# Expression of a β-1,3-glucanase from a biocontrol fungus in transgenic pearl millet

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## Abstract

*Sclerospora graminicola* is an oomycete (heterotrophic Stramenopiles), fungal-like obligate phytopathogen, the causal agent of downy mildew in pearl millet (*Pennisetum glaucum* [L.] R. Br.), and a major constraint in the production of this cereal crop. In this study a hydrolytic enzyme, β-1,3-glucanase (*gluc78*), from the biocontrol fungus *Trichoderma atroviride*, was introduced into the genome of a pearl millet breeding line, 842B, by particle bombardment. Constructs were prepared containing the *gluc78* gene, encoding the 78 kDa β-1,3-glucanase protein, downstream of either the constitutive ubiquitin promoter or the wound inducible potato proteinase inhibitor IIK gene promoter (*pin2*). The positive selectable marker gene, *man*A, encoding mannose-6-phosphate isomerase (phosphomannose isomerase) under the control of the ubiquitin promoter, was used for co-transformation. Transgenic plants were obtained harbouring the *man*A selectable marker gene and the antifungal gene *gluc78* downstream of either the ubiquitin or *pin2* promoter. Full constructs or minimal transgene expression cassettes containing the genes of interest were successfully introduced into the genome of pearl millet. Progeny of stably transformed plants, harbouring the *gluc78* transgene which is driven by the *pin2* promoter and followed by the rice *Act1* intron sequences, was subjected to pathogenicity trials. One transgenic event exhibited a reduction of 58% in the incidence of *S. graminicola* infection, however other transgenic pearl millet events showed no resistance to this phytopathogen. The event conferring decreased susceptibility to *S. graminicola* had high levels of the glucanase transcript especially in transgenic plants showing higher levels of downy mildew infection.
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*Keywords:* Fungal resistance; *Sclerospora graminicola*; Transgenic pearl millet; β-1,3-Glucanase

## 1. Introduction

Pearl millet (*Pennisetum glaucum* formerly *Pennisetum americanum*) is a staple food for the world's poorest and most food-insecure people in Africa and India. Pearl millet is the only major staple cereal that reliably produces both grain and forage on poor, sandy soils under hot, dry conditions of Africa and Asia (Goldman et al., 2003). The worst scourge of pearl millet is the downy mildew disease caused by the fungal phytopathogen *Sclerospora graminicola* (Sacc.) J. Schröt. It causes systemic infection in pearl millet that manifests itself as foliar symptoms and as green ear disease in the panicle (Sarosh et al., 2005).

*Trichoderma atroviride* (Sacc.) J. Schröt. (previously *T. harzianum*) is a soil-borne filamentous fungus, capable of parasitising several plant pathogenic fungi, and is a well characterised biocontrol agent (Ait-Lahsen et al., 2001; Emani

et al., 2003). *T. atroviride* is therefore a potential source of powerful resistance genes against fungal induced plant diseases. Secretion of lytic enzymes, mainly glucanases and chitinases, is considered the most crucial step of the mycoparasitic process (Cohen-Kupiec et al., 1999). The lytic enzymes degrade the cell walls of the pathogenic fungi, enabling *Trichoderma* to utilize both their cell walls and cellular contents for nutritional purposes.

It is well documented that acidic and basic plant β-1,3-glucanases are associated with fungal infection (Gheong et al., 2000; Ji et al., 2000; Jung and Hwang, 2000). Induction of a β-1,3-glucanase occurs in seedlings of pearl millet in response to infection by *S. graminicola* and high levels of a 30 kDa β-1,3-glucanase are present in downy mildew resistant pearl millet cultivars but absent in susceptible cultivars (Kini et al., 2000a,b). These studies indicate that β-1,3-glucanases play an important role in the defence mechanism of pearl millet plants against *S. graminicola* infection. In addition, transgenic potato plants expressing a soybean β-1,3-endoglucanase gene exhibited increased resistance to the oomycete *Phytophthora infestans* (Mont.) de Bary (Borkowska et al., 1998).

Broad-spectrum resistance against soil-borne and foliar pathogens was achieved in potato plants expressing an antifungal endochitinase from *T. atroviride* (*chit*42) (Lorito et al., 1998). The high level and broad spectrum of resistance obtained against these pathogens with a single constitutively expressed endochitinase gene, *chit*42, improved the previously limited efficacy of transgenic expression of chitinase genes from plants and bacteria in plants (Lorito et al., 1998). In addition, a 78 kDa β-1,3-glucanase (EC 3.2.1.58) from *T. atroviride* exhibited activity (ED$_{50}$ of 0.06–0.26 µg/ml) against *Phytophthora* spp. in an antifungal bioassay when applied in combination with microbial toxins and chemical fungicides (Fogliano et al., 2002). The aim of the presented study was to introduce *gluc*78, a 78 kDa β-1,3-glucanase enzyme from *T. atroviride* into the genome of pearl millet to improve resistance to *S. graminicola*. Since *gluc*78 had no sequence homology to plant or bacterium glucanases, it was hypothesised that stable integration and expression of this single glucanase gene in pearl millet could significantly contribute to increased resistance to downy mildew.

*Agrobacterium*-mediated transformation of crops usually generates transgenic plants carrying a single-copy transgene, flanked by the left and the right T-DNA border sequences. Nevertheless, a number of reports have shown the presence of multiple transgene copies and vector sequences in up to 75% of transformants (Kononov et al., 1997; Lange et al., 2006). Therefore, the approach of introducing minimal transgene expression cassettes (MTECs) by biolistic transformation with the goal of enhancing low copy number integration of transgenes, and minimising transgene rearrangements and gene silencing (Fu et al., 2000; Kohli et al., 1998), was employed. Vector backbones have the tendency to promote transgene rearrangements, since the vector backbone has been shown to provide a number of recombination hotspots. The removal of these sequences would possibly limit the influence of recombinogenic elements on the process of integration (Fu et al., 2000).

In this study, scutellum cells of pearl millet immature zygotic embryos (IZEs) were co-transformed with the positive select-able marker gene, *man*A and the *gluc*78 transgene either under the control of the *pin*2 or ubiquitin promoter. Seedlings produced from selfed seed of transgenic T$_2$ pearl millet of selected transformation events were tested for resistance to *S. graminicola*.

