic lines were confirmed to carry the transgene. Transgenic flax seedlings were analysed for resistance against fungal pathogens by inoculating with *Fusarium oxysporum* and *Fusarium culmorum.* The transgenic plants showed a 2–3-fold increase in resistance to the *Fusarium* infection when compared to the control plants.

Mondal *et al* (2007) reported the transformation of transgenic *Brassica juncea* (cv. RLM 198) with glucanase of tomato flanked by the CaMV 35S constitutive promoter and OCS terminator. The recombinant construct was mobilized into *A. tumefaciens* strain and was used for transformation of hypocotyl explants from 4-day-old seedlings of *Brassica juncea.* The putative transgenics were analysed by PCR and Southern bloting for integration of transgene. Northern hybridization indicated detectable and variable levels of expression of *glucanase* mRNA in the transformed lines. Under pathogen-challenged conditions in poly house, the transgenics showed restricted number, size and spread of lesions caused by *Alternaria brassicae*. Thus indicating towards the potential of glucanase in developing *Alternaria* leaf spot resistance in Indian mustard.

Sridevi (2008) made an effort to combine expression of both chitinase and *β-1, 3-glucanase* genes so as to achieve higher levels of resistance against sheath blight. For that scutellum derived callus of *Oryza sativa* L. subsp. indica variety Pusa Basmati 1 (PB1) was transformed by using *A. tumefaciens* LBA4404 (pSB1) harbouring the binary vector pNSP3 with rice chitinase and tobacco *β-1,3-glucanase* genes. The integration of chitinase and *β-1,3-glucanase* genes was confirmed by Southern blotting by using probes of the 1.1 kb *chi* gene and the 2.5 kb *β-1,3-glucanase* gene, and revealed that four of the six T_0 plants had single copies of complete T-DNAs. Northern and western blot analyses of T_1 plants revealed constitutive expression of chitinase and *β-1,3-glucanase* genes. The enzyme activity was assayed in both transgenic and control plants and demonstrated that homozygous T_2 plants of the single-copy lines CG20, CG27 and CG53 showed 62-fold, 9.6-fold and 11-fold higher chitinase activity over the control plants and *β-1,3-glucanase* activity was 1.1 to 2.5 fold higher in the transgenic plants. The assay of resistance levels of transgenic plants against *R. solani* revealed a 60 % reduction in sheath blight Disease Index in homozygous T_2 plants in the first week whereas disease Index increased from 61.8 in the first week to 90.6 in the third week in control plants, while it remained low (26.8-34.2) in the transgenic T_3 plants in the corresponding period. The results revealed the persistence of sheath blight resistance for a longer period in transgenics.

Wally *et al* (2009) conducted an experiment in which three PR protein genes (chitinase, *β-1, 3-glucanase* and *peroxidase*) were compared for their ability to enhance disease resistance in transgenic carrots, either expressed singly or in combination. Co-transformations of genes encoding an acidic wheat class IV chitinase (383), an acidic wheat *β 1,3-glucanase* (638) and a rice cationic *peroxidase* (*POC1*) were introduced into carrot in different combinations by *Agrobacterium*-mediated transformation. Surviving plants were analysed for the presence of the *bar* gene using PCR and PCR positive plants were screened for expression of the three transgenes using Northern hybridisation. The transgenic plants were analysed for disease resistance by inoculating the leaves of 4-6 month-old plants with either *Botrytis cinerea* or *Sclerotinia sclerotiorum*. The extent of leaf necrosis was determined by counting the number of individual lobed segments per leaflet diseased. The results revealed that disease symptoms were slower to develop and symptoms were reduced by up to 90 % for *B. cinerea* and 70 % for *S. sclerotiorum* in petioles of plants over-expressing POC1 and *β 1, 3-glucanase* (638).

Kheiri *et al* (2014) transformed five days old cotyledonary petioles of the *B. napus* with *β-glucanase* gene (*bgn*13.1) from *Trichoderma virens*-10 by *Agrobacterium*-mediated transformation. The insertion of transgene was verified by the polymerase chain reaction

(PCR) and Southern dot blotting in T_0 generation. The antifungal activity of the expressed *Bgn13.1* from transgenic plants on the actively growing phytopathogenic fungus *S. sclerotiorum*, was tested and RT-PCR positive transgenic lines showed inhibition against hyphal growth of *S. sclerotiorum* whereas crude protein from non transgenic plant did not inhibit hyphal growth of the fungal pathogen tested.

Fortiz *et al* (2013) transformed embryogenic calli of garlic by *Agrobacterium tumefaciens* strain LBA4404 strain having pC2301CHGLU plasmid with *TaCh*, *glu*, *gus* and *nptII* genes (coding for chitinase, glucanase, β-glucuronidase and neomycin phosphotransferase, respectively). The putative transgenics were analysed by PCR for the integration of 800 bp *TaCh* gene and further *in vitro* bioassays were conducted in which transgenic plantlets were challenged by fungal sclerotia. The results revealed that percentage of mycelial invasion (PMI) of *S. cepivorum* varied from 41 to 60 % in transgenic plantlets, whereas in non-transgenic plantlets (control), it was 80 % indicating that the transformed plants were not completely resistant but showed a delay in fungal infection.

Mercado et al (2015) developed transgenic strawberry plants expressing the *β-1,3-glucanase* gene *bgn13.1* from *T. harzianum*, under the control of the CaMV35S promoter by *Agrobacterium* mediated transformation. Several transgenic lines showing higher glucanase activity in leaves than control plants were selected for pathogenicity tests and inoculated with *C. acutatum*, one of the most important strawberry pathogens. The transgenic lines showed lower anthracnose symptoms in leaf and crown than control, thus indicated that *bgn13.1* from *T. harzianum* can be used to increase strawberry tolerance to crown rot diseases.

2.6 *In planta Agrobacterium* mediated genetic transformation

The first "*in planta*" method was reported by Feldmann and Marks (1987) that consisted of the imbibition of *Arabidopsis* seeds in fresh cultures of *Agrobacterium* led to stable integration of T-DNA in the *Arabidopsis* genome and the majority of about 14,000 transformants screened represented independent and unique events. *In planta* transformation is an alternative method which does not involve *in vitro* culture of plant cells or tissues, thereby reducing time, labor cost and most importantly avoiding somaclonal variation encountered during *in vitro* culture-mediated genetic transformation and regeneration.

Mayavan *et al* (2013) gave an efficient, reproducible and genotype independent *in planta* transformation using sugarcane seed as explant. Transgenic sugarcane production through *Agrobacterium* infection followed by *in vitro* regeneration is a time-consuming and highly genotype dependent process. Therefore to obtain more number of transformed sugarcane plants in a relatively short duration, sugarcane seeds were infected with *A. tumefaciens* EHA 105 harboring pCAMBIA 1304-*bar* and transformed plants were

successfully established without undergoing *in vitro* regeneration. The transformed sugarcane plants were selected against BASTA and screened by GUS and GFP visual assay, PCR and Southern hybridization. Highest transformation efficiency was shown when 12-h pre-cultured seeds were sonicated for 10 min, 3 min vacuum infiltered in 100 lM acetosyringone; and 0.1 % Silwett L-77 containing *Agrobacterium* suspension were cocultivated for 72-h. The amenability of the standardized protocol was tested on five genotypes and all the tested genotypes responded favorably, though CoC671 proved to be the best responding cultivar with 45.4 % transformation efficiency.

Mayavan *et al* (2015) gave an efficient, reproducible, and genotype independent *in planta* transformation using sugarcane setts from 6-month-old plants as explant. The sugarcane setts (nodal cuttings) were infected with three *A. tumefaciens* strains harbouring pCAMBIA 1301–*bar* plasmid, and the putative transformants were selected against BASTA and were screened by GUS histochemical assay. Highest transformation efficiency of 29.6 % (with var. Co 62175) was reported when sugarcane setts were pricked and sonicated for 6 min and vacuum infiltered for 2 min at 500 mmHg in *A. tumefaciens* C58C1 suspension containing 100 lM acetosyringone and 0.1 % Silwett L-77. The three-stage selection process completely eliminated the development of chimeric transgenic sugarcane plants. Among the five sugarcane varieties evaluated var. Co 6907 showed the maximum transformation efficiency (32.6 %) and the *in planta* transformation protocol developed could be applicable to transfer the economically important genes into different varieties of sugarcane in relatively short time.

Subramanyam *et al* (2015) reported a tissue culture-independent genetic transformation system for snake gourd using seed as an explant. Snake gourd seeds were infected with three *A. tumefaciens* strains harbouring pGA 492 plasmid, and the putative transformants were selected against BASTA. The maximum transformation efficiency (19.6 %) was recorded when the pre-cultured snake gourd seeds were sonicated for 30-min and vacuum infiltrated for 3-min at 750 mm of Hg in *A. tumefaciens* EHA 105 suspension containing 100 lM of acetosyringone. PCR analysis and Southern blotting confirmed the integration of *bar* gene and the transgene was successfully inherited into the T_1 progeny plants. They reported that *in planta* transformation method could produce transgenic snake gourd plants in a relatively short time (35-days).

Manickavasagam *et al* (2015) reported tissue culture-independent genetic transformation system for okra using seed as an explants and *A. tumefaciens* EHA 105 harbouring the binary vector pCAMBIA 1301–*bar* was used to infect the okra seeds. Maximum transformation efficiency of 18.3 % was recorded when the pre-cultured okra seeds

were sonicated for 30 min, vacuum infiltrated for 3 min in *Agrobacterium* suspension containing 100 lM acetosyringone and co-cultivated for 3 days on a medium containing 100 lM acetosyringone. The GUS histochemical analysis confirmed the *gus* A gene integration and expression, whereas PCR and Southern blot hybridization confirmed the *bar* gene integration and copy number in the transformed okra genome. The transgene was successfully segregated into the progeny plants with a Mendelian inheritance ratio of 3:1 and the *in planta* transformation protocol developed was applicable to transform the okra plants with disease-resistant traits, and the transformed plants can be generated within 60 days.

Shah *et al* (2014) reported transformation of cold tolerant gene (*DREB1A*) driven by Lip9 promoter, into three tomato genotypes through *in planta* mediated *A. tumefaciens* transformation strategy. Overnight imbibed seedlings were surface sterilized and, 3-day old shoot apical meristem of the developing seedling were pierced and incubated for 20 minutes with *A. tumefaciens* strain and then were co-cultivated for 2 days. The regenerated shoots were subjected to hygromycin for selection and the presence of *DREB1A* and *hpt* genes in the hygromycin resistant (T_0) transgenic plants was analysed by PCR. The transgene expression and copy number was detected in T_1 plants by Reverse Transcriptase PCR (RT-PCR) and Southern blotting that showed the stable integration of the transgene to the next generation. Physiological analysis of T_2 transgenic plants such as chilling stresses, stomatal conductance, transpiration rate and relative water contents revealed the increased expression as compared to NT plants.

Subramanyam *et al* (2013) reported an efficient and reproducible *in planta* transformation method using brinjal seed as an explant. The brinjal seeds were infected with *A. tumefaciens* EHA 105 harbouring pCAMBIA 1301-*bar* plasmid, and the transformants were selected against BASTA and were screened by GUS histochemical analysis. Maximum transformation efficiency was shown when the 18-h pre-cultured brinjal seeds were sonicated for 20 min and vacuum infiltered for 3 min at 500 mm of Hg in *Agrobacterium* suspension containing 100 μM acetosyringone and 0.2% Silwett L-77. Among the five brinjal varieties evaluated, Arka Samhitha showed maximum transformation efficiency at 45.66 %. The transgene was successfully transmitted to progeny plants (T_1) which was confirmed by GUS histochemical analysis, PCR and Southern hybridisation.

Rao *et al* (2008) developed a non-tissue culture based method of generating transgenic pigeonpea plants using *Agrobacterium*-Ti plasmid mediated transformation. The protocol involved raising of whole plant transformants (T_0 plants) directly from *Agrobacterium*-infected young seedlings. The plumular and inter-cotyledonary meristems of the seedling axes were targeted for transformation. The transformation conditions involved

pricking of the apical and inter-cotyledonary region of the seedling axes of two-day old germinating seedlings with a needle. Infection with *Agrobacterium* (LBA4404/pKIWI105 carrying *uidA* and *nptII* genes) in a medium containing wounded tobacco leaf extract, co-cultivation in the same medium for 1 h and transfer of seedlings to solirite for further growth and hardening, and subsequent transfer of seedlings to soil in pots in the greenhouse. Out of the 22-25 primary transformants that survived infection-hardening treatments from each of the three experiments, 15 plants on an average got established in the soil under greenhouse conditions. Of the several seeds harvested from all the T_o plants, 600 plants were sown to obtain progeny (T_1) plants and 350 of these were randomly analysed to determine their transgenic nature. PCR was performed for both *gus* (*uidA*) and *nptII* genes. The amplification of both transgenes was observed in 48 of the 350 T_1 plants. Southern blot analysis substantiated the integration and transmission of these genes. The protocol is applicable across different genotypes or cultivars of the crop and offers immense potential as a supplemental or an alternative protocol for generating transgenic plants of difficult-to-regenerate pigeon pea. Further, the protocol offers the option of doing away with a selection step in the procedure and so facilitates transformation, which is free of marker genes.

Abhishek *et al* (2014) developed the tissue culture independent *in planta* transformation protocol for tropical maize by transferring plumular meristem cells of germinating seeds of tropical maize genotype through *A. tumefaciens* infection. *Agrobacterium* strain EHA105 containing vector pCAMBIA3301 carrying *cry1Ab*, *gus* and *bar* gene was used. The expression of transgene *gus* in T_0 plants was confirmed by measuring the hydrolysis rate of the fluorescent substrate 4-methylumbelliferyl-beta-D-glucuronide (MUG) assay whereas the presence of *cry1Ab* gene was analysed by PCR and T_0 plants were allowed to grow in glass house into whole plant until maturity and were selfed to produce seeds of T_1 generation. The presence of transgene and its segregation was studied in T_1 generation through Southern blotting and ELISA indicating the presence of transgene and its expression respectively.

2.7 Characterization of putative transgenics through RT-PCR and qRT-PCR

In plant transgenic lines, the determination of level of transgene expression is essential. Northern blot analysis and RT-PCR are commonly used to analyse transgene expression. These techniques enable qualitative and semi-quantitative analysis of specific RNAs. Northern blot analysis has been used since 1977 to study gene expression. The method includes the use of harmful chemicals and takes several days to complete. RT-PCR is a qualitative method rather than quantitative. Thus, several approaches have been proposed to modify PCR to make it quantitative.

Real time PCR is widely used now for DNA and RNA quantification in different fields. The real-time PCR has advantage of wide dynamic range of quantification, high sensitivity, and high precision, with no post PCR handling, thus avoiding the possibility of cross-contamination and enabling high throughput. The fluorogenic real time PCR technique, is applied in plant molecular biology for determining transgene expression, transgene copy numbers, for analyzing zygosity in transgenic plants and for determining GUS expression. The method uses dual labeled fluorogenic hybridization probes and provided very accurate and reproducible quantitation of gene copies.

The data obtained from quantitative real time PCR experiments is analysed by two different methods: Absolute quantification and Relative quantification. Absolute quantification determines the input copy number of transcript of interest whereas relative quantification describes the change in expression of target gene relative to some reference group. The $2^{-\Delta\Delta C_T}$method is used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Livak and Schmittgen (2001) presented the derivation, assumptions, and applications of $2^{-\Delta\Delta C_T}$ method. The amplification efficiencies of target and reference must be equal. Usually housekeeping genes are used as reference genes. Suitable reference genes for real time quantitative PCR are *GADPH, β-actin, β2-microglobulin* and *rRNA*. Iskander *et al* (2004) developed assays for qRT-PCR analysis of sugarcane to compare *GADPH, β-tubulin,* and *β-actin* in addition to *25S rRNA* gene. Expression levels of *25S rRNA* and *GADPH* were found to be higher and more consistent across tissues as compared with *β-tubulin,* and *β-actin.* Besides the determination of expression level of transgenes, qRT-PCR has been used for transgene copy number studies in cotton, sugarcane, maize, soyabean and wheat. Southern blot analysis is traditionally used to determine transgene copy number, but the method is time consuming, laborious and requires large amount of DNA

A sorghum genotype, KAT 412 was genetically transformed with chitinase (*harchit*) and chitosanase (*harcho*) genes isolated from *T. harzianum* using particle bombardment (Kosambo-Ayoo *et al* 2011). Immature zygotic embryos of sorghum were particle bombarded with vector containing the both genes under the control of the constitutive ubiquitin1 promoter (*ubi*1 promoter) and the nopaline synthase terminator (T*nos*). Stable integration of *harchit* and *harcho* transgenes was confirmed by southern blot and qRT-PCR. Excised leaf segments and seedlings were used in ex-planta and in-planta tests to study the response of T_1 transgenic line to anthracnose caused by *Colletotrichum sublineolum*. Ex-planta studies with leaf segments revealed that, there were differences in the timing and severity of the disease

symptoms among the cultivars studied. Comparison of the leaf segments showed that, the transgenic plants developed less and late symptoms upon infection than the parent Wt plants.

Transgenic potato (*Solanum tuberosum* L.) was developed by the co-transfer of chitinase (*chit*42) and *β-1,3-glucanase* (*bgn*13.1) genes through *Agrobacterium*-mediated transformation (Esfahani *et al* 2010). Micro-tuber slices (approximately 6 months old), containing at least one bud, were infected with *A. tumefaciens* culture containing the plasmid with double gene cassette. Eight transgenic lines were regenerated on selection media which were further analysed for the presence of transgenes through PCR. Five lines out of eight were found to carry both transgene using gene specific primer through PCR. Expression of both chitinase and glucanase genes at the transcriptional level in the transformed potato lines were proven by means of RT-PCR. The amplification of cDNA fragment of expected size was found in only two transgenic lines which confirmed the the stable integration and expression of T-DNA in the genome of transgenic lines. Further, the antifungal activity of transgenes were tested against the phytopathogenic fungus *R. solani* using the radial diffusion assay which showed the resistance in RT-PCR positive plants.

Relative expression analysis of transgene using qRT-PCR and $2^{-\Delta\Delta CT}$ method in sugarcane showed (2000-fold or more) upregulation of EaHSP70 transgene compared to the NC plants. In addition, the upregulation of the abiotic stress-responsive genes, i.e. *DREB2*, *DNA helicase 45*, *LEA*, *RD29*, *ERD*, *ERF*, *Cor15* and *BRICK*, was more than 100-fold in each transgenic event when compared to NC plants imparting tolerance to drought and salinity stress (Augustine *et al* 2015).

Sandhu *et al* (2016) pre-cultured leaf segments of cultivar CoJ 83 with *Agrobacterium* suspension carrying antifungal gene encoding *β-1,3-glucanase* under the control of CaMV 35S promoter and NOS terminator, in presence of acetosyringone. The integration of transgene and its expression in T_0 plants were determined by reverse transcription-PCR and quantitative real time- PCR, respectively. The results revealed 4.15 and 3.89-fold increase in *β-1,3-glucanase* gene expression in plant numbers CoJ 83-11 and CoJ 83-12, respectively as compared to the non-transgenic plant, confirming insertion of the transgene in their genomes.

Assem and Hassan (2008) transformed 500 maize immature embryos using biolistic gene gun and the *Agrobacterium*-mediated transformation systems. The putative transgenics were analysed using PCR for the presence of 484 bp fragment corresponding to *bar* transgene in herbicide resistant events. The level of transgene expression and the copy number of transgene in transformed plant samples was determined by qRT-PCR analysis. Transgene copy number was calculated using $2^{-\Delta\Delta C_T}$ method by calculating the amount of *bar* transcript

in comparison to events transformed by bombardment method. The transgene was present at various levels in different samples with a range of approximately 10 fold. The *Agrobacterium* derived lines exhibited lower copy number for bar transgene per genome (3-7) while bombardment derived lines revealed high copy numbers (8-14 copies).

Toplak *et al* 2004 introduced a real-time PCR method for quantitative determination of transgene expression level in transgenic potato plants containing the gene for coat protein (CP) of potato virus Y strain NTN. The method was compared with northern blot analysis and semi-quantitative RT-PCR in terms of precision and sensitivity and was found to be accurate, sensitive, rapid and reproducible for further application in high-throughput molecular characterization of transgenic lines.

Relative expression analysis of transgene using qRT-PCR and $2^{-\Delta\Delta Ct}$ method in maxican lime showed that overall relative quantification level was highest in plants expressing transgene driven by the 35S promoter followed by RTBV, *rolC*, *AtSUC2* and *RSs1* and it varied among the individual lines tested (Dutt *et al* 2012). Similar study was done in two transgenic lemon clones carrying *chit42* gene from *T. harzianum*. The qRT-PCR analysis showed that the overexpression of *chit42* gene in transgenic clones enhanced transcript levels of other genes involved in disease defence response (Distefano *et al* 2008).

2.8 Bioassay for red rot resistance

Khan (2012) transformed sugarcane with antifungal genes (Har*Chit* and Har*Cho*) by *Agrobacterium* mediated transformation and putative transgenic plants obtained were confirmed and verified at genomic level by PCR analysis, southern blot analysis and reverse transcriptase PCR analysis. After the confirmation at genomic levels, bioassay for red rot resistance was performed in which four transgenic lines, P1, P5, P7 and P9 as well as wild type (non transformed regenerated plants) were completely sprayed with spore solution (1x 106 spores /ml). Equal volume (5ml) of spores was also applied with irrigation water to the roots. Plants were incubated at 24-25°C in phytotron by providing 70-80% relative humidity under 8/16 hrs light and dark rotation. Pathogenicity index was developed on the basis of leaf tip dryness, dead heart of leaf surface, mid rib redness and red leisions and results revealed that P7 showed more resistance against *C. falcatum* as compared to others i.e. 75.36% resistance against C. falcatum as compared to 100% susceptibility for non transgenic plants.

Sundar *et al* (2008) demonstrated the induction of PR proteins in sugarcane leaves and suspension-cultured cells of red rot resistant (BO 91) and susceptible (CoC 671) sugarcane cultivars in response to treatment with *C. falcatum* elicitor. The elicitor was extracted and purified by growing the fungus *C. falcatum* on oatmeal broth under room temperature (28 ± 2 °C) and cell suspension cultures were established from one month old

embryogenic calli of red rot susceptible (CoC 671) and resistant (BO 91) sugarcane cultivars. The cell cultures were treated with elicitor and the samples were collected at different intervals for analysis of chitinase and *β-1,3-glucanase*. A significant increase was observed in the activities of chitinase and *β-1,3-glucanase* in red rot resistant (BO 91) than susceptible (CoC 671) sugarcane cultivar. Changes in the protein profile of sugarcane leaves and cultured cells were analyzed by SDS-PAGE revealed the accumulation of 25 kDa protein predominantly in case of suspension cultured cells of resistant cultivar (BO 91) and 27 kDa protein in the cells of susceptible cultivar. Western blot analysis revealed the induction of a 25 kDa thaumatin-like protein (TLP) in elicitor treated suspension-cultured cells and leaves. The induced TLP in sugarcane treated with elicitor might have involved in resistance against *C. falcatum* and suggested a possible role of PR proteins in defence mechanism of sugarcane against the red rot pathogen.

Singh et al (2014) reported the role of endochitinase gene and efficacy of *trichoderma* against *C. falcatum* went. in sugarcane by inoculating *C. falcatum* (Cf09, Cf08 and Cf401) in the presence of five bioagents (*T. harzianum*, *T. viride*, *T. actoviride*, *T. virens* and *A. awamori*) and data were recorded after 6th days of incubation. The results revealed that *C. falcatum* pathotypes showed the suppression in their radial growth when co-cultured with the bioagents. Radial growths of *C. falcatum* pathotypes in control were 50.0–53.7 mm after 6th day of incubation, while in presence of bioagents radial growth of *C. falcatum* pathotypes ranged between 26.7–29.3, 31.3–33.7, 24.7–25.3, 30.3–33.3 and 30.3–33.0 mm for *T. viride*, *T. virens*, *T. harzianum*, *T. atroviride* and *A. awamori*, respectively. The results revealed that the treatments of healthy setts with spore suspension of *T. harzianum* and *T. viride* along with soil treatment with bioagents were observed to increase germination by 49.35 and 46.7 % respectively.

CHAPTER – III

MATERIAL AND METHODS

The experiments conducted in present study entitled "CHARACTERISATION OF TRANSFORMED SUGARCANE CARRYING ANTI-FUNGAL *β-1,3-GLUCANASE* GENE" were carried out in the Tissue Culture and Transformation Laboratories of School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana during year 2014-2016. This chapter describes the materials used and methods followed for various experiments.

3.1 Plant material

Agrobacterium mediated *in planta* transformed putative transgenic plants (10) of sugarcane cultivar CoJ 83 generated by Kaur (2014) carrying *β-1, 3-glucanase* transgene under the control of CaMV 35S promoter and NOS terminator grown in transgenic glass house of School of Agricultural Biotechnology, Ludhiana were used as experimental plant material. Plant to row progenies of the 10 plants were revived from nodal cuttings (bud setts).

3.2 Clonal propagation of *in planta* transformed T_0 putative transgenic plants

Axillary bud setts from 10-month old *Agrobacterium* mediated *in planta* transformed putative transgenic plants (10) were collected by cutting nodal segments called setts carrying one axillary bud. Single bud setts were prepared by cutting above growth ring and leaving 8-10 cm of the internode below the bud. Subsequently, the setts were surface sterilized with 1% bavistin by shaking on shaker at 120 rpm and 0.1 % $HgCl_2$ for 10-20 min. This was followed by two washings with sterile distilled water. The setts were transferred to polythene bags containing field soil and farm yard manure (3:1) with the bud in top position. The polythene bags were kept in transgenic glass house, maintained at a temperature of 30 °C with relative humidity 80 %. They were regularly watered twice in a day during summers and once in a day during winters.

3.3 Maintenance of gene construct

The gene construct referred as H1 was maintained in *Agrobacterium* strain LBA4404 consisting of pBI121 backbone containing cauliflower mosaic virus (CaMV) 35S promoter, *β-1,3-glucanase* gene (Accession no. GU300766), NOS terminator sequence and *npt II* selectable marker (Figure 2). It was grown on Yeast extract mannitol (YEM) medium supplemented with kanamycin (50 mg/l) and streptomycin (50 mg/l). YEM media was prepared by dissolving 0.4 g yeast extract, 10 g mannitol, 0.1 g sodium chloride (NaCl), 0.2 g magnesium sulfate heptahydrate ($MgSO_4.7H_20$) and 0.5 g dipotassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_20$) in 700 ml distilled water. The pH was adjusted to 7.0 by adding 1 N NaOH/HCl and the final volume was scaled to one litre. Solid YEM media was prepared by

adding bacteriological agar (1.6 %) to liquid YEM media. The media was sterilized by autoclaving and antibiotics were added to media in laminar flow. The stock solutions of kanamycin and streptomycin were prepared as follows:

Kanamycin: Five hundred mg of kanamycin sulphate (Hi-Media, India) was dissolved in 10 ml of distilled water to make a stock solution of 50 ppm concentration. The solution was filter sterilized using syringe mounted 0.2 µm nylon membrane filters and dispensed in to eppendorf tubes and stored at -20 °C.

Streptomycin: Five hundred mg of streptomycin (Hi-Media, India) was dissolved in 10 ml of distilled water to make a stock solution of 50 ppm concentration. Then the solution was filter sterilized using 0.2 µm nylon syringe filter, dispensed in to eppendorf tubes and stored at -20°C.

3.4 Plasmid DNA isolation

The *Agrobacterium* strain LBA4404 carrying H1 plasmid was streaked on YEM-agar plates. The streaked plates were incubated at 28 °C for 40 h. An isolated colony was lifted and suspended in 10 ml of liquid YEM media supplemented with kanamycin (50 mg/ml) and streptomycin (50 mg/ml) and incubated at 28 °C for 40 h with continuous shaking at 200 rpm for primary culture production. After 24 h of incubation, primary culture was transferred to 500 ml of fresh liquid YEM media with added antibiotics and was incubated at 28 °C for another 24 h with continous shaking at 230 rpm. After 48 h of incubation, plasmid DNA was isolated from the broth using "alkaline lysis method" of Birnboim and Doly (1979) with minor modifications as given below:

1. The broth was transferred to Oakridge tubes and bacterial cells were pelleted by centrifugation (Eppendorf, Germany) at 4000 rpm for 15 min at 4°C.
2. The supernatant was discarded and the pellet was resuspended in 10 ml alkaline solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) by vortexing.
3. To this, 20 ml of solution II (0.2 M NaOH; 1 % SDS) was added and mixed thoroughly by inverting 4-5 times. The mixture was incubated at room temperature for 5-10 min.
4. Then 20 ml of solution III (3 M potassium acetate, pH 4.8) was added. The contents were mixed gently and incubated on ice for 10 min.
5. After 10 min the mixture was centrifuged at 11, 000 rpm for 30 min at 4 °C.
6. In order to obtain a tight pellet, centrifugation was done again for 15 min at 4 °C.
7. The supernatant was transferred to the fresh tube and to this 0.6 volume of chilled isopropanol was added. The contents were mixed properly and incubated at room temperature for 10 min.

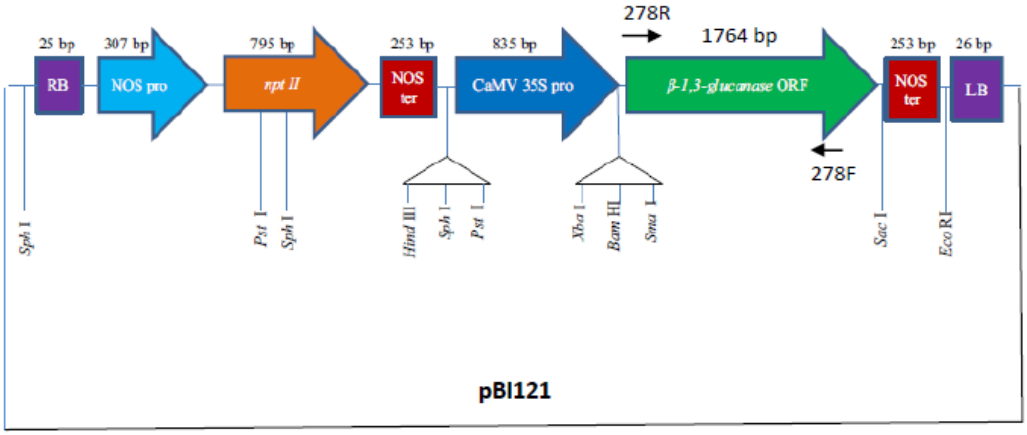


Figure 2: H1 plasmid containing *β-1, 3-glucanase* gene cassette

8. The mixture was again centrifuged at 8000 rpm for 15 min at room temperature.
9. The supernatant was decanted and pellet was rinsed with 70% ethanol at room temperature.
10. Ethanol was drained off and pellet was air-dried.
11. The pellet was dissolved in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and was eventually verified by polymerase chain reaction (PCR).

3.5 Verification of gene construct in H_1 plasmid through PCR

The isolated H1 plasmid DNA was verified for the presence of the *β-1,3-glucanase* transgene by PCR analysis. The analysis was carried out using gene specific primers designed using oligo analysis tools (Table 1).

Table1: Nucleotide sequence of *β-1,3-glucanase* transgene specific primers

ORF	Primer	Nucleotide Sequence (5'-3')	Tm (^oC)	Annealing temperature (^oC)	Expected Amplicon size (bp)	Genbank Accession #
β-1,3-glucanase	Forward 278F	ATGGGTGACCCAACCAATCCTCCCATT	69.5	67	1764	GU300766
	Reverse 278R	TTGACATTCGAATTGAGCGCAGCCCTG	69.8			

The PCR reaction mix (20 µl) contained the 100 ng template DNA (2 µl), 25 µM $MgCl_2$ (1.6 µl), 10 µM forward and reverse primers (0.6 µl), 10 mM dNTPS (0.6 µl), 5x PCR buffer (4.0 µl), 5 units/µl *Taq* DNA Polymerase (1 µl) and sterile water (10.2 µl). Amplifications were carried out in a programmable DNA thermocycler (Mastercycler Gradient-eppendorfTM, Germany) using the following program:

Step I Initial denaturation at 95°C for 5 min.

Step II Denaturation at 95°C for 1 min.

Step III Primer annealing at 67°C for 2 min.

Step IV Extension at 72°C for 2 min.

Step V Repeat steps II to IV for 30 cycles

Step VI Final extension at 72°C for 5 min.

Step VII Store at 4°C

The PCR product was analysed on 1 % agarose gel. The agarose gel was prepared by dissolving 1 g of agarose (MP Biomedical, USA) in 100 ml of 1X TBE buffer (45 mM Tris base, 45 mM Boric acid and 1 mM EDTA). The mixture was heated till agarose dissolved

completely, i.e. when the solution became clear. It was cooled to 60 °C with constant stirring and 5 µl of ethidium bromide (10 µg/ml) was added at final concentration of 0.5 µg/ml buffer. The agarose solution was then poured into gel casting tray and was left for 30-40 min for solidification at room temperature. After solidification comb was removed and 10 µl of each PCR amplified product was loaded in individual wells of solidified agarose gel along with standard 1 kb DNA ladder (Promega, Cat. No. G5711). The amplicons were resolved by running the gel at 5 V/cm for 1 h. The gel was visualized using photo gel documentation system (Avegene, USA).