## 2. Materials and methods

### 2.1. Plasmid construction

A gene (*gluc*78) encoding an antifungal glucan 1,3-β-glucosidase was cloned from strain P1 of the biocontrol fungus *T. atroviride* (Donzelli et al., 2001). The gene encodes for a protein of 770 aa, including a 40 aa signal peptide. The *gluc*78/nos terminator fragment was ligated downstream of the constitutive ubiquitin promoter within pAHC25 (Christensen and Quail, 1996) and the product was designated pUBI*gluc*78 (10 131 bp) (Fig. 1). A construct was also prepared containing the *gluc*78 gene driven by the *pin*2 promoter followed by an AMV leader sequence (pGEM*gluc*78; 6584 bp). The cassette containing the *pin*2 promoter, AMV leader sequence, *gluc*78 gene and nos terminator was isolated from pBIN-GLUC (Donzelli et al., 2001) and inserted in the Hind III/Eco RI restriction sites of the multiple cloning region of pGEM-7Zf(−) (Promega). Construct pPA*gluc*78 (7148 bp) was prepared with the *pin*2 promoter, and the rice *Act*1 intron sequence upstream of the *gluc*78 gene (Fig. 1). The *pin*2 promoter followed by the rice actin intron was isolated from pDX109 (Xu et al., 1993) as a *Sma* I and Hind III sticky end fragment. The ubiquitin promoter was inserted upstream of the *man*A gene in the multiple cloning region of the promoterless construct pNOV3604 (Syngenta Seeds AG). The resulting 6210 bp construct was designated pNOV3604ubi (Fig. 1).

In order to assess the sequence integrity of expression vectors, plasmid DNA was digested with various restriction enzymes both individually and in combinations to verify that the expected fragment sizes were obtained for the four prepared constructs. In addition, sequence integrity of the vectors and inserts across ligation regions was verified by sequence analysis (ABI PRISM™ dye terminator cycle sequencing) using the ready reaction kit with AmpliTaq® DNA polymerase (The Perkin Elmer Corporation, Norwalk, USA).

### 2.2. Pearl millet plant transformation

Medium J was used for all pearl millet transformations as previously described (O'Kennedy et al., 2004a). MTECs were isolated as follows: Hind III isolated fragment of 4613 bp from pUBI*gluc*78, Hind III/*Sph* I isolated fragments of 3845 bp or 4109 bp from pGEM*gluc*78 or pPAgluc78, respectively and a Hind III/*Asp* 718 fragment of 3559 bp from pNOV3604ubi. The fragments were gel purified with a QIAquick (QIAGEN, Hilden, Germany) gel extraction kit or Geneclean II kit (Q BIOgene, USA) under sterile conditions. Proliferating IZEs were co-bombarded with pNOV3604ubi and one of the constructs containing the *gluc*78 gene (Table 1) at a ratio (molar) of 1:2 respectively. IZEs were pre-cultured for 6–8 days, bombarded at
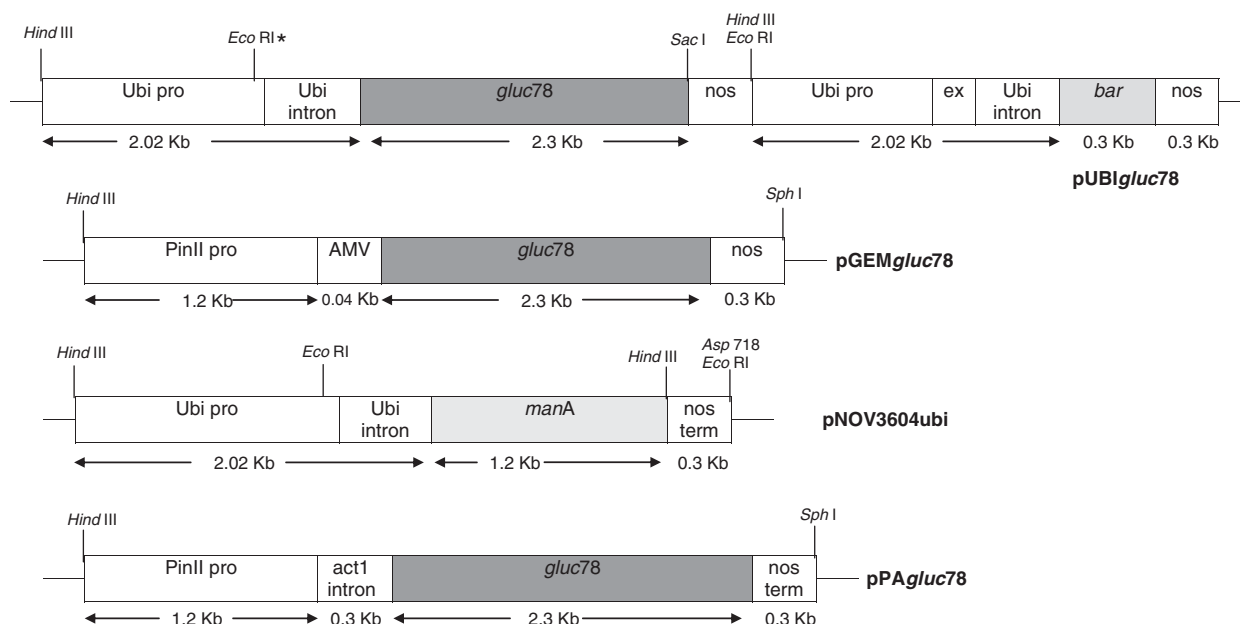
Fig. 1. Constructs containing either the selectable marker genes *man*A or *bar*, or the antifungal gene *gluc*78. The Eco RI site in the ubiquitin intron upstream of gluc78 is potentially methylated (indicated by an asterisks, *) as was the case for construct pAHC25 it originates from Christensen and Quail (1996).

a helium pressure of 900–1200 kPa and then cultured for 4–6 days on osmoticum free medium before being transferred to mannose-containing selection medium, of 2 g/l maltose and 15 g/l mannose.

## 2.3. PCR analysis of putative transgenic plants

The *man*A specific primers (PMI fwd: 5′-CGT TGA CTG AAC TTT ATG GTA TGG-3′ and PMI as: CAC TCT GCT GGC TAA TGG TG-3′) were used to amplify a 965 kb mannose gene fragment, from genomic DNA preparations of putative transgenic pearl millet plantlets. The *gluc*78 specific primers (*gluc*78 fwd: 5′-ATG ATG GGT CTC TCA ACC GTC-3′ and *gluc*78 as: 5′-TAG CAC CGC CAT TGA GAA TGG-3′) were used to amplify a 1100 kb fragment, from

### Table 1
Conditions for bombardment of pearl millet IZEs of genotype 842B pre-cultured on medium J and subsequently bombarded with either full constructs (experiments 1 and 2) or minimal transgene expression cassette (experiments 3 and 4) of pUbi-*gluc*78 (ubi *gluc*), pGEM *gluc*78 (AMV *gluc*) or pPA *gluc*78 (PA *gluc*) (containing the beta-1,3-glucanase 78) and the *man*A gene as selectable marker gene.

| Exp. # | Helium pressure (kPa) | Constructs | DNA introduced (ng/shot) | Transgenic events |
|---|---|---|---|---|
| 1 | 1000 | *man*A:pPA*gluc* | 64:104 | 7.7.2 |
| | | | | 7.9 |
| | 1200 | | | 7.10 (1–3) |
| | | | | 7.10 (A–C) |
| 2 | 1000 | *man*A:ubi gluc | 85:170 | 2.1A |
| 3 | 900 | *man*A:ubi gluc | 70:113 | 7.6B |
| 4 | 900 | *man*A:AMV gluc | 66:182 | 10.4A |

genomic DNA preparations. The *bla* gene (conferring ampicillin resistance) present in the vector backbones was amplified with the following primers: bla fw 5′-TGCTTAATCAGT-GAGGCACC-3′ and bla as1 5′-AGATCAGTTGGGTG-CACG-3′ to produce a PCR product of 757 bp.

## 2.4. Southern blot analysis of transgenic plants

Genomic DNA was extracted from putative transgenic pearl millet leaf material using the mini extraction procedure of Dellaporta et al. (1983). Five micrograms of pearl millet genomic DNA was digested with selected restriction enzymes and the fragments separated on an agarose gel and analysed by southern blot analysis as previously described (O'Kennedy et al., 2004b). Internal fragments of the *man*A gene of pNO-V3604ubi and the *gluc*78 gene of pPA*gluc*78 were labelled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche Diagnostics GmbH, Mannheim, Germany). The DIG labelled molecular weight marker III (Roche Diagnostics GmbH, Mannheim, Germany) was included on all blots in order to estimate fragment sizes. An internal DNA probe of *gluc*78 was also prepared using redivue™ $^{32}$P-dCTP labelling and the mega prime™ labelling kit (RPN1605) (Amersham Biosciences, UK) and southern blot analysis (DNA target) conducted as described for northern blot analysis.

## 2.5. Northern and western blot analyses of transgenic pearl millet plants

In order to determine expression levels of the *gluc*78 transgene, RNA and protein extractions, of selected non-

transgenic control plants and transgenic pearl millet inoculated with *S. graminicola*, were prepared 21 days after inoculation and probed with a labelled DNA fragment or antibody, respectively. Wound elicited expression of *gluc*78 was assessed in similar age transgenic and non-transgenic control plants by wounding with forceps at 1 cm intervals along a selected leaf. Leaves were harvested 24 h after wounding.

Total RNA was extracted using the QIAGEN RNeasy plant mini kit (QIAGEN) according to the manufacturer's recommendation. The RNA was separated on a denaturing formaldehyde gel as described in the booklet of the QIAGEN RNeasy plant mini kit. Eight microgram of RNA was loaded per lane. The RNA was transferred overnight via capillary action to a positively charged nylon membrane (Roche Diagnostics GmbH) using 10× SSC (Sambrook et al., 1989) and then fixed to the dried membrane for 30 min at 80 °C followed by exposure to UV light for 3 min.

An internal DNA probe of *gluc*78 was prepared using redivue™ $^{32}$P-dCTP labelling and the mega prime™ labelling kit (RPN1605) (Amersham Biosciences, UK). Unincorporated nucleotides were removed with a QIAquick® Nucleotide Removal kit (QIAGEN) and the probe was denatured by boiling before addition to the blots. Blots were treated for 4 h in prehybridisation solution (100 mg/l denatured Salmon sperm DNA, 50 mM Tris pH 8.0, 10 mM EDTA, 5× SSC, 20 mg each of BSA, Ficoll 400 and PVPP, 0.2% SDS [m/v]) followed by 16 h in hybridisation solution (100 mg/l denatured Salmon sperm DNA, 500 g/l [m/v] dextran sulfate, 50 mM Tris pH 8.0, 10 mM EDTA, 5× SSC, 20 mg/l each of BSA, Ficoll 400 and PVPP, 0.2% SDS [m/v]) at 65 °C in a Techne Hybridiser HB-1D (Techne). Post hybridisation washes were performed as follows: 5 min at room temperature (2× SSC, 0.5% SDS [m/v]), a second more stringent wash for 5 min at room temperature (0.1× SSC, 0.1% SDS) and 10–15 min at 65 °C (0.1× SSC, 0.1% SDS). Blots were exposed to X-ray film (Hyperfilm™ MP, Amersham Pharmacia Biotech) in the presence of intensifier screens for 3–5 days at −80 °C.

Protein extractions were carried out according to Kini et al. (2000a) with the addition of PVPP and protease inhibitor cocktail (Sigma P9599) for protection against protease activity. Leaf material was homogenised with a mortar and pestle in extraction buffer (50 mM sodium acetate buffer pH 5.2), containing PVPP and protease inhibitor cocktail, according to the manufacturer's recommendation. The extracts were centrifuged at 4 °C for 10 min at 12 000 rpm. The supernatant was frozen at −20 °C until SDS-PAGE analysis. The protein concentration of each sample was determined by Bio-Rad protein dye (Bio-Rad), reagent, with bovine serum albumin as the standard. For each sample, 2 μg aliquots of protein were separated by polyacrylamide gel electrophoresis (PAGE) on a 12% separating, and 6% stacking gel according to the method of Laemmli (1970). Proteins were transferred to MSI PVDF-plus transfer membranes (0.45 μm) (Amersham Biosciences) by means of a Hoefer TE22 blot system. Western blots were probed with antiserum (1:750) to the glucanase protein using the ECL RPN2108 western blotting kit (Amersham Biosciences). For biotinylated molecular marker detection (ECL RPN2107, Amersham Biosciences) streptavidin-

horseradish peroxidase conjugate (ECL RPN1231, Amersham Biosciences) was used.

### 2.6. Pathogenicity trials of transgenic progeny

Transgenic pearl millet seedlings containing the antifungal gene, *gluc*78, from *T. atroviride* (Donzelli et al., 2001), and selectable marker gene, *man*A (Syngenta Crop Protection AG), were identified by germination on mannose-containing medium. The transgenic germinating plantlets were transplanted to pots of peat and sand compost at the glasshouse facility at Pen-y-Ffridd Experimental Station, Bangor, Wales, prior to inoculation with *S. graminicola*. *S. graminicola* isolates from Niger, Senegal and Mali were maintained in the glasshouse on the highly susceptible pearl millet cultivar 7042(S).