3.6 Characterization of T_0 putative transgenic plants of clonal generation 1(CG_1)

3.6.1 Genomic DNA isolation of T_0 putative transgenic plants

The genomic DNA was isolated from leaf tissue of 10 month old 127 sprouted clones using miniprep CTAB extraction method (Murray and Thompson 1980):

1. Young leaves from were collected from 10 month old putative transgenic plants.
2. The leaves were crushed in liquid nitrogen using mortar and pestle to obtain powder. The powder so obtained was collected in 2 ml centrifuge tubes.
3. To the powder 800 µl of CTAB buffer (2 % CTAB, 1.4 M NaCl, 100 mM TrisCl, 20 mM EDTA, 0.5 % sodium bisulphate) containing 1 % mercaptoethanol was added and the tubes were incubated for 1 hour at 65 °C in water bath.
4. After incubation, 800 µl of chloroform:isoamyl in the ratio 24:1 was added to the centrifuge tubes and placed on shaker at 70 rpm for 30-45 min.
5. After shaking, the samples were centrifuged at 10,000 rpm for 15 min in microcentrifuge machine.
6. The supernatant was separated from tissue debris in fresh 1.5 ml centrifuge tube using pipette and given RNase treatment by adding 10 µl of RNase to each tube followed by incubation at 37 °C for 30 min.
7. To this solution, 800 µl of chilled isopropyl alcohol was added and mixed by gentle inversions. Tubes were incubated at 4 °C for 15 min.
8. The tubes were centrifuged at 10,000 rpm for 10 min.
9. The supernatant was discarded from each tube and pellet was washed with 70 % ethanol.
10. The pellet was air dried and DNA pellet was dissolved in 50 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) for overnight.
11. The DNA samples were stored at 4 °C and after proper dissolving placed at -20 °C for long term storage.

3.6.2 PCR analysis of isolated DNA from T_0 putative transgenic plants of clonal generation 1 (CG_1)

Genomic DNA isolated from 127 sprouted clones representing clonal generation 1 (CG_1) were confirmed for the presence of *β-1,3-glucanase* transgene through PCR by using gene specific primers 278F and 278R primers (Table 1). A PCR reaction of 20 µl reaction mix containing 40 ng template DNA (2 µl), 25 µM $MgCl_2$ (1.5 µl), 5 µM forward and reverse primers (1.0 µl), 10 mM dNTPS (4.0 µl), 5x PCR buffer (4.0 µl), 5 units/µl *Taq* DNA Polymerase (0.5 µl) and sterile water (6.0 µl). The amplifications were carried out using the same profile as given in Section 3.5. The amplicons were visualized on 1 % agarose gel.

3.6.3 Reverse Transcriptase PCR (RT-PCR) analysis on PCR positive T_0 plants of CG_1

The putative transgenic plants that were found positive by PCR analysis were further analyzed for gene expression studies using RT-PCR. The protocols followed for RNA extraction, quantification, cDNA synthesis and RT-PCR are described below:

3.6.3.1 Total RNA isolation

The total RNA was isolated using MP fast RNA Pro kit from the young leaves of PCR positive plants as follows:

1. Young leaves from 10 month old healthy PCR postitive plants were collected.
2. The leaves were crushed in liquid nitrogen to form a fine powder using a pre-chilled mortar and pestle. The powder so obtained (300-500 mg) was transferred to lysing matrix tubes.
3. To the powder, 1 ml of RNApro^{TM} solution was added and homogenized thoroughly by using a vortex.
4. The lysing matrix tubes containing the mixture were centrifuged for 5 min at 12,000 rpm at 4 °C.
5. The supernatant so obtained was transferred to fresh centrifuge tubes and incubated for 5 min at room temperature.
6. Then 300 µl of chloroform was added into the supernatant and vortex for 10 min. This was followed by incubation for 5 min at room temperature.
7. The tubes were centrifuged at 12000 rpm for 5 min at 4 °C. After centrifugation, the aqueous phase was transferred to clean tubes.
8. After that 500 µl of 100 % ethanol was added and mixed by inversion. The tubes were incubated for 30 min to 1 h at -20 °C.
9. The tubes were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was removed and the pellet was washed with 75 % ethanol (made in Diethylpyrolcarbonate (DEPC) water). DEPC water was prepared by adding 1 ml of commercially available DEPC into 1 L distilled water and autocalving it at 15 psi for 20 min.

10. Ethanol was removed and the pellet was air dried for 10 min at room temperature.
11. The pellet was resuspended in 100 µl DEPC water and incubated for 5 min at room temperature.
12. The RNA samples were stored at -80 °C until further use.

3.6.3.2 Quantitative analysis of RNA

The quantification of isolated RNA was done using a spectrophotometer U2900 (Hitachi, Japan) by measuring the absorbance at 260 nm in quartz cuvettes. The RNA samples were diluted 100X (dilution factor) by dissolving 1 µl of isolated RNA solution in 99 µl of nuclease free water and absorbance was analysed. The RNA concentration was determined using following formula:

$$\text{RNA concentration } (\mu g/\mu l) = \frac{O.D_{260} \times \text{Dilution Factor} \times 40}{1000}$$

3.6.3.3 Qualitative analysis of RNA

RNA integrity was verified on denaturing-agarose gel. Denaturing gel was prepared by dissolving 1.2 g agarose (MP Biomedical, USA) in 100 ml of 1 X MOPS buffer [MOPS (41.9 g), Sodium Acetate (8.2 g) and EDTA (3.72 g)]. The 10X MOPS buffer (pH 7.0) was prepared in autoclaved distilled water which was subsequenty diluted to 1X using 0.1% DEPC water for preparation of gel. The gel mixture was heated till agarose dissolved completely, i.e. when the solution became clear. It was cooled to 60 °C with constant stirring. To this solution, 5 µl of ethidium bromide and 1.78 ml of formaldehyde was added. The agarose solution was then poured into gel casting tray and was left for 30-40 min for solidification at room temperature. After solidification, 5 µl of each RNA sample was mixed with 5 µl of 50 % glycerol and was loaded in individual wells of denaturing agarose gel. The RNA samples were resolved by running the gel in 1 X MOPS buffer (made in 0.1 % DEPC water) at 5 V/cm. The gel was visualized under UV light and photographed using photo gel documentation system.

3.6.3.4 Synthesis of cDNA from RNA

The cDNA was synthesised from total RNA using 1st strand cDNA synthesis kit (Promega, USA) using 0.1-1 µg RNA as template for each sample. The cDNA was synthesized according to the manufacture's instructions as follows:

Procedure:

1. The components of cDNA synthesis kit were thawed, mixed thoroughly, centrifuged briefly and were placed on ice.
2. Template RNA primer mixture was prepared by adding 50 mM oligodT primer (1µl), 10 mM dNTP mix (1 µl), template RNA (1µl), RNase free water (7 µl) in microtubes.

3. This mixture was incubated at 65° C for 5 min and then cooled immediately using ice.
4. To the reaction mixture 5X Primescript buffer (4 µl), RNase inhibitor (0.5 µl) and 200 U Primescript RTase (1 µl) was added. The volume of the reaction mixture was scaled to 20 µl by adding RNase free water. The components of the mixture were mixed gently.
5. The reaction mixture was then incubated at 30 °C for 10 min followed by incubation at 42 °C for 30-60 min.
6. Finally the enzymes were inactivated by incubation at 95 °C for 5 min followed by cooling on ice.
7. The cDNA product was stored at -20 °C.

3.6.3.5 Confirmation of cDNA using 26S rRNA primers

The synthesized cDNA from the RNA of the PCR positive plants was confirmed using 26S rRNA gene specific primers (Table 2).

Table 2: Nucleotide sequence of 26S rRNA gene specific primers

Gene	Primer	Nucleotide Sequence (5'-3')	Tm (^oC)	Annealing temperature (^oC)	Expected Amplicon size (bp)	Genbank Accession #	Reference
26S rRNA	Forward 26SF	CACAATGATAGG AGGAGCCGAC	63.4	60	534	AY283368	Singh *et al* (2004)
	Reverse 26SR	CAAGGGAACGGG CTTGGCAGAATC	63.2				

The PCR reaction mix (20 µl) contained the cDNA template (2 µl), 25 µM $MgCl_2$ (1.5 µl), 5 µM forward and reverse primers (1.0 µl), 10 mM dNTPS (4.0 µl), 5x PCR buffer (4.0 µl), 5 units/µl *Taq* DNA Polymerase (0.5 µl) and sterile water (6.0 µl). Amplifications were carried out in a programmable DNA thermocycler (Mastercycler Gradient-eppendorf™, Germany) using the following program:

Step I Initial denaturation at 95°C for 5 min.

Step II Denaturation at 95°C for 1 min.

Step III Primer annealing at 60°C for 1 min.

Step IV Extension at 72°C for 1 min.

Step V Repeat steps II to IV for 30 cycles

Step VI Final extension at 72°C for 7 min.

Step VII Store at 4°C.

The amplicons were visualised on 1 % agarose gel under UV light and photographed using photo gel documentation system (Avegene, USA).

3.6.3.6 Reverse transcriptase PCR

The cDNA samples confirmed by PCR using 26S rRNA primers were then analysed by RT-PCR for the presence of *β-1,3-glucanase* transgene. RT-PCR was carried out using *β-1,3-glucanase* transgene gene specific primers 278F and 278R (refer to Table 1). RT-PCR was performed using the same reaction volume and PCR profile as given in the section 3.5.

3.6.4 Quantitative Reverse Transcriptase PCR (qRT-PCR) analysis on RT-PCR positive T_0 plants of CG_1

The relative expression of the *β-1,3-glucanase* transgene in the RT-PCR positive plants was analysed by Roche LightCycler® 96 Real-Time PCR System machine. In this experiment, *β-1,3-glucanase* specific primer (MKQ1071F and MKQ1071R) (Table 3) and *β-tubulin* gene specific primer (Ling *et al* 2014) were used for analysis of relative transgene expression (Table 4).

Table 3: Nucleotide sequence of *β-1,3- glucanase* specific primers for qRT-PCR

Gene	Primer	Nucleotide Sequence (5'-3')	Tm (°C)	Annealing Temp. (°C)	Expected Amplicon size (bp)	Genbank Accession #
β-1,3-glucanase	Forward (MKQ1071F)	ATCGCTGAGGACTTCAATGG	62	60	107	GU300766
	Reverse (MKQ1071R)	TGCTCACTTCCTATCGCATAAA	61.9			

Table 4: Nucleotide sequence of *β-tubulin* specific primers for qRT-PCR

Gene	Primer	Nucleotide Sequence (5'-3')	Tm (°C)	Annealing Temp. (°C)	Expected Amplicon size (bp)	Genbank Accession #
β-tubulin	Forward (*tubulin* F)	CCAAGTTCTGGGAGGTGATCTG	64.1	60	103	CA222437
	Reverse (*tubulin* R)	TTGTAGTAGACGTTGATGCGCTC	64.4			

3.6.4.1 Calibration of Roche LightCycler® 96 Real-Time PCR System machine

A standard curve was generated using 10 fold serial dilutions of cDNA for assessing the reaction efficiency of *β-1,3-glucanase* transgene and the *β-tubulin* gene. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of Ct value against log of input nucleic acid. A 100 % efficient reaction would yield a 10-fold increase in PCR

amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$). A calculation for estimating the efficiency (E) of a real-time PCR assay is:

$$E = (10^{-1/slope} - 1)*100$$

The slope of a standard curve is used to estimate the PCR amplification efficiency of a real-time PCR reaction. A standard curve slope of -3.32 indicates a PCR reaction with 100 % efficiency.

qRT-PCR was performed in triplicates using cDNA from transgenic and non-transformed plants (NC). The PCR reaction mix (20 µl) contained the cDNA template (2 µl), forward and reverse primers (1 µl), 2X PCR SYBR green ready mix (10 µl) and sterile water (7 µl). Amplifications were carried out in LightCycler® 96 Real-Time PCR System machine using the following program:

Step I Initial denaturation at 94˚C for 3 min.

Step II Denaturation at 94˚C for 10 sec.

Step III Primer annealing at 60˚C for 30 sec.

Step IV Extension at 72˚C for 30 sec.

Step V Repeat steps II to IV for 40 cycles

Step VI Melt curve at 55˚C for 30 sec

The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiment (Livak and Schmittgen 2001). According to ΔΔCT method:

$\Delta C_T = \Delta C_{TTarget} - \Delta C_{Treference}$ i.e. $\Delta CT = \Delta C_{T\ \beta\text{-}1,\ 3\text{-}glucanase} - \Delta C_{Ttubulin}$

$\Delta\Delta CT = \Delta C_{Ttest\ sample} - \Delta C_{Tcalibrator}$ sample i.e. $\Delta\Delta CT = \Delta C_{Ttransgenic} - \Delta C_{Tnon\text{-}transgenic}$

Where C_t is the threshold cycle number at which the fluorescence generated within a reaction crosses the background fluorescence signal emitted during the early cycles of the PCR reaction before the real-time PCR instrument detects the amplification of the PCR product. A lower C_T value corresponds to faster significant increase in fluorescence signal due to high quantity of target template in the sample and thus lower expression. The ΔCT refers to the change in the C_T value after treatment and $2^{-\Delta\Delta CT}$ represents the fold change in gene expression relative to untreated control.

3.7 Bioassay on qRT-PCR positive T_0 plants of CG_1 for red rot resistance

Transgenic plants were screened for red rot using the plug method (Srinivasan and Bhat 1961). The standing cane was used for red rot screening and the inoculation was carried

out in July so that incubation period coincides with high humidity conditions suitable for disease development. Protocol followed is described below:

1. Cultures of *C. falcatum* was grown on dextrose agar media were used for inoculation.
2. About 10 days old culture of *C. falcatum* (Cf 08 and Cf 09) with profuse sporulation (70,000 conidia/ml) was used for nodal infection.
3. A bore hole was punched in the middle of the 3rd internode from bottom with a cork borer and the plug of cane tissue with the rind removed.
4. The spore suspension (2ml) was injected into the bore hole, the hole replugged with removed cane tissue and sealed tight with parafilm.
5. After an incubation period of 60 days, the inoculated canes were split open longitudinally and severity of red rot symptoms was scored on basis of condition of top leaves, progress of the red rot lesion along the length of the cane, width of the lesions, nodal transgression and amount of white spots present in inoculated canes (Table 5). The values for all symptoms were added and the score was given on the scale 0-9 (Table 6).

Table 5: Red rot screening

Symptoms	Numerical scale	Severity of Symptoms
Condition on top	0	Green
	1	Yellow or Drying
Nodal transgression	0	No lesion spread. Lesion restricted to inoculated cane
	1	Lesion transgression of one node above the inoculated cane
	2	Lesion transgression of 2 node above the inoculated cane
	3	Lesion transgression of more than 2 node above the inoculated cane
Lesion width	0	No lesion spread
	1	Lesion spread to about 25% of the width of the stalk
	2	Lesion spread to about half the cane width
	3	Lesion spread to more than half the cane width
White spots	0	No white spots
	1	Sparse presence of white spots
	2	Moderate to profuse presence of white spots

Table 6: Categorisation of red rot reactions (Srinivasan and Bhat 1961)

Score on (0-9) category	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)

CHAPTER – IV

RESULTS AND DISCUSSION

The present study was undertaken to characterize *in planta* transformed sugarcane plants carrying anti-fungal *β-1,3-glucanase* transgene developed with an objective for inducing red rot resistance caused by fungal pathogen *C. falcatum*. The results obtained have been presented in this chapter under the following headings:

4.1 Clonal propagation of *in planta* transformed T_0 putative transgenic plants

4.2 PCR analysis on T_0 putative transgenic plants of clonal generation 1 (CG_1)

4.3 RT-PCR analysis on PCR positive T_0 plants of CG_1

4.4 qRT-PCR analysis on RT-PCR positive T_0 plants of CG_1

4.5 Bioassay on qRT-PCR positive T_0 plants of CG_1 for red rot resistance

4.6 CG_2 plant analysis for chimerism

4.1 Clonal propagation of *in planta* transformed T_0 putative transgenic plants

Agrobacterium mediated *in planta* transformed putative transgenic plants (10) of sugarcane cultivar CoJ 83 developed by Kaur (2014) carrying *β-1,3-glucanase* transgene under the control of CaMV 35S promoter and NOS terminator were grown in transgenic glass house of School of Agricultural Biotechnology, Ludhiana for 10 months without molecular characterization. *In planta* transformation is a tissue culture independent method of introducing transgene directly into the genome of organised tissue thereby reducing time, labour cost and avoiding somaclonal variation encountered during *in vitro* mediated genetic transformation and regeneration (Subramanyam *et al* 2015). The method mimics the natural infection process of *A. tumefaciens* resulting in high (upto five-fold) transformation efficiency as compared to conventional tissue culture-based transformation (Supartana *et al* 2006), however leading to chimerism.

In the present study, plant to row progenies of the 10 plants were revived from nodal cuttings (axillary bud setts). A total of 295 single bud setts were planted in polythene bags containing field soil, out of which 127 setts sprouted (Table 7). A large number of transgenic plants have been reported to be produced through axillary bud transformation and clonal propagation in a short time period (Manickavasagam *et al* 2004). *In planta* transformation strategy targeting *Agrobacterium* to the meristem of axillary buds has been successfully standardized for kenaf (Kojima *et al* 2004) and mulberry (Ping *et al* 2003) leading to generation of stable transgenic plants.