All the transgenic plants were derived from the cultivar 842B. The seedlings were planted in low-nutrient compost consisting of 2 parts fine peat to one part sharp sand with added nutrients (Chempak Seed Base, Chempak Products, UK: NPK 25–39–30 mg/l). Pots were placed on flood benching in a controlled environment glasshouse providing a 16-hour day length with light intensity between 500 and 1200 μmol Em$^{-2}$ s$^{-1}$ and a temperature of 25–30 °C from 06:00 to 18:00 and 20 °C from 18:00 to 06:00. The plants were watered by filling the benches to a depth of 1–2 cm for 30 min. Plants were fed twice weekly with a liquid fertilizer (Vitax Vitafeed 214, Leicester, UK). When the seedlings were at the coleoptile to one-leaf stage, inoculum was prepared. Leaves from 2 to 3 month old infected plants (Niger, Senegal and Mali isolates in a 1:1:1 ratio) were collected mid-morning, after plants had been photosynthesising for at least 4 h, wiped clean of old sporangia using moist laboratory roll (Kimwipes Roll, Kimberly Clark, Kent, UK) and incubated in sealed plastic boxes lined with moist laboratory roll for 7–8 h at 20 °C in the dark. Freshly produced sporangia were collected by shaking the leaves in cold distilled water (0–2 °C). The resulting sporangial suspension was filtered through muslin to remove debris and adjusted to $2 \times 10^{-5}$ sporangia/ml with cold distilled water. Each pot of seedlings was sprayed with approximately 4 ml of the inoculum using a compressed-air cylinder-fed sprayer (Kestrel Eqpt. Ltd., London, UK). The inoculum was maintained on ice throughout inoculation to prevent zoospore release and so ensure a uniform sporangial concentration over time (Jones et al., 2001). The pots were covered with a polythene sheet to maintain a high level of humidity and incubated in the glasshouse at 20 °C overnight. The development of downy mildew on treated plants was determined 21 days post infection and compared to wild-type control plants. All residual plant material was incinerated after assessment. Care was taken to destroy the plant material before flowering occurred.

### 2.7. Reverse transcription quantitative PCR (RT-qPCR) analysis of transgenic plants

Gene specific primers were synthesised for an endogenous pearl millet β-glucanase (AF488414) and *gluc*78 (AF253421) from *T. atroviride*. Primers designed to a pearl millet 18S RNA gene were used as an internal reference control for normalisation (Crampton et

al., 2009). Optimal primer design was performed using Primer3 software (http://frodo.wi.mit.edu), and internal primer secondary structure (hairpins, dimers, palindromes, and repeats) was assessed using Net Primer software (www.PremierBioSoft.com). Primers used in qRT-PCR are as follow: 18S rRNA (18S fw 5'-GCCATCGCTCTGGAT ACATT-3' and 18S as 5'-TCAT-TACTCCGATCCCGAAG-3'), endogenous glucanase (Gluc fw 5'-GCTTCTGGTCCTCGTTGAAG -3' and Gluc as 5'-GAACTTGATGCGGATGGTG-3') and *T. atroviride* transgene glucanase expression (Gluc78 fw 5-CTGACCCTGACTTTTCC AAGTG-3' and Gluc78 as 5'-GAAGAAGCTGTACATGC-CAGTG-3'), producing PCR products of 83, 101 and 113 bp, respectively.

DNAse 1-treated (QIAGEN) total RNA from each of the treated plants was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Each 20 µl reaction contained 200 ng total RNA, 60 µM random hexamer primer, 1× Transcriptor RT reaction buffer, 20 U Protector RNase Inhibitor, 1 mM dNTP mix, 10 U Transcriptor reverse transcriptase. Reactions were incubated at 55 °C for 30 min, after which the Transcriptor reverse transcriptase was inactivated by heating to 85 °C for 5 min. Reactions were stored at −20 °C until use in real time PCR reactions.

Conditions for all PCR reactions were optimised in a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA, USA) with regard to forward and reverse primer concentrations and annealing temperature. Optimised results were applied to the LightCycler PCR protocol. Real time PCR reactions were prepared using a LightCycler® FastStart DNA Master$^{PLUS}$ SYBR Green 1 kit (Roche Diagnostics). Each 20 µl reaction contained 0.25 µM forward primer, 0.25 µM reverse primer, 1× LightCycler Master Mix and 2 µl cDNA (undiluted). Reactions were added to glass capillaries and placed into the LightCycler rotor (Roche Diagnostics). The following LightCycler experimental run protocol was used: denaturation cycle (95 °C for 10 min), amplification and quantification cycle repeated 45 times (95 °C for 10 s, 60 °C for 5 s, and 72 °C for 6 s with a single fluorescence measurement), melting curve cycle (65–95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement), and finally a cooling step to 40 °C. LightCycler software 3.3 (Roche Diagnostics) was employed to calculate crossing points ($C_T$) for each transcript. $C_T$ is defined as the point at which the fluorescence rises appreciably above the background fluorescence (Pfaffl, 2001).

## 3. Results

### 3.1. Production of transgenic pearl millet

The *gluc*78 gene, encoding β-1,3-glucanase from *T. atroviride* (*gluc*78), in constructs pGEM*gluc*78 and pPA*gluc*78 was driven by the potato proteinase inhibitor IIK wound inducible promoter, (Fig. 1) to elicit expression during wounding and/or pathogen attack. In addition, the ubiquitin constitutive promoter, was ligated upstream of the selectable marker gene, *man*A (pNO-V3604ubi), or *gluc*78 (pUBI*gluc*78). Two-direction sequence analysis confirmed that sequence integrity was maintained across

ligation sites and that the ATG start codon of *gluc*78 was in the desired reading frame. The *gluc*78 gene was introduced as part of complete plasmids or as MTECs (fragments containing only the promoter, open reading frame and terminator sequences). The *gluc*78 genes were co-transformed with the *man*A selectable marker gene.

Ten transgenic plants contained the *gluc*78 transgene from *T. atroviride* (Table 1), driven either by the constitutive ubiquitin promoter (pUBI*gluc*78) or the *pin*2 wound inducible promoter (pPA*gluc*78), whereas one plant contained only the selectable marker gene, *man*A (event 10.4A, data not shown). Genomic southern blot analysis of $T_0$ and $T_1$ transformation events showed that events 7.10 1,2,3 and A,B,C were clones, whereas 7.7–2, 7.9, 2.1A, 10.4A and 7.6B were unique transformation events (Figs. 2 and 3). Twenty two and 111 pg of plasmid DNA represent 2 and 10 copies of the transgenes, respectively. Identical integration patterns were obtained for respective $T_1$ progeny (data not shown). The integration pattern of transformation events 7.10 1–3 and A–C was also confirmed by hybridisation with a radioactive labelled internal fragment of gluc78 (Fig. 2c). Transformation events 2.1A and 7.6B were obtained by co-transformation of the *man*A selectable marker gene and the antifungal gene *gluc*78 driven by the constitutive promoter, ubiquitin (Fig. 3). Transformation events 2.1A and 7.6B were obtained introducing either full constructs or minimal transgene expression cassettes (MTECs), respectively. Restriction enzymes Hind III alone or Hind III in combination with Eco RI isolates the gene of interest including the promoter and terminator sequence (Figs. 1 and 3). Southern blot fragments larger and smaller than the expected sizes are potentially concatamers or truncated versions of the introduced genes (Fig. 3, lanes 11–13). DNA of transformation event 2.1A was digested with Hind III, which theoretically releases a 4.6 kb fragment containing ubiquitin promoter plus *gluc*78 plus nos terminator (lanes 4–8, Fig. 3a) and 2.2 kb fragment (Hind III/ Eco RI digest) containing the ubiquitin intron plus *man*A transgene (lane 9, Fig. 3b). The Eco RI site in the ubiquitin intron upstream of *gluc*78 is potentially methylated as was the case for construct pAHC25 it originates from (Christensen and Quail, 1996). Therefore, the Hind III/Eco RI digest of transformation event 2.1A also resulted in a 4.6 kb fragment (lane 9, Fig. 3a) instead of the expected 3 kb fragment. Transformation event 10.4A contained only the selectable marker gene *man*A, but not the *gluc*78 transgene (data not shown). A co-transformation efficiency of 91% was therefore obtained.

An estimated 6–12 copies of the minimal transgene expression cassette containing *gluc*78 were inserted in transformation event 7.6B. Apart from the expected 4.6 kb fragment, numerous larger and smaller band sizes were obtained indicating truncated versions, concatamers, tandem repeat inserts or rearrangement of the transgene *gluc*78 (Fig. 3a). PCR amplification of the antibiotic resistance gene, *bla*, was negative for transgenic line 7.6B, illustrating that vector backbone was absent and only the linear minimal cassette integrated.

In order to determine the Mendelian segregation of the transgenic events ($T_1$ to $T_4$ progeny) obtained in this study,
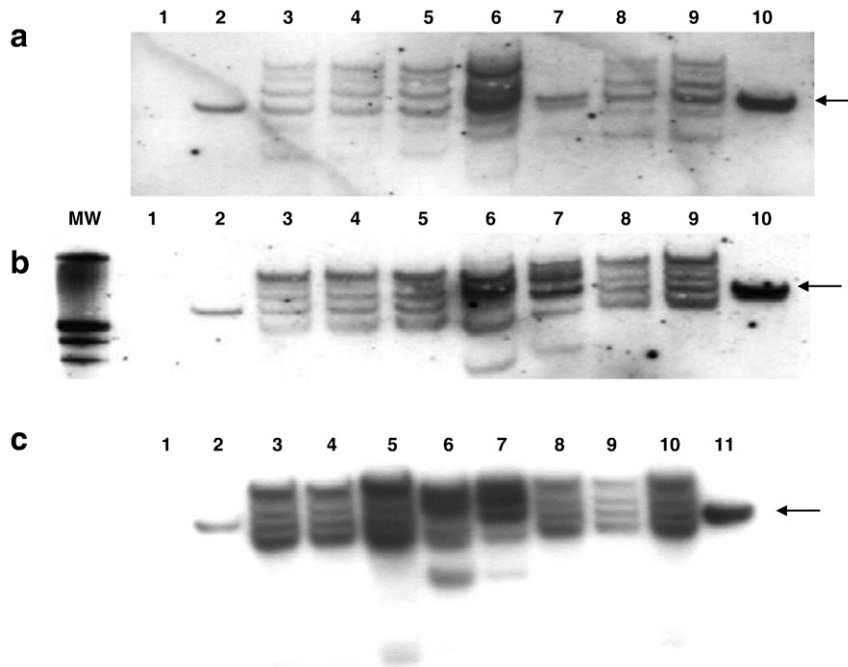
Fig. 2. Southern blot analysis of independent $T_0$ transformation events of pearl millet genotype 842B. Genomic DNA was purified from plant leaf material, restricted with Hind III and resolved in a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal DNA fragment of the *man*A (blot a) or *gluc*78 (blots b and c) transgene, either DIG labelled (blots a and b) or radioactive labelling (blot c). Blots a and b: MW, DIG labelled molecular weight marker III (Roche Diagnostics South Africa); lane 1, untransformed pearl millet leaf material of genotype 842B; lanes 2 and 10, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lane 3, plant 7.10A; lane 4, plant 7.10B; lane 5, plant 7.10C; lane 6, plant 7.7.2; lane 7, plant 7.9; lane 8, plant 7.10.1; lane 9, plant 7.10.3. Blot c: lane 1, untransformed control; lanes 2 and 11, represent untransformed pearl millet spiked with 2 and 10 transgene copies respectively; lane 3, plant 7.10A; lane 4, plant 7.10B; lane 5, plant 7.10C; lane 6, plant 7.7.2; lane 7, plant 7.9; lane 8, plant 7.10.1; lane 9, plant 7.10.2; lane 10, plant 7.10.3. The arrows indicate the size of pNOV3604ubi (6.2 kb) or pPA*gluc*78 (7.15 kb) linearised with Hind III.

progeny of transgenic events was germinated on mannose-containing medium (Table 2) as described previously (O'Kennedy et al., 2004b). Often less than 50% of the seeds germinated on the selection medium, therefore not reflecting a typical Mendelian segregation during the first four generations.