4.2 PCR analysis on T_0 putative transgenic plants of clonal generation (CG_1)

The genomic DNA was isolated from leaf tissue of six week-old 127 sprouted clones (Figure 3), and analysed by PCR for the presence of *β-1,3-glucanase* transgene. The genomic

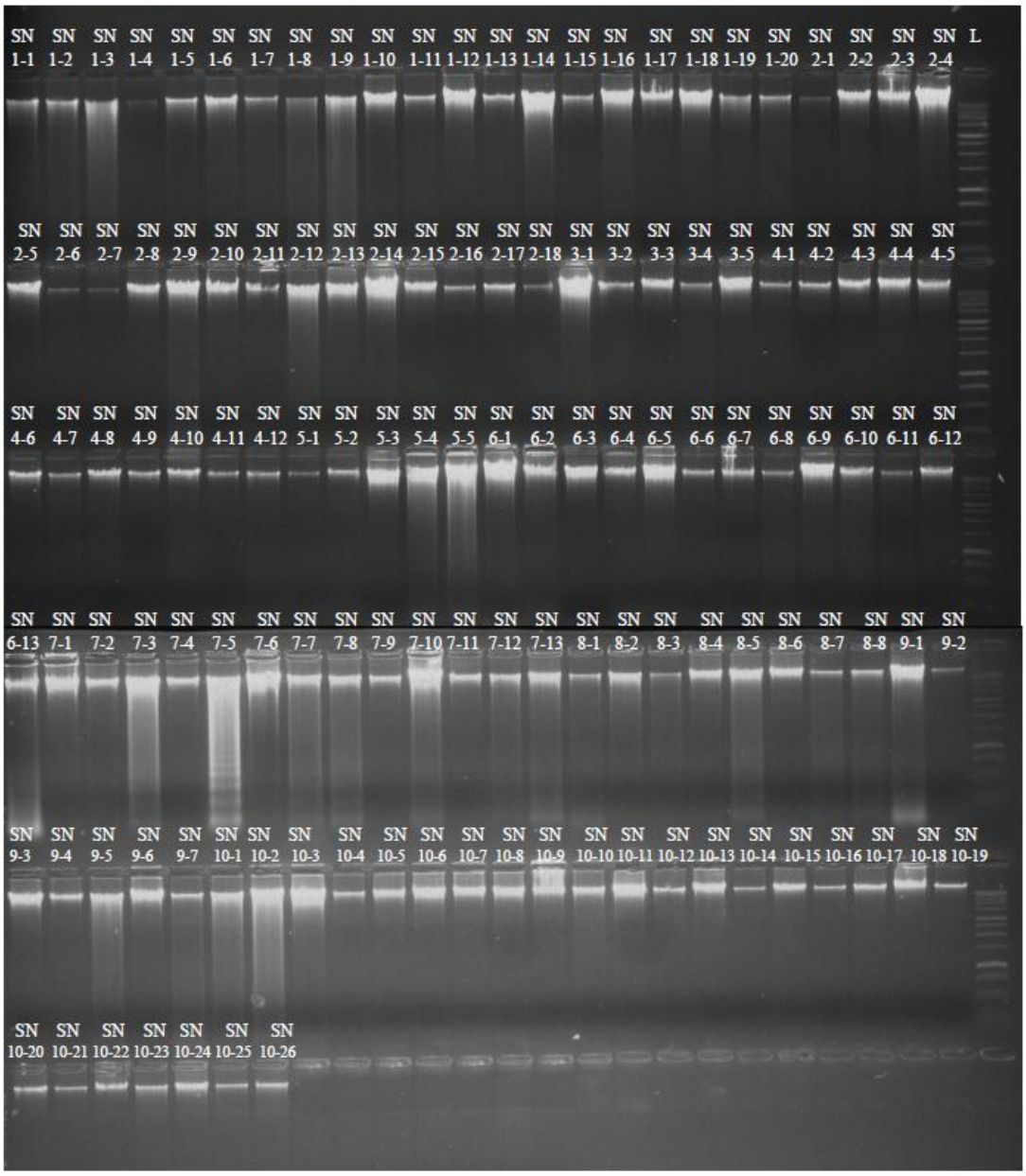


Figure 3: Genomic DNA isolation from clones of T_0 putative transgenic plants

L refers to 1 Kb ladder (Promega, Cat. No. G5711); For the numbering with SN series, please refer Table 7

DNA of non-transformed plant (CoJ 83) was used as negative control (NC); and H1 gene cassette containing 1764 bp *β-1,3-glucanase* transgene under the control of CaMV 35S promoter and NOS terminator in *Agrobacterium* strain LBA4404 was used as positive control (PC).

Table 7: Clonal propagation of *in planta* transformed T_0 putative transgenic plants and their PCR analysis

Putative T_0 transgenic plant designation (by Kaur 2014)	Number of bud setts planted	Number of bud setts sprouted	Designation of CG_1 (present study)	No. of PCR positive clones	Designation of PCR positive clones
IP1	22	20	SN1-1 to SN1-20	2	(SN1-12; SN1-19)
IP2	20	18	SN2-1 to SN2-18	-	-
IP3	41	5	SN3-1 to SN3-5	-	-
IP4	22	12	SN4-1 to SN4-12	-	-
IP5	31	5	SN5-1 to SN5-5	-	-
IP6	38	13	SN6-1 to SN6-13	2	(SN6-2; SN6-5)
IP7	30	13	SN7-1 to SN7-13	-	-
IP8	20	8	SN8-1 to SN8-8	-	-
IP9	36	7	SN9-1 to SN9-7	-	-
IP10	35	26	SN10-1 to SN10-26	2	(SN10-12; SN10-13)
TOTAL	**295**	**127**	**-**	**6**	**-**

The PCR analysis of 127 clones revealed the amplification of 1764 bp product corresponding to *β-1,3-glucanase* transgene in six putative transgenic plants designated as SN1-12, SN1-19, SN6-2, SN6-5, SN10-12 and SN10-13 (Figure 4). PCR analysis on the six putative plants was repeated to validate the integrity of *β-1,3-glucanase* transgene (Figure 5). PCR is a simple and routinely used method for preliminary screening of sugarcane transformants (Supartana *et al* 2005; Mayavan *et al* 2013; Mayavan *et al* 2015; Sandhu *et al* 2016). The integration of transgene into the plant genome has been successfully verified through PCR screening in T_0 generation of various vegetatively propagated crops like tea (Mondal *et al* 2001), peach (Rosa *et al* 2004), pomegranate (Terakami *et al* 2007) and banana (Subramanyam *et al* 2011).

In the present study, only six axillary bud setts i.e. two each from SN1-1 to SN1-20, SN6-1 to SN6-13 and SN10-1 to SN10-26 out of 127 sprouted setts were identified to carry *β-1,3-glucanase* transgene pointing towards chimerism. This is mainly ascribed to the transformation of differentiated tissue that resulted in T_0 plants being chimeric, and stable transformants can be obtained by applying selection agent and selection pressure that would

help in screening of chimeras in the *in planta* transformation (Subramanyam *et al* 2013; Manickavasagam *et al* 2015; Mayavan *et al* 2015). The T_0 plants were not subjected to selection due to their chimeric nature. Chimerism has been reported in several *Agrobacterium* mediated *in planta* transformed transgenic plants such as groundnut (Rohini and Rao 2000), pigeon pea (Rao *et al* 2008), rice (Lin *et al* 2009), bell pepper (Kumar *et al* 2009), grapevine (Fujita et al 2009), field bean (Keshamma *et al* 2012), brinjal (Subramanyam *et al* 2013), sugarcane (Mayavan *et al* 2013), maize (Abhishek *et al* 2014), snake gourd (Subramanyam *et al* 2015) and okra (Manickavasagam *et al* 2015). The chimeras were screened in T_0 generation through stringent selection using *bar* gene (Lin *et al* 2009; Mayavan *et al* 2013; Subramanyam *et al* 2013; Subramanyam *et al* 2015; Manickavasagam *et al* 2015) and *gus* gene (Rohini and Rao 2000; Kumar *et al* 2009; Abhishek *et al* 2014). Mayavan *et al* (2015) identified transformants from *in planta* transformed sugarcane setts through BASTA selection at three developmental stages i.e. initially at the stage of sett sprouting, then during tiller formation, and finally at the green house stage where plants were grown for 45 days.

4.3 Reverse Transcriptase PCR (RT-PCR) analysis on PCR positive T_0 plants of CG_1

The expression of transgene in six PCR positive plants (SN1-12, SN1-19, SN6-2, SN6-5, SN10-12 and SN10-13) was analysed at transcriptional level using RT-PCR. The total RNA of six PCR positive plants and non-transformed plant (NC) was isolated using MP fast RNA Pro kit as explained in Section 3.6.3. The quantification of isolated RNA was carried out spectrophotometerically by diluting the sample 100-fold (Table 8). The RNA integrity was verified on denaturing agarose gel. The results showed that the isolated RNA contained fragments corresponding to 28S and 18S RNA (Figure 6), and was used for further experiments.

Table 8: Quantification of RNA

Clone No.	$O.D._{260}$	RNA concentration (µg/µl)
SN1-12	1.090	3.647
SN1-19	1.050	3.256
SN6-2	1.080	2.678
SN6-5	0.990	3.933
SN10-12	0.935	4.563
SN10-13	0.978	4.312
NC	0.962	3.365

The isolated RNA (1 µg) was used as a template to synthesize cDNA using 1st strand cDNA synthesis kit (Promega, USA). The cDNA integrity was verified by PCR using 26S rRNA primers. The amplicons were resolved on 1 % agarose gel and a 534 bp fragment

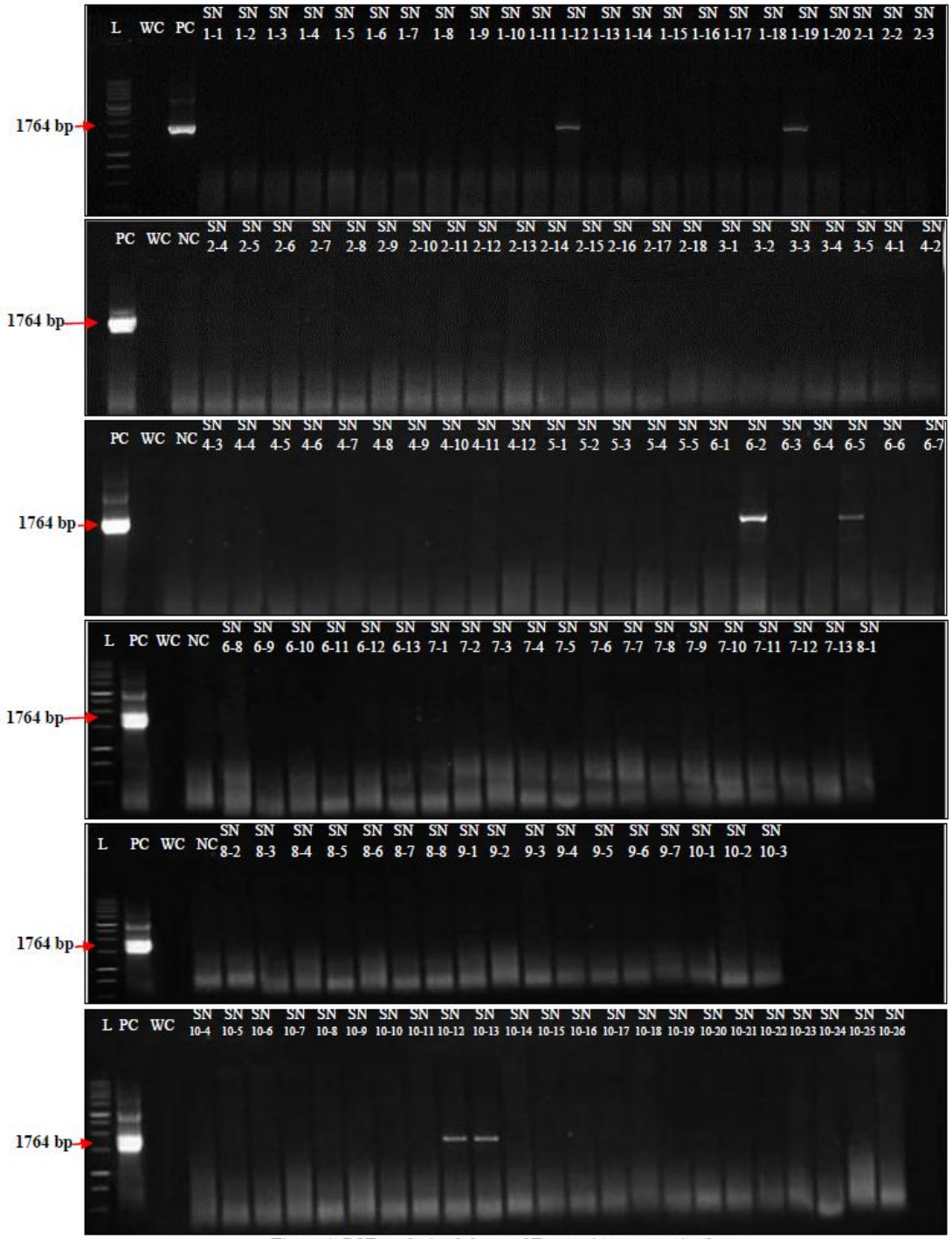


Figure 4: PCR analysis of clones of T_0 putative transgenic plants

L refers to 1 Kb ladder (Promega, Cat. No. G5711); SN1-1 to SN10-26 represents clones of T_0 putative transgenic plants; PC - Positive Control; WC- Water Control; NC - Negative Control from non-transformed plant

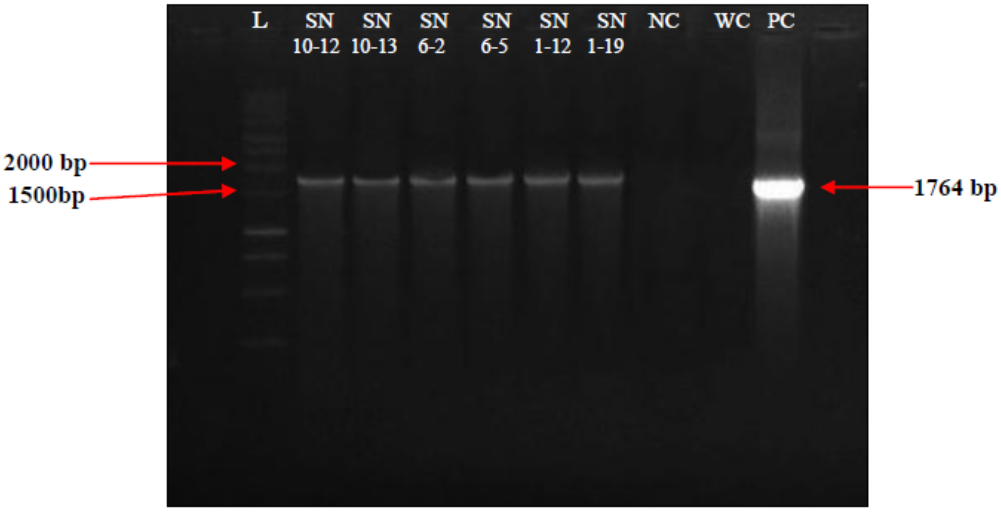


Figure 5: PCR positive T_0 putative transgenic plants

L refers to 1 Kb ladder (Promega, Cat. No. G5711); SN10-12, SN10-13, SN6-2, SN6-5, SN1-12 and SN1-19 represents PCR positive T_0 putative transgenic plants; PC - positive control harbouring H1 gene cassette containing 1764 bp *β-1,3-glucanase* transgene in *Agrobacterium* strain LBA4404; B - Blank well; NC - Negative Control from non-transformed plant

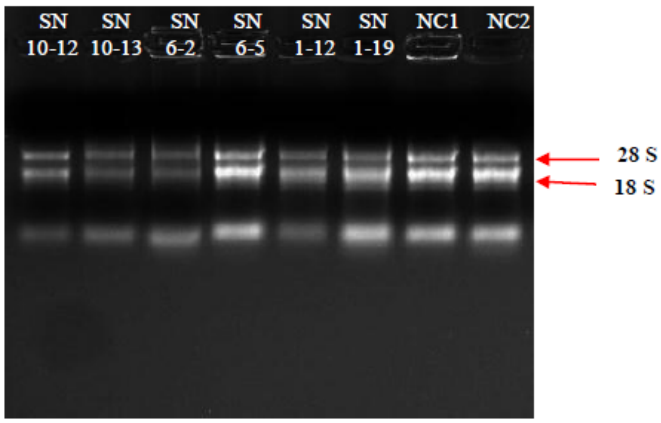


Fig 6: RNA integrity of PCR positive T_0 plants of CG_1

The numbers SN10-12, SN10-13, SN6-2, SN6-5, SN1-12 and SN1-19 refer to RNA isolated from six PCR positive T_0 putative transgenic plants of CG_1; NC1 and NC2 - RNA isolated from the Negative Control (non-transformed plants)

corresponding to 26S rRNA was observed in six PCR positive plants indicating the authenticity of RNA isolation and cDNA synthesis (Figure 7). Analysis of cDNA integrity through the amplification pattern of housekeeping gene such as 26S rRNA is a common practice and has been used in various expression studies (Goidin *et al* 2001).