### 3.2. Pathogenicity trials with S. graminicola

Selected transgenic plants were subsequently inoculated with *S. graminicola*. $T_2$ transgenic pearl millet plants designated 7.10B, 7.10-1 and 7.10-2 were inoculated by *S. graminicola* to screen for downy mildew resistance. Downy mildew disease
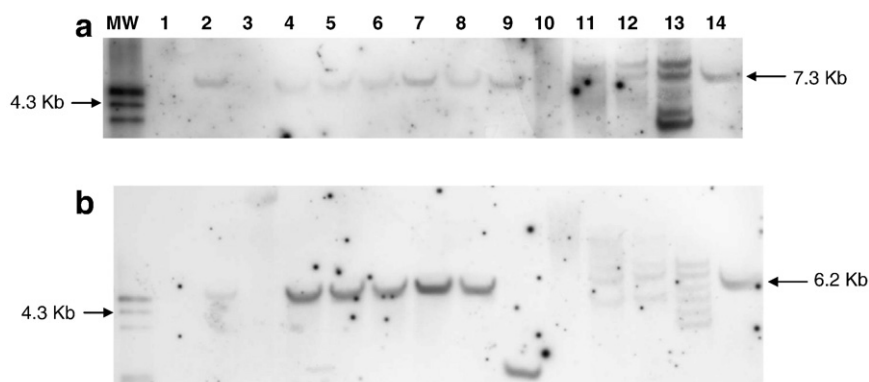


Fig. 3. Southern blot analysis of independent $T_0$ transformation events of pearl millet genotype 842B transformed with full constructs (2.1A) or minimal transgene cassettes (7.6B). Genomic DNA was purified from plant leaf material, resolved in a 0.8% agarose gel, transferred to a nylon membrane and then probed with a DIG labelled internal DNA fragment of *gluc*78 (blot a) or *man*A (blot b). MW, DIG labelled molecular weight marker III; lane 1, DNA of untransformed pearl millet leaf material of genotype 842B; lanes 2 and 14, represent DNA of untransformed 842B spiked with 2 and 10 transgene copies respectively; lane 3, undigested DNA of plant 2.1A $T_0$; lane 4, 2.1A $T_0$ digested with Hind III; lanes 5–8, genomic DNA of individual $T_1$ progeny plants of 2.1A digested with Hind III; lane 9, genomic DNA of 2.1A digested with Hind III/Eco RI; lane 10, genomic DNA of $T_1$ 7.6B undigested; lanes 11 and 12, genomic DNA of individual $T_1$ progeny plants of 7.6B digested with Hind III; lane 13, genomic DNA of an individual $T_1$ plant of 7.6B digested with Hind III/Eco RI.

was assessed 21 days after inoculation based on the incidence of infected plants within each pot (Table 3). The disease score for each treatment was the infection percentage for individual pot replicates. Thus, the disease score for each entry could range from 0 to 100% and was a continuous variable. Symptoms of systemic infection were seen as distinct chlorosis of infected leaves, white downy growth on both leaf surfaces (the asexual sporangiophores emerging from stomata) and stunted growth.

Transgenic event 7.10-2 ($T_2$ progeny) inoculated with *S. graminicola*, gave a disease score reduction of almost 58% when compared to inoculated control 842B plants (Table 3). Progeny of transgenic events 7.10B and 7.10-1 however, gave a higher disease incidence (1.5 and 1.7 fold, respectively) when compared to control plants.

## 3.3. Molecular analysis of the expression of the gluc78 transgene

RNA and protein analyses were conducted to determine if a correlation existed between the expression levels of *gluc*78, and altered *S. graminicola* disease susceptibility. Expression of the *gluc*78 transgene in leaves was analysed by RNA gel northern blotting and by protein gel western blotting for selected control and transgenic plants (Figs. 4 and 5). Wound-induced expression of the glucanase transcript of transformation event 7.10-2 was 2–4 times the intensity of the unwounded control leaf material 2, 6, 18, 24 and 48 h after wounding (northern blot analysis not shown). Under the stated assay conditions, faint signals of the *gluc*78 transcript were detected in both inoculated and non-inoculated non-transgenic plants (Fig. 4a, b and c; lanes 1 and 2). High levels of expression were obtained in

Table 2
Seed germination on mannose-containing medium (1 g/l maltose and 15 g/l mannose) or maltose alone for 842B control plants.

| Pearl millet transformation events | Number of seed *man*A pos:number of seeds *man*A neg | | | |
|---|---|---|---|---|
| | $T_1$ | $T_2$ | $T_3$ | $T_4$ |
| | +:− | +:− | +:− | +:− |
| 842B control | | | | 44:14 |
| | | | | 19:21 |
| 7.6B | | 59:377 | | |
| 2.1A | | 23:21 | 7:5 | |
| 7.10A | 4:142 | 24:76 | | |
| 7.10B | 1:11 | 3:6 | 26:14 | 30:15 |
| | | | | 11:29 |
| 7.10C | 7:163 | 12:22 | | 33:10 |
| | | 21:29 | | 21:19 |
| 7.10.1 | 70:772 | 24:76 | | 18:27 |
| | | 33:16 | | 10:30 |
| 7.10.2 | 16:408 | 50:165 | | 34:11 |
| | | 5:72 | | 16:24 |
| 7.10.3 | 21:196 | 12:88 | | |
| | | 9:32 | | |
| | | 8:32 | | |
| 7.9 | 1:32 | 36:421 | 3:9 | |
| | | 20:80 | | |
| 7.7.2 | 2:74 | 35:36 | 10:38 | |
| | | 2:34 | 2:10 | |

Table 3
Transgenic and non-transgenic control plants screened for downy mildew susceptibility by inoculation with *S. graminicola*.

| Experimental pearl millet plant material | Total number of plants treated | Number of plants infected | Overall disease incidence (%) |
|---|---|---|---|
| Uninoculated, non-transgenic, 842B | 36 | 0 | 0.0 |
| Inoculated, non-transgenic, 842B | 41 | 17 | 41.5 |
| Inoculated, transgenic event 7.10.B | 34 | 24 | 70.6 |
| Inoculated, transgenic event 7.10.1 | 5 | 3 | 60.0 |
| Inoculated, transgenic event 7.10.2 | 57 | 10 | 17.5 |

infected transgenic plants, and slightly lower levels of expression were obtained in inoculated uninfected transgenic plants, when compared to control plants (Fig. 4). In addition, the protein encoded by *gluc*78 was clearly expressed in transgenic plants inoculated with *S. graminicola* (Fig. 5), whereas the protein was absent in non-transgenic control plants whether the plants were inoculated with the pathogen or not.

RT-qPCR confirmed the occurrence of an increased level of transcript of the transgene in individually infected transgenic pearl millet events when infected by *S. graminicola* (Fig. 6). Transgene *gluc*78 expression levels in control 842B (plant 1, non-challenged; plant 4, challenged but non-infected and plant 5, challenged and infected with *S. graminicola*) were absent. Plants 10, 12, 15 and 16 indicated by the downward diagonal pattern on the bars showed *S. graminicola* infection symptoms whereas the rest did not show any signs of downy mildew infection although challenged with the pathogen. RT-qPCR results (Fig. 6) indicate that *S. graminicola* infected transgenic plants displayed higher gluc78 expression levels than non-infected plants.