Subsequently, the expression of *β-1,3-glucanase* transgene was confirmed using transgene specific primers 278F and 278R. The results obtained by RT-PCR analysis revealed the differential expression of 1764 bp *β-1,3-glucanase* transgene in all the six PCR positive plants (Figure 8). This indicated transcription of *β-1,3-glucanase* transgene in transgenic plants. The differential level of transgene expression through RT-PCR analysis has been reported in transgenic tabacoo carrying *BoRS1* transgene (Tang *et al* 2005), banana carrying hepatitis B surface antigen encoding gene (Kumar *et al* 2005) and sugarcane carrying antifungal gene (Khan 2012; Khamrit *et al* 2013; Sandhu *et al* 2016).

4.4 Quantitative Reverse Transcriptase PCR (qRT-PCR) analysis on RT-PCR positive T_0 plants of CG_1

The relative *β-1,3-glucanase* transgene expression in six RT-PCR positive transgenic plants was analysed by qRT-PCR. The cDNA synthesised from isolated RNA of six RT-PCR positive transgenic plants was amplified using qRT-PCR primers for *β-1,3-glucanase* transgene (MKQ1071F and MKQ1071R) and *tubulin* gene. The *tubulin* gene was used as an endogenous control with an objective to normalize the expression values of *β-1,3-glucanase* transgene.

As a preliminary test the standard curves for the assessment of efficiencies of *β-1,3-glucanase* transgene and *tubulin* gene were plotted individually for the validation of comparative Ct method of relative quantification. The standard curve slope of -3.124 for *tubulin* gene indicated the efficiency of reaction to be 108.98 % (Amplification factor = 2.09) [Figure 9]. A standard curve slope of -3.10 indicated the performance of reaction with 110.17 % efficiency (Amplification factor = 2.10) for *β-1,3-glucanase* transgene (Figure 10). The validation experiment_indicated that the amplification efficienies of the *tubulin* gene and *β-1,3-glucanase* transgene were approximately equal. Therefore, $\Delta\Delta C_T$ method was used to analyze the relative expression of *β-1,3-glucanase* transgene (Livak and Schmittgen 2001). The reference genes are presumed to be expressed constitutively and display stable expression under a variety of experimental conditions and are therefore used for correct data normalization and gene expression measurement (Stephens *et al* 2011; Yan *et al* 2012; Gantasala *et al* 2013). A study conducted by Faize *et al* (2010) while generating transgenic apricot and tobacco lines transformed with the binary vector pBin19-35SGus intron carrying *nptII* gene demonstrated that serially diluted DNA used to obtain a standard curve for the

endogenous *β-actin* and *nptII* genes revealed a correlation of 0.99 between the Ct value and log (DNA) for the two genes, and the linear relationship indicated that Ct value is suitable for the estimation of relative amount of the transgene.

The qRT-PCR analysis of six RT-PCR positive transgenic plants (SN1-12, SN1-19, SN6-2, SN6-5, SN10-12, SN10-13) and non-transformed plant (NC) were performed in triplicates. The amplification chart and melt peak chart obtained are shown in Figure 11 and 12. The clusters in the amplification chart and Ct values for individual reactions with two different primers (*tubulin*; MKQ1071F and MKQ1071R) are presented in sigmoid curve pattern. The sigmoid curve pattern gave a clear representation of the four phases of sigmoidal curve suggesting that there was no error in the run process of any reaction. The four phases of sigmoidal curve are (1) linear ground phase (2) early exponential phase (3) log linear phase (4) plateau phase representing different levels of run procsss (Tichopad *et al* 2003). The melt peak chart analysis confirmed the specificity of reaction, single peak with each primer represented that the primer was annealing at a particular temperature in all the reactions and absence of any non-specific amplification (Yan *et al* 2012; Gantasala *et al* 2013). The melt peak obtained in present study showed single individual peak for each primer representing that the primers were annealing at a specific temperature in all the reactions and there was no non-specific amplification.

The threshold cycle (Ct) values for individual reactions and the mean Ct value for a set of triplicates are shown in Appendix-I. The Ct values were analysed using the mathematical model ($2^{-\Delta\Delta CT}$) given by Livak and Schmittgen (2001). The relative expression of *β-1,3-glucanase* transgene and *tubulin* gene in six plants revealed that three plants designated as SN1-19, SN10-12 and SN10-13 had $2^{-\Delta\Delta C_T}$ equal to 3.197, 4.280 and 4.438 respectively, which meant that in case of SN1-19 there was three-fold increase and four-fold increase higher for SN10-12 and SN10-13 in the expression of *β-1,3-glucanase* transgene as compared to to non-transformed plant (Table 9). In case of plant no. SN6-2 and SN1-12, $2^{-\Delta\Delta C_T}$ was equal to 1.75 and 1.94 respectively, which meant that there was about two-fold increase in the expression of *β-1,3-glucanase* transgene as compared to non-transformed plant. In case of plant no. SN6-5, the $2^{-\Delta\Delta C_T}$ was equal to 0.423 suggesting that the expression of *β-1,3-glucanase* transgene was down-regulated in this plant in comparison to non-transformed plant.

qRT-PCR analysis has many advantages such as high sensitivity, high precision, no post PCR handling, thus avoiding the possibility of cross-contamination and enabling high

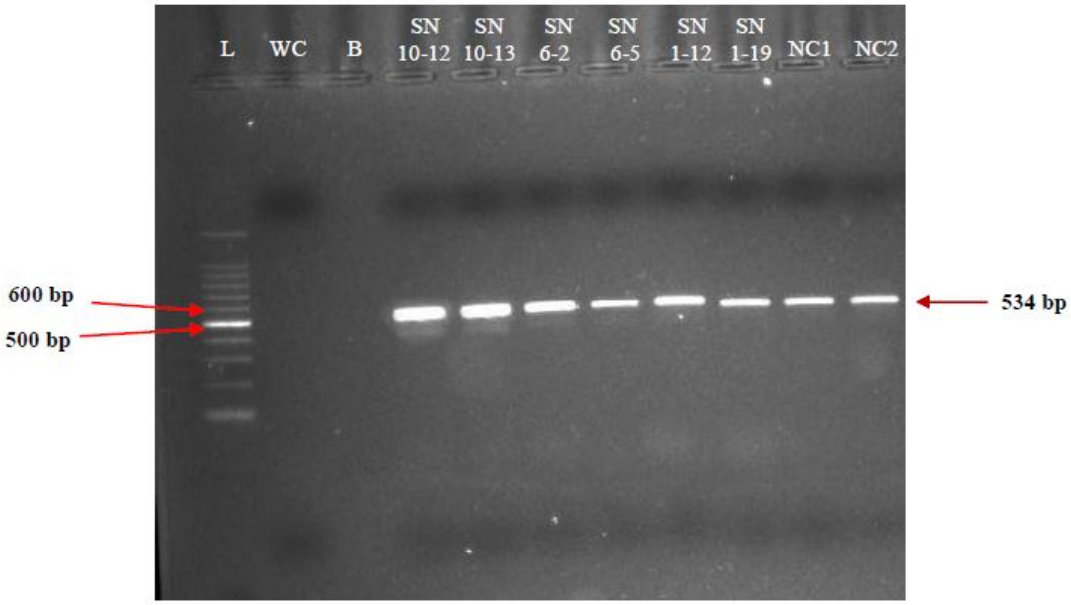


Fig. 7: Confirmation of cDNA integrity with 26Sr RNA primers

L refers to 100 bp ladder (Takara, Cat. No. 3407A); SN10-12, SN10-13, SN6-2, SN6-5, SN1-12 and SN1-19 represents the amplification of 26S rRNA gene in six PCR positive T_0 transgenic plants of CG_1; PC - Positive Control harbouring H1 gene cassette containing 1764 bp *β-1,3-glucanase* transgene in *Agrobacterium* strain LBA4404; B – Blank well; WC – Water Control; NC1 and NC2 represents the amplification of 26S rRNA gene in Negative Control (non-transformed plants)

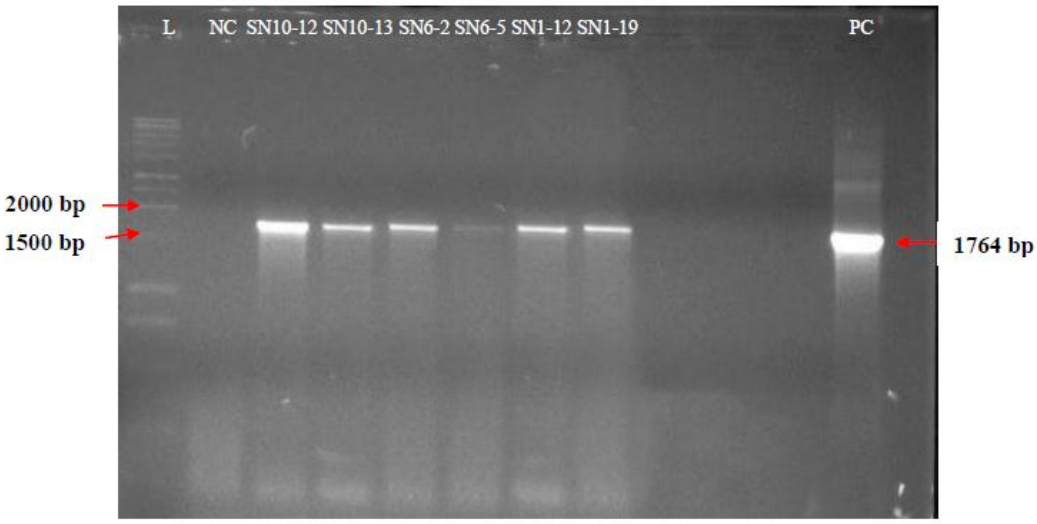


Fig 8: Confirmation of the expression of *β-1, 3-glucanase* transgene through RT-PCR using gene specific primers

L refers to 1 Kb ladder (Promega, Cat. No.G5711); SN10-12, SN10-13, SN6-2, SN6-5, SN1-12 and SN1-19 represents RT-PCR positive T_0 putative transgenic plants carrying *β-1,3-glucanase* transgene; PC - Positive Control harbouring H1 gene cassette containing 1764 bp *β-1,3-glucanase* transgene in *Agrobacterium* strain LBA4404; NC - Negative Control from non-transformed plant

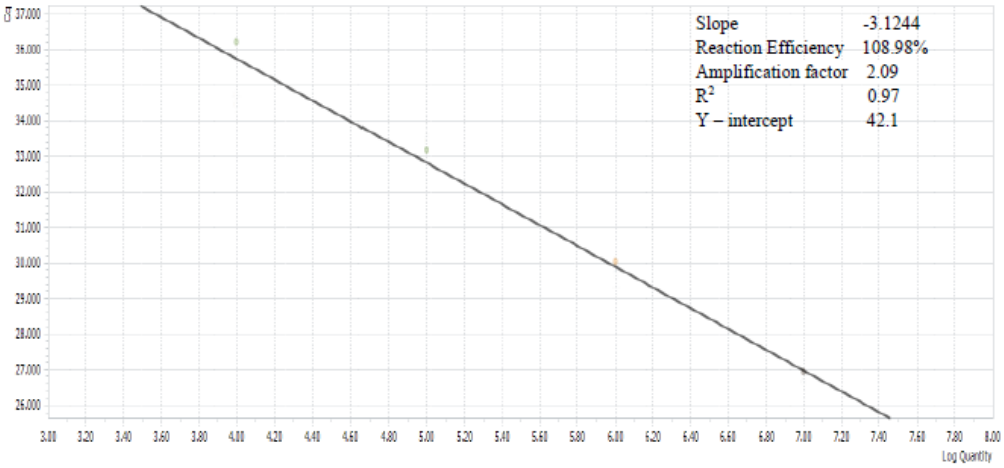


Figure 9: Amplification efficiency of *tubulin* gene in qRT-PCR determined by plotting cDNA dilution against Ct value

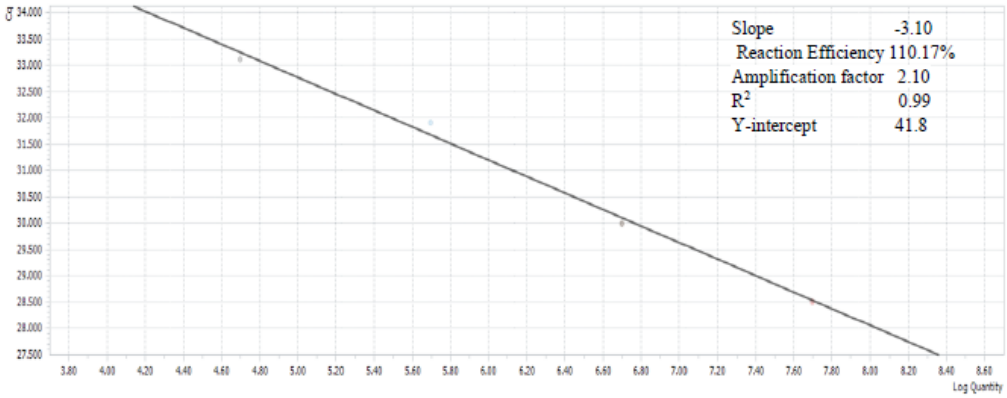


Figure 10: Amplification efficiency of *β-1, 3-glucanase* transgene in qRT-PCR determined by plotting cDNA dilution against Ct value

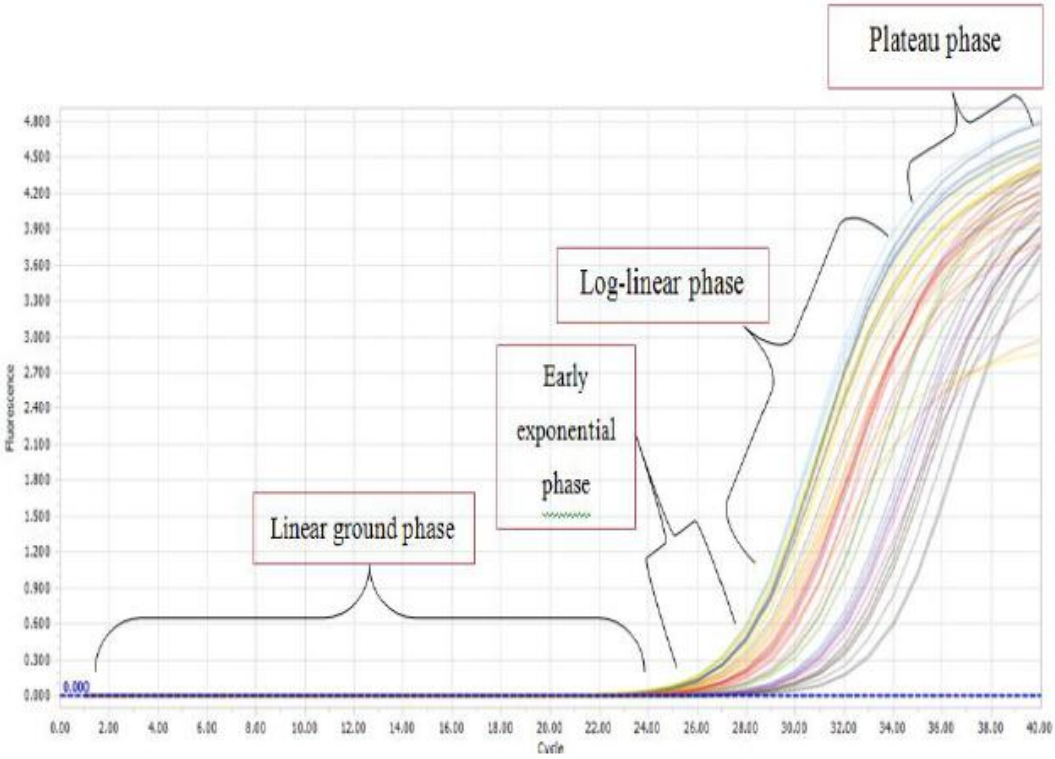


Fig 11: Amplification chart obtained from qRT-PCR analysis

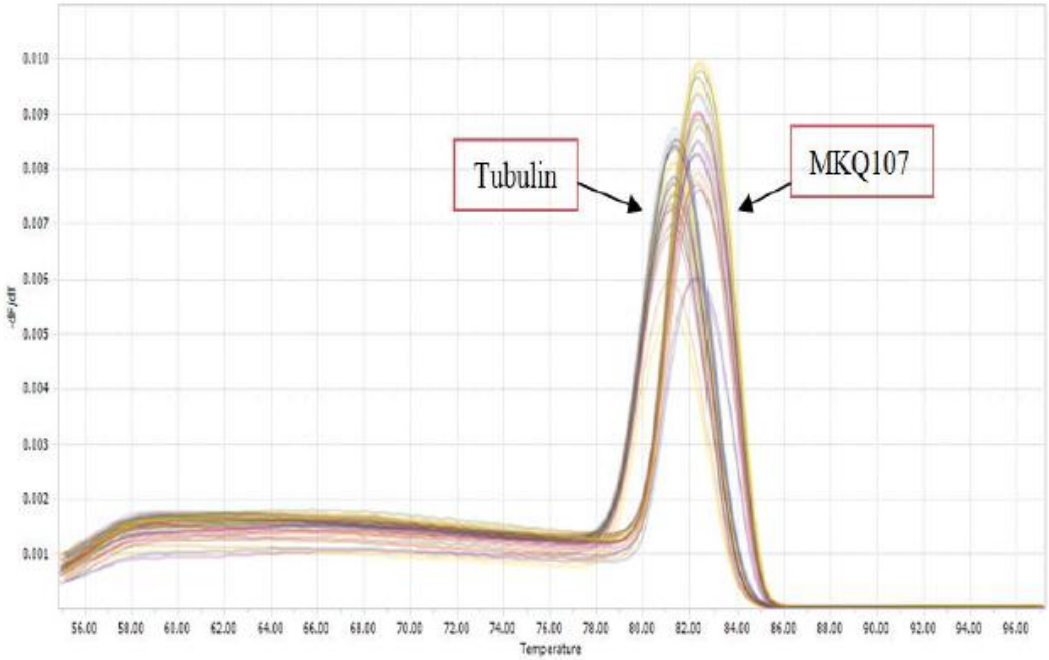


Fig. 12: Melt peak chart obtained from qRT-PCR analysis

throughput (Bustin 2002; Ginzinger 2002; Wall and Edwards 2002). The relative expression analysis through qRT-PCR has been used to analyse the transgene expression in potato (Toplak *et al* 2004), lemon (Distefano *et al* 2008), maize (Assem and Hassan *et al* 2008), rice (Kumari and Pandey *et al* 2013) and sugarcane (Iskandar *et al* 2004; Su et al 2013; Augustine *et al* 2014; Sandhu *et al* 2016). Likewise, the relative expression analysis of trangene in present study through qRT-PCR led to the identification of transgenic plants carrying relatively higher *β-1,3-glucanase* transgene expression, thus confirming the stable integration of transgene in the genomes of transgenic plants.

Table 9: Fold change expression of *β-1,3-glucanase* transgene

Clone no.	Average Ct (*β-1,3glucanase*)	Average Ct (*tubulin*)	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
NC	27.96±0.09	27.02±0.17	0.94±0.08	0±0.08	0.99 (0.94-1.05)
SN1-12	26.31±0.17	26.33±0.02	-0.02±0.15	-0.96±0.15	1.95 (1.75-2.15)
SN1-19	30.66±0.12	31.69±0.35	-1.03±0.23	-1.97±0.23	3.96 (3.34-4.59)
SN6-2	26.78±0.26	26.65±0.06	0.13±0.2	-0.81±0.2	1.76 (1.52-2.01)
SN6-5	30.09±0.15	27.91±0.20	2.18±0.05	1.24±0.08	0.42 (0.40-0.44)
SN10-12	27.37±0.15	28.53±0.26	-1.16±0.11	-2.1±0.11	4.14 (3.97-4.31)
SN10-13	29.10±0.07	30.31±0.15	-1.21±0.08	-2.15±0.08	4.44 (4.19-4.69)

4.5 Bioassay on qRT-PCR positive T_0 plants of CG_1 for red rot resistance

The transgenic sugarcane plants carrying *β-1,3-glucanase* transgene and non-transformed plant (NC) were screened for red rot resistance using plug method (Srinivasan and Bhat 1961) in the transgenic glasshouse. A bore hole was punched in the middle of the 3rd internode from bottom of each cane and spore suspension of *C. falcatum* (Cf08 pathotype) was injected into the bore hole and sealed with parafilm. After an incubation period of 60 days, the inoculated canes were split open longitudinally and severity of red rot symptoms was scored on basis of condition of top leaves, progress of the red rot lesion along the length of the cane, width of the lesions, nodal transgression and amount of white spots present in inoculated canes (Table 10). The values for all symptoms were added and the score was given on the scale 0-9 (refer to Section 3.7). Transgenic sugarcane plants designated as SN1-19, SN10-12 and SN10-13 did not show any white spots or lesion spread (i.e. lesion was restricted at the inoculation site of the cane) and green leaves on top (Figure 13). The plant no. SN1-12, SN6-2 and SN6-5 showed lesion transgression of more than 2 node above the inoculated cane. The results revealed that transgenic sugarcane plants designated as SN1-19, SN10-12 and SN10-13 displayed resistance against red rot as compared to non-transformed plant (NC).

In the subsequent year, same plants were inoculated with Cf 09 pathotype and results revealed that transgenic sugarcane plant SN10-12 and SN10-13 were moderately resistant to red rot as compared to NC (Figure 13).

Table 10: Bioassay on transgenic sugarcane plants carrying *β-1,3-glucanase* transgene for screening of red rot resistance

Clone no.	C. falcatum pathotype	Condition on top[1]	Lesion width[2]	White spots[3]	Nodal transgression[4]	Scale (0-9)[5]	Category[6]
NC	Cf08	0	2	1	3	6	S
	Cf09	1	2	1	3	7	HS
SN1-12	Cf08	0	1	0	2	3	MR
	Cf09	1	1	0	2	4	MS
SN1-19	Cf08	0	1	0	0	1	R
	Cf09	1	1	0	2	4	MS
SN6-2	Cf08	0	1	0	2	3	MR
	Cf09	1	2	1	2	6	HS
SN6-5	Cf08	0	1	0	3	4	S
	Cf09	1	2	1	2	6	HS
SN 10-12	Cf08	0	1	0	0	1	R
	Cf09	0	1	0	2	3	MR
SN 10-13	Cf08	0	1	0	0	1	R
	Cf09	0	1	0	2	3	MR

1, 2, 3, 4, 5 and 6 are explained in Table 5 and 6 (Section 3.7); S- Susceptible, HS-Highly Susceptible, R- Resistant, MR- Moderately Resistant, MS- Moderately Susceptible

Saksena *et al* (2014) reported that Cf08 pathotype was the most virulent among various pathotypes present in Punjab and had the ability to infect newly developed varieties. Khan (2012) performed pathological bioassay on transgenic sugarcane plants carrying Har*Chit* and Har*Cho* transgene with *C. falcatum* spores and revealed that all transgenic lines showed minimal damage caused by *C. falcatum*. Sundar et al (2008) reported that treatment of sugarcane leaves with glycoprotein elicitor isolated from *C. falcatum* lead to marked increase in the activities of chitinase and *β-1,3-glucanase* in red rot resistant (BO 91) as compared to susceptible (CoC 671) sugarcane cultivar. Su *et al* (2013) reported *β-1,3-glucanase* enzyme activity in the resistant variety increased faster and lasted longer than in the susceptible one when inoculated with *Sporisorium scitamineum,* along with a positive correlation between the activity of the *β-1,3-glucanase* and smut resistance. In the present study, upon inoculation of transgenic sugarcane plants with Cf08 inoculum, the plants with high transgene expression (SN1-19, SN10-12 and SN10-13) displayed enhanced red rot resistance. The relative transgene expression in the roots and leaves of six transgenic plants before and after inoculation demonstrated induction of transgene (Table 11 and 12). Amplification and melt peak chart from qRT-PCR analysis is shown as Figure 14 (A, B, C, D, E and F). The raw data of qRT-PCR in triplicates for relative transgene expression from roots and leaves before and after is shown in Appendix II.

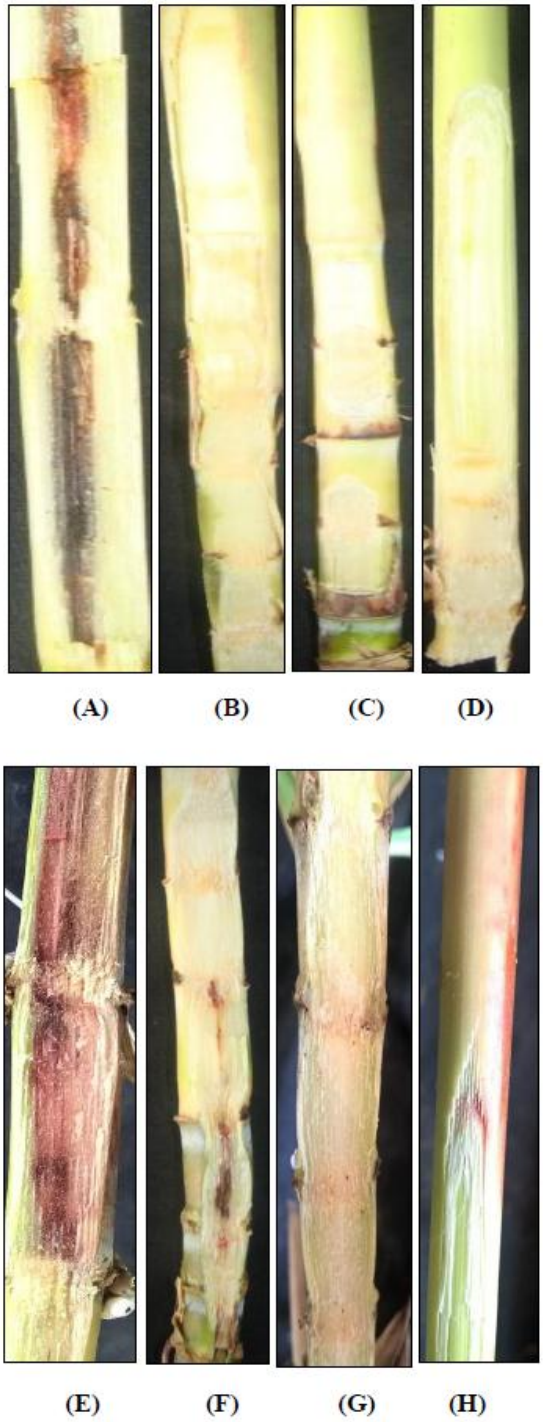


Figure 13: Red rot incidence

Reaction to Cf08 (A) Non transformed plant (NC) - Susceptible (B) SN1-19 - Resistant (C) SN10-12 - Resistant (D) SN10-13 - Resistant; Reaction to Cf09 (E) Non transformed plant (NC) - Highly Susceptible (F) Non transformed plant (NC) - Susceptible (G) SN1-19 - Moderately Susceptible (H) SN10-12 - Moderately Resistant (I) SN10-13 - Moderately Resistant

Table 11: Fold change expression of *β-1,3-glucanase* transgene (Before and After inoculation in roots)

Clone no.	Average Ct (*β-1, 3 -glucanase*)		Average Ct (*tubulin*)		ΔCT		ΔΔCT		$2^{-\Delta\Delta CT}$	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
NC	26.90 ±0.15	26.02 ±0.12	26.83 ±0.20	25.42 ±0.15	0.07 ±0.05	0.60 ±0.03	0 ±0.05	0 ±0.03	0.99 (0.96-1.03)	0.99 (0.97-1.02)
SN10-12	23.80 ±0.12	25.09 ±0.07	24.60 ±0.10	27.17 ±0.23	-0.80 ±0.02	-2.08 ±0.16	-0.87 ±0.02	-2.68 ±0.16	1.82 (1.80-1.85)	6.44 (5.73-7.16)
SN10-13	23.80	25.09	24.60	27.17	-0.80	-2.08	-0.87	-2.68	1.82	6.04
SN1-12	24.79 ±0.16	25.79 ±0.15	24.82 ±0.11	26.84 ±0.25	-0.03 ±0.05	-1.05 ±0.10	-0.10 ±0.05	-1.65 ±0.05	1.07 (1.03-1.10)	3.27 (2.92-3.63)
SN1-19	26.82 ±0.13	25.04 ±0.05	27.42 ±0.12	26.20 ±0.10	-0.60 ±0.01	-1.16 ±0.05	-0.67 ±0.01	-1.76 ±0.01	1.59 (1.58-1.60)	3.38 (3.37-3.50)
SN6-2	26.21 ±0.20	28.56 ±0.20	26.04 ±0.15	28.67 ±0.05	0.17 ±0.05	-0.11 ±0.15	0.10 ±0.05	-0.71 ±0.05	0.93 (0.90-0.96)	1.64 (1.47-1.81)
SN6-5	26.02 ±0.12	30.75 ±0.06	25.52 ±0.11	29.95 ±0.07	0.50 ±0.01	0.80 ±0.01	0.43 ±0.01	0.20 ±0.01	0.73 (0.73-0.74)	0.87 (0.86-0.87)

Table 12: Fold change expression of *β-1,3-glucanase* transgene (Before and After inoculation in leaves)

Clone no.	Average Ct (*β-1, 3glucanase*)		Average Ct (*tubulin*)		ΔCT		ΔΔCT		$2^{-\Delta\Delta CT}$	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
NC	27.40 ±0.92	25.42 ±0.18	27.49 ±0.34	25.39 ±0.25	-0.09 ±0.58	0.03 ±0.07	0 ±0.58	0 ±0.92	1.07 (0.66-1.49)	0.99 (0.95-1.04)
SN6-5	28.60 ±0.21	30.75 ±0.18	25.96 ±0.15	27.95 ±0.21	2.64 ±0.06	2.80 ±0.03	2.73 ±0.06	2.83 ±0.03	0.15 (0.14-0.15)	0.14 (0.13-0.14)
SN6-2	29.29 ±0.32	28.37 ±0.10	30.05 ±0.10	29.22 ±0.32	-0.76 ±0.22	-0.85 ±0.22	-0.67 ±0.22	-0.88 ±0.22	1.60 (1.36-1.85)	1.86 (1.58-2.14)
SN1-12	29.31 ±0.15	26.55 ±0.14	29.57 ±0.18	27.77 ±0.20	-0.26 ±0.03	-1.22 ±0.06	-0.17 ±0.03	-1.25 ±0.06	1.12 (1.10-1.14)	2.37 (2.28-2.47)
SN1-19	28.41 ±0.10	28.01 ±0.24	29.85 ±0.36	29.10 ±0.22	-1.44 ±0.26	-1.09 ±0.02	-1.35 ±0.26	-1.12 ±0.02	2.58 (2.12-3.05)	2.17 (2.14-2.20)
SN10-12	27.25 ±0.10	26.07 ±0.14	29.10 ±0.36	28.93 ±0.22	-1.85 ±0.26	-2.86 ±0.08	-1.76 ±0.26	-2.89 ±0.08	3.43 (2.82-4.05)	7.42 (7.01-7.42)
SN10-13	27.25 ±0.10	26.07 ±0.14	29.10 ±0.36	28.93 ±0.22	-1.85 ±0.26	-2.86 ±0.08	-1.76 ±0.26	-2.89 ±0.08	3.43 (2.82-4.05)	7.42 (7.01-7.42)