## 4. Discussion

This is the first report introducing antifungal genes in a pearl millet parental breeding line which were previously used in breeding programmes for resistance to downy mildew. Our rationale was to further improve the moderately resistant breeding line 842B through the introduction of the antifungal gene *gluc*78. In previous studies, antifungal genes were introduced in a highly susceptible inbred line 7042S (Girgi et al., 2006; Latha et al., 2006).

Using a biolistic-mediated transformation protocol both complete constructs and MTECs were successfully introduced into line 842B. The overall goal of using MTECs was to enhance low copy number integration of transgenes, and minimise transgene rearrangements and gene silencing as previously reported (Fu et al., 2000; Kohli et al., 1998). Vector backbones have the tendency to promote transgene rearrangements, since the vector backbone has been shown to provide a number of recombination hotspots (Fu et al., 2000). The
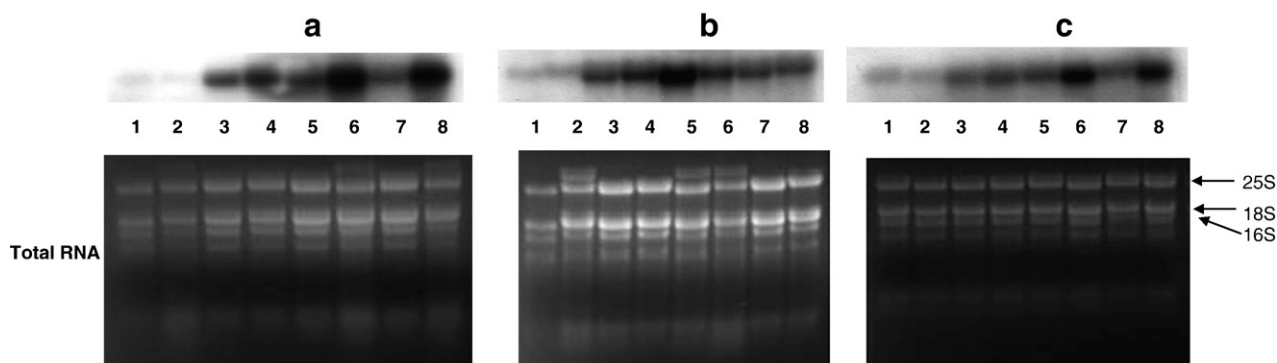
Fig. 4. Detection of the *gluc*78 *T. atroviride* glucanase transcript in infected and non-infected selfed progeny T$_2$ generation transgenic and control untransformed pearl millet genotype 842B. Total RNA was extracted from leaf material and 8 µg of RNA loaded/lane and blotted to a nylon membrane. The RNA gel blot was hybridised with a *gluc*78 internal DNA fragment probe. Northern blot analysis was performed on a: lane 1, non-infected non-challenged with *S. graminicola* control plant; lane 2, infected control plant; lanes 3 and 4, two individual infected plants from transformation event 7.10.1; lanes 5 and 7, non-infected 7.10B; lanes 6 and 8, infected 7.10B; b: lane 1, non-infected control plant; lane 2, infected control plant; lanes 3, 4, 7 and 8, non-infected challenged transgenic plants of event 7.10.2; lanes 5 and 6, infected challenged transgenic plants of event 7.10.2; c: lane 1, non-infected non-challenged control plant; lane 2, non-infected challenged control plant; lanes 3–8, non-infected challenged transgenic plants of event 7.10.2. Ethidium bromide stained gels show that equal amounts of total RNA were loaded in each lane, in each set a, b and c.

removal of these sequences would possibly limit the influence of recombinogenic elements during the process of integration (Fu et al., 2000). In this study, an estimated 6–12 copies of the transgene *gluc*78 were inserted in pearl millet transformation event 7.6B, probably due to the high DNA concentration used for bombardment (183 ng/shot). Recently, Lowe et al. (2009) produced more than 1600 maize (inbred H99) events consistently yielding single-copy events at high transformation frequencies using only 2.5 ng cassettes DNA/shot.

Generally, transgenic pearl millet plants expressing gluc78 were more susceptible to *S. graminicola* and displayed severe disease symptoms relative to wild-type 842B plants. *In vivo* and *in vitro* infection of the same transgenic pearl millet lines with *Puccinia substriata* also showed an increase in disease incidence (data not shown). However, T$_2$ progeny of transgenic event 7.10-2, inoculated with *S. graminicola*, resulted in a disease score reduction of almost 58%, whereas transgenic events 7.10B and 7.10-1 gave a significantly higher disease incidence when compared to control plants. Quantitative real time PCR confirmed the occurrence of an increased level of the gluc78 transcript of the transgene in individually infected transgenic pearl millet, as well as plants that were infected with *S. graminicola* but did not confer resistance to these pathogens. Sanders et al. (2002) reported that silencing of a heterologous glucanase, lead to diminishing levels of the plant's endogenous glucanase activity, and increased disease susceptibility. The authors reported that this could be due to post-transcriptional gene silencing (PTGS) induced among glucanase genes that share some homology. The glucanase from *T. atroviride* shares no sequence homology with glucanases of plants or bacteria and
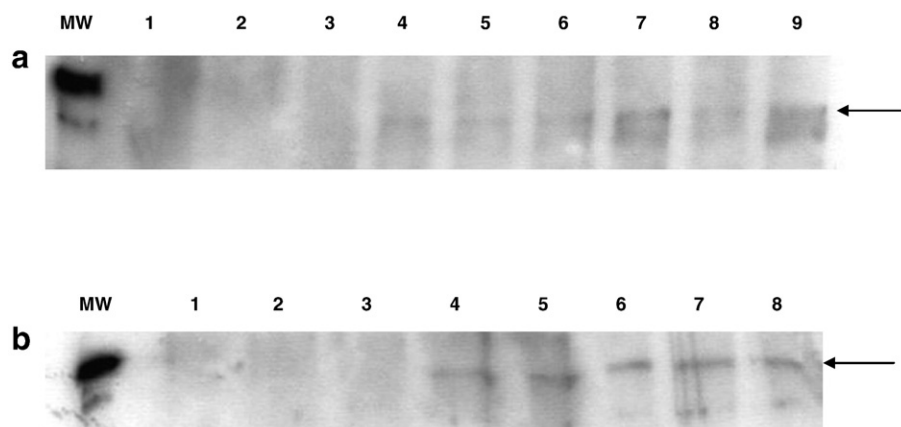