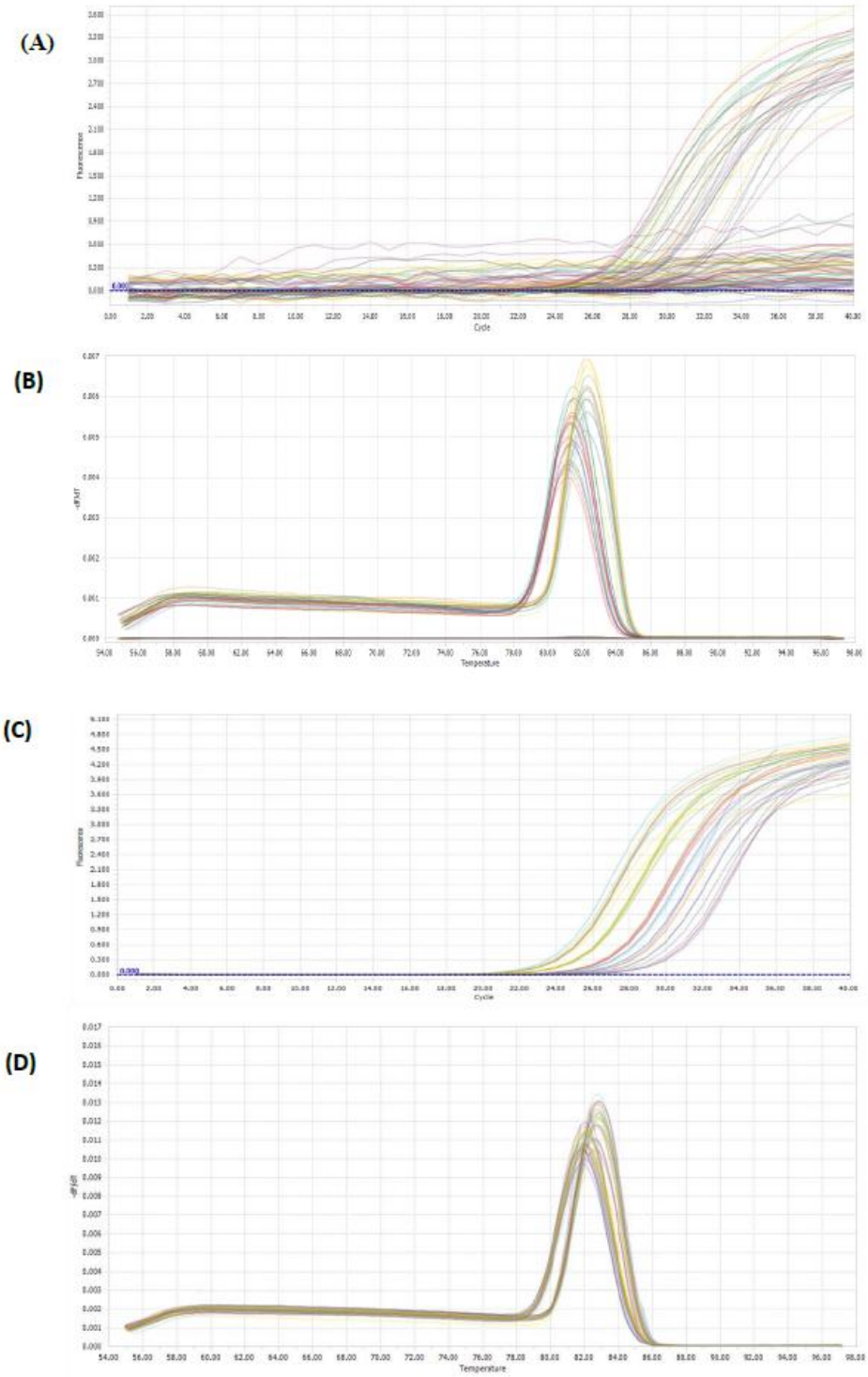


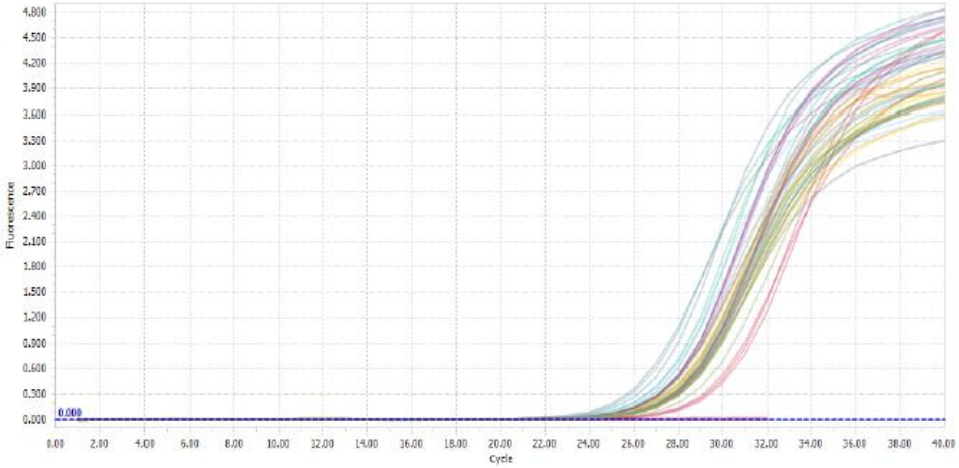
Figure 14 (A): Amplification chart from qRT-PCR analysis (Before inoculation in leaves)

Figure 14 (B): Melt peak chart from qRT-PCR analysis (Before inoculation in leaves)

Figure 14 (C): Amplification chart from qRT-PCR analysis (Before inoculation in roots)

Figure 14 (D): Melt peak chart from qRT-PCR analysis (Before inoculation in roots)

(E)



(F)

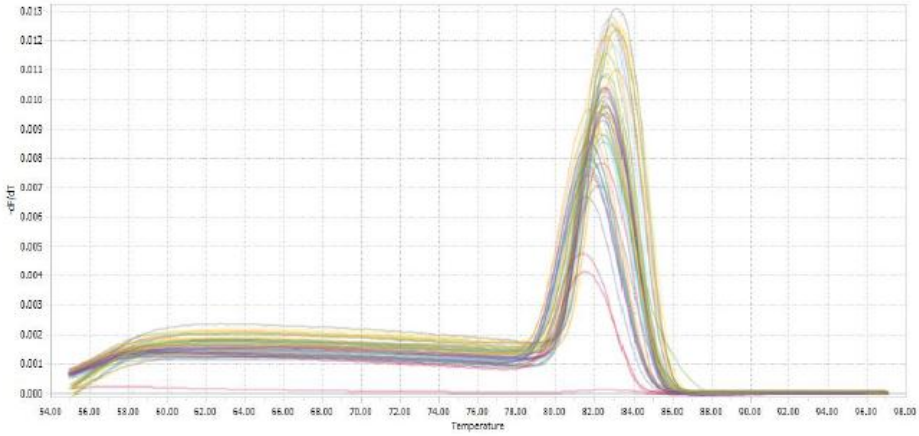


Figure 14 (E): Amplification chart from qRT-PCR analysis (After inoculation in leaves and roots)

Figure 14 (F): Melt peak chart from qRT-PCR analysis (After inoculation in leave and rootss)

4.6 CG_2 plant analysis for chimerism

The plant to row progenies of two T_0 putative transgenic plants of clonal generation 1 (CG_1) plants designated as SN10-12 and SN10-13 were grown to raise CG_2 to evaluate chimerism following T-DNA uptake through *Agrobacterium* mediated *inplanta* transformation. A total of 37 single buds from two T_0 putative transgenic plants of clonal generation 1 (CG_1) plants i.e. 17 single bud setts from SN10-12 and 20 single bud setts from SN10-13 were planted in polythene bags containing field soil, out of which 15 setts sprouted (Table 13).

Table 13: Clonal propagation of *in planta* transformed T_0 CG_2 putative transgenic plants and their PCR analysis

Putative T_0 transgenic plant designation (CG_1)	Number of bud setts planted	Number of bud setts sprouted	Designation of CG_2	No. of PCR positive clones	Designation of PCR positive clones
SN 10-12	17	7	C1-1 to C1-7	7	(C1-1; C1-2; C1-3; C1-4; C1-5; C1-6; C1-7)
SN 10-13	20	8	C2-1 to C2-8	8	(C2-1; C2-2; C2-3; C2-4; C2-5; C2-6; C2-7; C2-8)
TOTAL	**37**	**15**	**-**	**15**	**-**

The genomic DNA from each CG_2 plant was verified for *β-1,3-glucanase* transgene through PCR. The 1764 bp corresponding to transgene was observed in all 15 T_0 putative transgenic plants of clonal generation 2 (CG_2) (Figure 15). The *β-1,3-glucanase* transcription was confirmed in these T_0 putative CG_2 plants through semi-quantitative RT-PCR (Figure 16 A, B and C). qRT-PCR analysis revealed that relative transgene expression in clones of SN10-12 was statistically similar (Table 14). Likewise expression in SN10-13 clones were also statistically similar (Table 15).

Table 14: Fold change expression of *β-1,3-glucanase* transgene in clones of SN10-12 (CG2)

Clone no.	Average Ct (*β-1, 3 -glucanase*)	Average Ct (*tubulin*)	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
NC	27.90±0.09	26.83±0.17	1.07±0.08	0±0.08	0.99 (0.94-1.05)
C1-1	25.82±0.17	26.02±0.02	-0.20±0.15	-1.27±0.15	2.42 (2.17-2.67)
C1-2	29.60±0.12	29.79±0.35	-0.19±0.23	-1.26±0.23	2.42 (2.04-2.80)
C1-3	24.52±0.26	24.82±0.06	-0.3±0.2	-1.37±0.2	2.60 (2.25-2.96)
C1-4	26.92±0.15	27.42±0.20	-0.5±0.05	-1.57±0.05	2.97 (2.86-3.07)
C1-5	26.04±0.15	26.52±0.26	-0.48±0.11	-1.55±0.11	2.93 (2.71-3.16)
C1-6	24.25±0.07	24.54±0.15	-0.29±0.08	-1.36±0.08	2.56 (2.42-2.71)
C1-7	24.60±0.07	24.80±0.15	-0.2±0.08	-1.27±0.08	2.41 (2.28-2.54)

Table 15: Fold change expression of *β-1,3-glucanase* transgene in clones of SN10-13 (CG2)

Clone no.	Average Ct (*tubulin*)	Average Ct (*β-1, 3 -glucanase*)	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
NC	27.16±0.09	26.66±0.17	-0.50±0.08	0±0.08	0.99 (0.94-1.05)
C2-1	27.80±0.07	26.06±0.15	-1.74±0.08	-1.24±0.08	2.36 (2.23-2.49)
C2-2	27.84±0.15	26.15±0.20	-1.69±0.05	-1.19±0.05	2.28 (2.20-2.36)
C2-3	27.94±0.15	26.14±0.26	-1.80±0.11	-1.30±0.11	2.47 (2.28-2.65)
C2-4	27.81±0.12	26.02±0.35	-1.79±0.23	-1.29±0.23	2.47 (2.08-2.86)
C2-5	27.87±0.07	26.08±0.15	-1.79±0.08	-1.29±0.08	2.46 (2.31-2.58)
C2-6	27.90±0.26	26.01±0.06	-1.89±0.02	-1.39±0.02	2.62(2.58-2.65)
C2-7	27.96±0.15	26.17±0.20	-1.79±0.05	-1.29±0.05	2.44 (2.36-2.53)
C2-8	27.96±0.07	26.06±0.15	-1.90±0.08	-1.40±0.08	2.63 (2.49-2.78)

The qRT-PCR analysis of 15 transgenic clones and NC were performed in triplicates as shown in Appendix-II. The amplification chart and melt peak chart are shown in Fig 17 (A, B, C and D). These results confirmed successful integration of *β-1,3-glucanase* transgene in genome of T_0 putative transgenic plants of clonal generation 2 (CG_2) pointing towards absence of chimeras in CG_2.

The transgenic plants can be further analysed for copy number using Southern hybridization (Southern 1975), protein expression analysis using ELISA (Engvall and Perlmann 1971) and Western hybridisation (Towbin 1979). The hyphal growth in control and resistant plants can be analysed using electron microscopy.

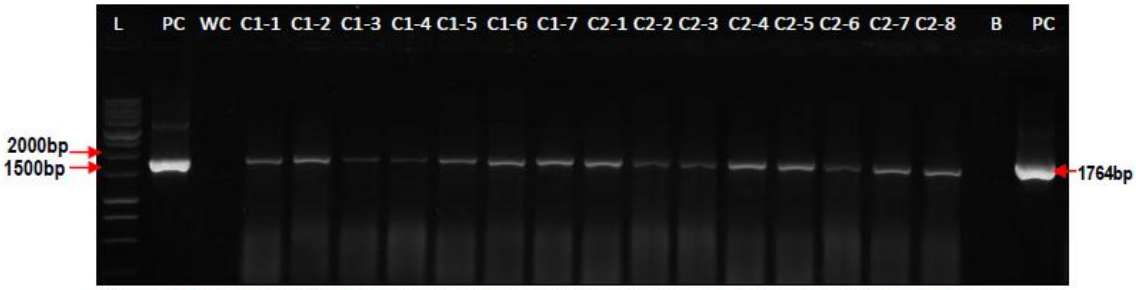


Figure 15: PCR positive T_0 putative transgenic plants CG_2 ; L refers to 1 Kb ladder (Promega, Cat. No. G5711); C1-1 to C1-7 and C2-1 to C2-8 represents PCR positive T_0 putative transgenic plants of CG_2; PC - positive control harbouring H1 gene cassette containing 1764 bp *β-1,3-glucanase* transgene in *Agrobacterium* strain LBA4404; B - Blank well; WC - Water Control

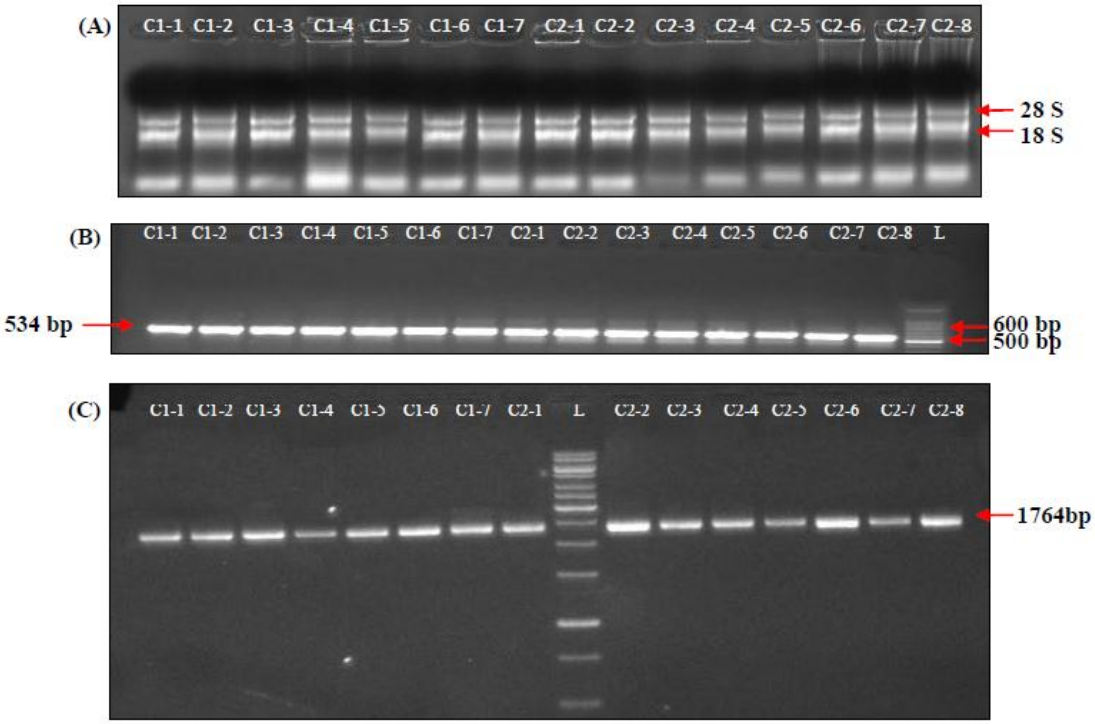


Fig 16 (A): RNA integrity of PCR positive T_0 plants of CG2; The numbers C1-1 to C-7 and C2-1 to C2-8 refer to RNA isolated from six PCR positive T_0 putative transgenic plants of CG_2

Fig 16 (B): Confirmation of cDNA integrity with 26Sr RNA primers; L refers to 100 bp ladder (Takara, Cat. No. 3407A); C1-1 to C-7 and C2-1 to C2-8 represents the amplification of 26S rRNA gene in 15 PCR positive T_0 transgenic plants of CG_2

Fig 16 (C): Confirmation of the expression of *β-1, 3-glucanase* transgene through RT-PCR using gene specific primers; L refers to 1 Kb ladder (Promega, Cat. No.G5711); C1-1 to C-7 and C2-1 to C2-8 represents RT-PCR positive T_0 putative transgenic plants carrying *β-1,3-glucanase* transgene

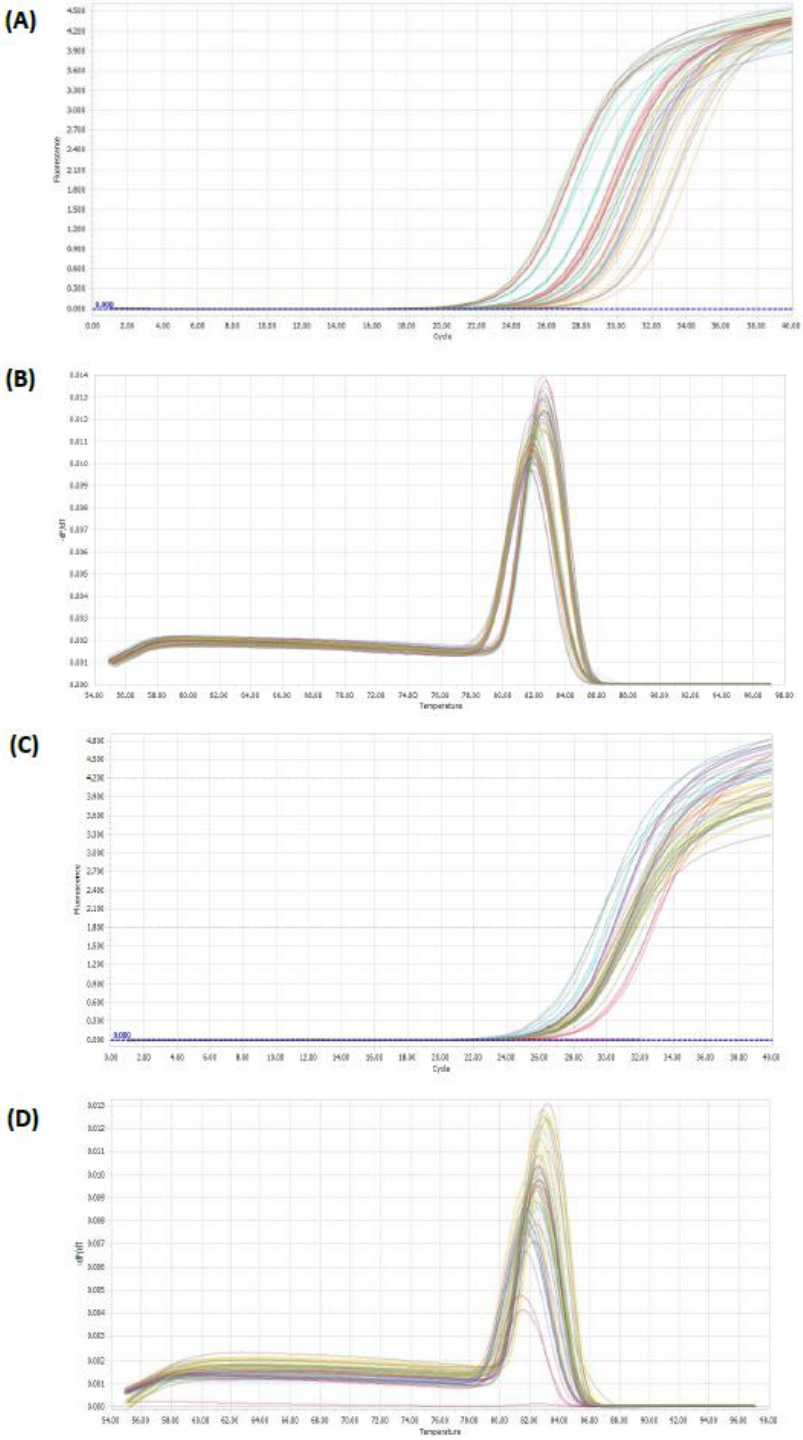


Figure 17 (A): Amplification chart obtained from qRT-PCR analysis of CG_2 (SN10-12)

Figure 17 (B): Melt peak chart obtained from qRT-PCR analysis of CG_2 (SN10-12)

Figure 17 (C): Amplification chart obtained from qRT-PCR analysis of CG_2 (SN10-13)

Figure 17 (D): Melt peak chart obtained from qRT-PCR analysis of CG_2 (SN10-13)

CHAPTER V

SUMMARY

Sugarcane (*Saccharum* spp. *officinarum*) is an important crop for food and energy. It's production is hampered by various biotic and abiotic stresses. Red rot caused by *C. falcatum* is the major reason for low productivity and production in India.

In the present study *in planta* transformed sugarcane plants carrying anti-fungal *β-1,3-glucanase* transgene under the control of CaMV 35S promoter and NOS terminator were evaluated for presence of transgene and red rot resistance. *In planta* transformed T_0 putative transgenic plants (10) were propagated through plant to row progenies leading to formation of 295 axillary bud setts, out of which 127 setts sprouted representing the clonal generation 1 (CG_1). The PCR analysis on T_0 putative transgenic plants of CG_1 (127 clones) revealed the amplification of 1764 bp product corresponding to *β-1,3-glucanase* transgene in six putative transgenic plants designated as SN1-12, SN1-19, SN6-2, SN6-5, SN10-12 and SN10-13. The expression of transgene in these six plants was analysed at transcriptional level using RT-PCR revealing the differential expression of transgene in all the six plants. Further, the relative *β-1,3-glucanase* transgene expression in six RT-PCR positive T_0 plants of CG_1 was confirmed by qRT-PCR. The cDNA of six positive transgenic plants was amplified using *β-1,3-glucanase* gene specific primers and primers for endogenous control (*tubulin*). The relative expression of *β-1,3-glucanase* transgene and *tubulin* gene revealed that three plants designated as SN1-19, SN10-12 and SN10-13 had $2^{-\Delta\Delta C_T}$ equal to 3.197, 4.280 and 4.438 respectively, as compared to to non-transformed control. These plants were maintained in transgenic glass house. Bioassay on six qRT-PCR positive T_0 plants was carried out for red rot disease incidence by infecting the canes with Cf08 pathotype of *C. falcatum*. The plants designated as SN1-19, SN10-12 and SN10-13 did not show any red rot symptoms as compared to non-transformed plant (NC).

Thus, the present study led to the identification of three CG_1 transgenic plants (SN1-19, SN10-12 and SN10-13) with high *β-1,3-glucanase* transgene expression and resistance to red rot. The CG_2 plant analysis through qRT-PCR revealed that relative transgene expression in clones of SN10-12 and SN10-13 was statistically similar suggesting absence of chimerism.

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APPENDIX-I

Replicate data obtained for six RT-PCR positive T_0 plants of CG_1 in qRT-PCR experiment

Well	Type	Threshold cycle (Ct)	Ct mean
E8	NT_*tubulin*	27.22	27.02
F8		26.93	27.02
G8		26.92	27.02
A8	NT_107	28.05	27.96
B8		27.87	27.96
C8		27.95	27.96
E5	SN1-12_*tubulin*	26.31	26.33
F5		26.32	26.33
G5		26.35	26.33
A5	SN1-12_107	26.25	26.31
B5		26.38	26.31
C5		26.31	26.31
E6	SN1-19_*tubulin*	31.64	31.69
F6		31.68	31.69
G6		31.76	31.69
A6	SN1-19_107	30.86	30.66
B6		30.52	30.66
C6		30.60	30.66
E2	SN6-2_*tubulin*	26.67	26.65
F2		26.59	26.65
G2		26.70	26.65
A2	SN6-2_107	27.08	26.78
B2		26.67	26.78
C2		26.60	26.78
E7	SN6-5_*tubulin*	28.14	27.91
F7		27.77	27.91
G7		27.81	27.91
A7	SN6-5_107	30.25	30.09
B7		29.96	30.09
C7		30.07	30.09
E3	SN10-12_*tubulin*	28.82	28.53
F3		28.46	28.53
G3		28.32	28.53
A3	SN10-12_107	27.50	27.37
B3		27.40	27.37
C3		27.20	27.37
E4	SN10-13_*tubulin*	30.75	30.31
F4		30.04	30.31
G4		30.16	30.31
A4	SN10-13_107	29.97	29.10
B4		29.29	29.10
C4		28.04	29.10

Note: NT_*tubulin* refers to non-transformed sample with *tubulin* primer and NT_1071 refers to non-transformed sample with MKQ1071 primer; SN refers to designation of CG_1

APPENDIX-II

Replicate data obtained for T_0 plants of CG_1 in qRT-PCR experiment (After inoculation in roots)

Well	Type	Threshold cycle (Ct)	Ct mean
E8	NT_*tubulin*	25.40	25.42
F8		25.40	25.42
G8		25.44	25.42
A8	NT_107	26.01	26.02
B8		26.05	26.02
C8		26.02	26.02
E5	SN1-12_*tubulin*	26.81	26.84
F5		26.83	26.84
G5		26.85	26.84
A5	SN1-12_107	25.80	25.79
B5		25.81	25.79
C5		25.78	25.79
E6	SN1-19_*tubulin*	26.19	26.20
F6		26.20	26.20
G6		26.19	26.20
A6	SN1-19_107	25.04	25.04
B6		25.04	25.04
C6		25.03	25.04
E2	SN6-2_*tubulin*	28.67	28.67
F2		28.65	28.67
G2		28.67	28.67
A2	SN6-2_107	28.52	28.56
B2		28.56	28.56
C2		28.57	28.56
E7	SN6-5_*tubulin*	29.96	29.95
F7		29.95	29.95
G7		29.95	29.95
A7	SN6-5_107	30.70	30.75
B7		29.74	30.75
C7		30.75	30.75
E3	SN10-12_*tubulin*	27.15	27.17
F3		27.16	27.17
G3		27.18	27.17
A3	SN10-12_107	25.07	25.09
B3		25.10	25.09
C3		25.09	25.09
E1	SN10-13_*tubulin*	27.15	27.17
F1		27.16	27.17
G1		27.18	27.17
A1	SN10-13_107	25.07	25.09
B1		25.10	25.09
C1		25.09	25.09

Replicate data obtained for T_0 plants of CG_1 in qRT-PCR experiment (Before inoculation in roots)

Well	Type	Threshold cycle (Ct)	Ct mean
E8	NT_*tubulin*	26.81	26.83
F8		26.84	26.83
G8		26.84	26.83
A8	NT_107	26.88	26.90
B8		26.92	26.90
C8		26.91	26.90
E5	SN1-12_*tubulin*	24.81	24.82
F5		24.83	24.82
G5		24.83	24.82
A5	SN1-12_107	24.80	24.79
B5		24.81	24.79
C5		24.78	24.79
E6	SN1-19_*tubulin*	27.41	27.42
F6		27.43	27.42
G6		27.43	27.42
A6	SN1-19_107	26.80	26.82
B6		26.84	26.82
C6		26.81	26.82
E2	SN6-2_*tubulin*	26.03	26.04
F2		26.05	26.04
G2		26.01	26.04
A2	SN6-2_107	26.18	26.21
B2		26.20	26.21
C2		26.22	26.21
E7	SN6-5_*tubulin*	25.52	25.52
F7		25.50	25.52
G7		25.51	25.52
A7	SN6-5_107	26.01	26.02
B7		26.02	26.02
C7		26.02	26.02
E3	SN10-12_*tubulin*	24.58	24.60
F3		24.61	24.60
G3		24.61	24.60
A3	SN10-12_107	23.78	23.80
B3		23.81	23.80
C3		23.81	23.80
E1	SN10-13_*tubulin*	24.58	24.60
F1		24.61	24.60
G1		24.61	24.60
A1	SN10-13_107	23.78	23.80
B1		23.81	23.80
C1		23.81	23.80

Replicate data obtained for T_0 plants of CG_1 in qRT-PCR experiment (Before inoculation in leaves)

Well	Type	Threshold cycle (Ct)	Ct mean
E8	NT_*tubulin*	27.51	27.49
F8		27.49	27.49
G8		27.49	27.49
A8	NT_107	27.39	27.40
B8		28.41	27.40
C8		27.40	27.40
E5	SN6-5_*tubulin*	25.96	25.96
F5		25.95	25.96
G5		25.95	25.96
A5	SN6-5_107	28.59	28.60
B5		28.61	28.60
C5		28.61	28.60
E6	SN6-2_*tubulin*	30.04	30.05
F6		30.08	30.05
G6		30.06	30.05
A6	SN6-2_107	29.30	29.29
B6		29.28	29.29
C6		29.28	29.29
E2	SN1-12_*tubulin*	29.56	29.57
F2		29.57	29.57
G2		29.57	29.57
A2	SN1-12_107	29.30	29.31
B2		29.32	29.31
C2		29.32	29.31
E7	SN1-19_*tubulin*	29.80	29.85
F7		29.84	29.85
G7		29.84	29.85
A7	SN1-19_107	28.40	28.41
B7		28.42	28.41
C7		28.41	28.41
E3	SN10-12_*tubulin*	29.08	29.10
F3		29.11	29.10
G3		29.10	29.10
A3	SN10-12_107	27.24	27.25
B3		27.25	27.25
C3		27.25	27.25
E1	SN10-13_*tubulin*	29.08	29.10
F1		29.11	29.10
G1		29.10	29.10
A1	SN10-13_107	27.24	27.25
B1		27.25	27.25
C1		27.25	27.25

Replicate data obtained for T_0 plants of CG_1 in qRT-PCR experiment
(After inoculation in leaves)

Well	Type	Threshold cycle (Ct)	Ct mean
E8	NT_*tubulin*	25.38	25.39
F8		25.39	25.39
G8		25.39	25.39
A8	NT_107	25.44	25.42
B8		25.42	25.42
C8		25.41	25.42
E5	SN1-12_*tubulin*	27.76	27.77
F5		27.76	27.77
G5		27.77	27.77
A5	SN1-12_107	26.54	26.55
B5		26.56	26.55
C5		26.54	26.55
E6	SN1-19_*tubulin*	29.10	29.10
F6		29.09	29.10
G6		29.10	29.10
A6	SN1-19_107	28.01	28.01
B6		28.02	28.01
C6		28.01	28.01
E2	SN6-2_*tubulin*	29.21	29.22
F2		29.22	29.22
G2		29.22	29.22
A2	SN6-2_107	28.37	28.37
B2		28.36	28.37
C2		28.36	28.37
E7	SN6-5_*tubulin*	27.94	27.95
F7		27.96	27.95
G7		27.94	27.95
A7	SN6-5_107	30.74	30.75
B7		30.75	30.75
C7		30.74	30.75
E3	SN10-12_*tubulin*	28.92	28.93
F3		28.93	28.93
G3		28.93	28.93
A3	SN10-12_107	26.06	26.07
B3		26.07	26.07
C3		26.07	26.07
E1	SN10-13_*tubulin*	28.92	28.93
F1		28.93	28.93
G1		28.93	28.93
A1	SN10-13_107	26.06	26.07
B1		26.07	26.07
C1		26.07	26.07

APPENDIX-III

Replicate data obtained for 15 RT-PCR positive T_0 plants of CG_2 in qRT-PCR experiment

Well	Type	Threshold cycle (Ct)	Ct mean
E8	NT_*tubulin*	26.75	26.83
F8		26.85	26.83
G8		26.90	26.83
A8	NT_107	27.86	27.90
B8		28.01	27.90
C8		27.88	27.90
E5	C1-1_*tubulin*	25.96	26.02
F5		26.08	26.02
G5		26.01	26.02
A5	C1-1_107	25.80	25.82
B5		25.85	25.82
C5		25.84	25.82
E6	C1-2_*tubulin*	29.80	29.79
F6		29.78	29.79
G6		29.81	29.79
A6	C1-2_107	29.61	29.60
B6		29.61	29.60
C6		29.57	29.60
E2	C1-3_*tubulin*	24.78	24.82
F2		24.85	24.82
G2		24.83	24.82
A2	C1-3_107	24.52	24.52
B2		24.58	24.52
C2		24.52	24.52
E7	C1-4_*tubulin*	27.38	27.42
F7		27.45	27.42
G7		27.43	27.42
A7	C1-4_107	26.90	26.92
B7		26.90	26.92
C7		26.95	26.92
E3	C1-5_*tubulin*	26.48	26.52
F3		26.60	26.52
G3		26.50	26.52
A3	C1-5_107	26.03	26.08
B3		26.01	26.08
C3		26.02	26.08
E4	C1-6_*tubulin*	24.54	24.54
F4		24.56	24.54
G4		24.53	24.54
A4	C1-6_107	24.26	24.25
B4		24.22	24.25
C4		24.26	24.25

Well	Type	Threshold cycle (Ct)	Ct mean
E1	C1-7_*tubulin*	24.82	24.80
F1		24.78	24.80
G1		24.82	24.80
A1	C1-7_107	24.62	24.60
B1		24.58	24.60
C1		24.62	24.60
E8	NT_*tubulin*	27.22	27.16
F8		27.14	27.16
G8		27.14	27.16
A8	NT_107	26.14	26.66
B8		27.08	26.66
C8		26.70	26.66
E5	C2-1_*tubulin*	27.81	27.80
F5		27.91	27.80
G5		27.81	27.80
A5	C2-1_107	26.06	26.06
B5		26.05	26.06
C5		26.06	26.06
E6	C2-2_*tubulin*	27.83	27.84
F6		27.84	27.84
G6		27.84	27.84
A6	C2-2_107	26.14	26.15
B6		26.15	26.15
C6		26.15	26.15
E2	C2-3_*tubulin*	27.92	27.94
F2		27.96	27.94
G2		27.95	27.94
A2	C2-3_107	26.10	26.14
B2		26.22	26.14
C2		26.12	26.14
E7	C2-4_*tubulin*	28.01	27.81
F7		27.70	27.81
G7		27.79	27.81
A7	C2-4_107	25.96	26.02
B7		26.08	26.02
C7		26.01	26.02
E3	C2-5_*tubulin*	27.89	27.87
F3		27.84	27.87
G3		27.88	27.87
A3	C2-5_107	26.04	26.08
B3		26.10	26.08
C3		26.11	26.08
E4	C2-6_*tubulin*	27.92	27.90
F4		27.88	27.90
G4		27.90	27.90

Well	Type	Threshold cycle (Ct)	Ct mean
A4	C2-6_107	26.03	26.01
B4		26.01	26.01
C4		26.03	26.01
E1	C2-7_*tubulin*	27.99	27.96
F1		27.94	27.96
G1		27.95	27.96
A1	C2-7_107	26.20	26.17
B1		26.15	26.17
C1		26.16	26.17
E9	C2-8_*tubulin*	27.97	27.96
F9		27.96	27.96
G9		27.95	27.96
A9	C2-8_107	26.06	26.06
B9		26.06	26.06
C9		26.05	26.06

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