Fig. 5. Western blot analysis of total soluble proteins from leaves of transgenic and non-transgenic control plants. A two microgram aliquot of protein was separated in each lane, the blot immunoprobed with polyclonal anti-*gluc*78 antibodies (dilution 1:750) and visualised with anti-rabbit secondary antibodies (dilution 1:5000). Blots a and b: MW, ECL molecular weight marker; lane 1, non-infected non-challenged with *S. graminicola* control plant; lane 2, non-infected *S. graminicola* challenged plant; lane 3, infected control plant. Blot a: lanes 4 and 5, two individual infected plants from transformation event 7.10.1; lanes 6 and 8, non-infected 7.10B; lanes 7 and 9, infected 7.10B. Blot b: lanes 4, 5 and 8, non-infected challenged transgenic plants of event 7.10.2; lanes 6 and 7, infected challenged transgenic plants of event 7.10.2. The arrows indicate the protein size of approximately 78 kDa.
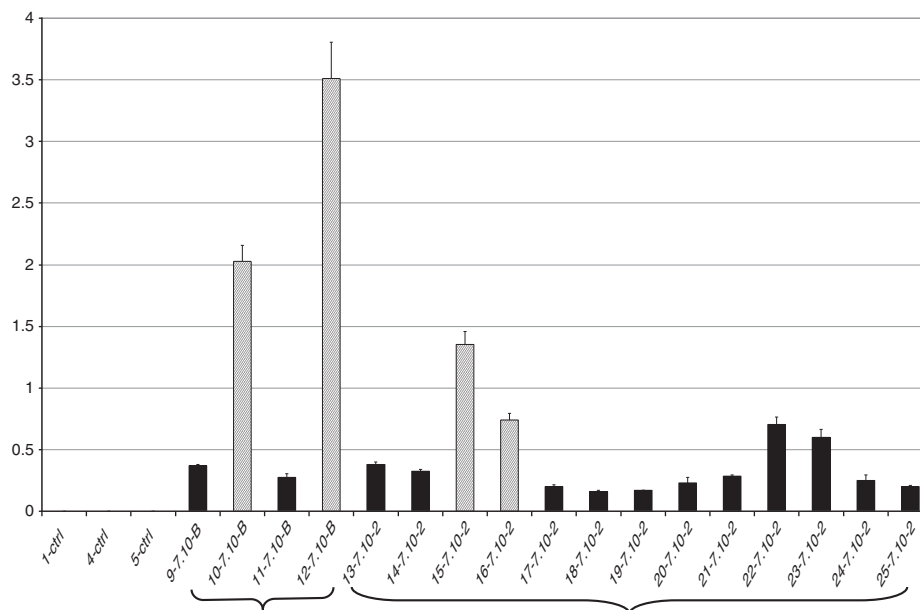
Fig. 6. Transcript levels of the transgene *gluc*78 from *T. atroviride* determined by RT-qPCR in transgenic pearl millet and non-transgenic 842B control plants following infections. The Y-axis indicates the β-glucanase expression relative to 18S RNA levels and the X-axis shows the 842B control plants followed by individual infected plants. Individual progeny plants (bars) of transgenic events (7.10-2 and 7.10B) are grouped together by brackets and compared to control 842B plants. The plant numbering for each bar is given as the plant number for each individual experiment followed by the transgenic event number (7.10-2 or 7.10B). The error bars represent three technical replicates. β-Glucanase expression in control 842B (plant 1, non-challenged; plant 4, challenged but non-infected and plant 5, challenged and infected with *S. graminicola*) compared to transgenic events 7.10B and 7.10-2, four and thirteen plants respectively, inoculated with *S. graminicola*. Plants 10, 12, 15 and 16 indicated by the downward diagonal pattern on the bars (▨) showed symptoms of *S. graminicola* infection, whereas plants indicated by the black boxes showed no symptoms of *S. graminicola* infection.

therefore PTGS is unlikely to be the cause of inefficiency of the introduced gluc78.

Callose, a sugar polymer that is composed of (1,3)-β-D-glucan, is deposited between the plasma membrane and the cell wall in close proximity to the invading pathogen as part of the HR to infection by fungi and viruses (Donofrio and Delaney, 2001). Accumulation of callose in response to insect and pathogenic attacks has been well documented (Flors et al., 2007; Kruger et al., 2002; Saheed et al., 2009), and indicates that callose deposition constitutes a defence mechanism against insect and pathogen invasion. Iglesias and Meins (2000) proved that the movement of plant viruses is delayed in a β-1,3-glucanase-deficient tobacco mutant, showing an enhanced resistance related to augmented deposition of callose. Inhibition of callose synthesis by infiltrating 2-DDG, a callose synthesis inhibitor (Flors et al., 2005; Ton and Mauch-Mani, 2004), reversed the enhanced resistance of the transgenic plants lacking β-1,4-glucanases when compared to the wild-type levels. Similarly, transformation of tobacco and *Nicotiana sylvestris* expressing an antisense construct of a class I β-1,3-glucanase from the latter species (homologous to tobacco PR-2d) became less diseased than non-transformed control plants. This result is interpreted as being due to enhanced callose accumulation at the plasmodesmata, leading to more effective blockage of virus transport from cell to cell (Bucher et al., 2001; Fridborg et al., 2003). In the present study, it is therefore possible that expression of the gluc78 transgene negatively modulates callose deposition through

gluc78 hydrolysation of β-1,3 glycosidic bonds in linear or branched glucans of the plant.

Production of lytic enzymes by *Trichoderma* spp. and their effect on the growth of phytopathogenic fungi is well established (Fogliano et al., 2002; Lorito et al., 1998; Schirmböck et al., 1994) but not the effect on plant glucan and callose formation. The effect of using *Trichoderma* spp. on the endogenous glucan and callose levels in plants still needs to be elucidated.

This is the first report of an antifungal β-1,3-glucanase from *T. atroviride* that is stably expressed in a transgenic cereal crop. Preliminary pathogenicity trials and molecular analysis indicated that the expression of this protein during wounding, *P. substriata* and *S. graminicola* infection, increased the glucanase transcript levels, but promotes disease susceptibility rather than improve disease tolerance. Preliminary results expressing β-1,3-glucanase from *T. atroviride* (*gluc*78) in transgenic pearl millet, indicate a failure to confer enhanced resistance to *S. graminicola*. Despite significant progress in elucidating defence mechanisms in cereal crops (Ayliffe and Lagudah, 2004; Salzman et al., 2005; Zu-Salzman et al., 2004), and particularly in pearl millet (Crampton et al., 2009), the mechanism that governs resistance to downy mildew remains an enigma.

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