Universität Hamburg, Department Biologie

GENETIC TRANSFORMATION OF KENYAN SORGHUM (*SORGHUM BICOLOR* (L.) MOENCH) WITH ANTI-FUNGAL GENES AND RESPONSE TO *COLLETOTRICHUM SUBLINEOLUM* INFECTION

Dissertation

Submitted to the Department of Biology

Faculty of Mathematics, Informatics and Natural Sciences

University of Hamburg, Germany

for the degree of

Doctor rerum naturalium

(Dr. rer. nat.)

Linus Moses Kosambo Ayoo

from Kisumu, Kenya

Hamburg, 2008

AUTHORITY

Genehmigt vom Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Professor Dr. E. KRANZ
Weiterer Gutachter der Dissertation:
Herr Professor Dr. H.-P. MÜHLBACH
Tag der Disputation: 26. September 2008

Hamburg, den 10. September 2008

Professor Dr. Jörg Ganzhorn
Leiter des Departments Biologie

ENGLISH CERTIFICATE

University of Hamburg
Department of Biology

ZMK
ZENTRUM FÜR MEERES- UND KLIMAFORSCHUNG

July 8th, 2008

This is to certify that the quality of the English employed in the PhD thesis submitted by the candidate Linus Kosambo Ayoo entitled "Genetic Transformation of Kenyan Sorghum (*Sorghum bicolor* L. Moench) Cultivars with Anti-Fungal Genes and Response to *Colletotrichum sublineolum* Infection" is of a standard sufficient to fulfil the language requirements of the University of Hamburg

Sincerely

Prof. Dr. Michael St. John
Institute for Hydrobiology and Fisheries Science
University of Hamburg
Grosse Elbestrasse 133
22767 Hamburg
Germany
Tel. +49404283 86600
FAX +49404283 86618
email michael.st john @uni-hamburg.de

DEDICATION

To Atieno, Aluoch, Ajwang
and Frömming-Kosambo

SUMMARY

Sorghum (*Sorghum bicolor* (L.) Moench) is an important food crop in Kenya as well as scores of other African and Asian countries. It ranks fifth worldwide in production among cereals and it is unique in its adaptation to adverse environmental conditions. Anthracnose, caused by *Colletotrichum sublineolum,* is one of the destructive fungal diseases of sorghum that cause extensive annual yield losses. Classical breeding and genetic engineering for traits conferring tolerance and resistance against fungal pathogens is one of the strategies of boosting production. Genetic engineering could be used to exploit the natural anti-fungal proteins produced by saprophytic fungi, such as *Trichoderma harzianum*. Lytic antifungal proteins, like the chitinases and chitosanases*,* degrade chitin and chitosan that are components fungal cell walls. This renders the cell walls osmotically sensitive and ultimately destroys target fungi. Constitutive expression of the *chitinase* (*HarChit*) and *chitosanase* (*HarCho*) genes from *T. harzianum* in transgenic plants could confer resistance to fungal diseases. Particle Bombardment and *Agrobacterium tumefaciens* were used to genetically transform sorghum lines sampled from Kenya with *HarChit* and *HarCho* genes from *T. harzianum*. Three stable transgenic lines, KOSA-1, KOSA-2 and KOSA-3, integrated the two anti-fungal genes were generated from the wild type line KAT 412 through particle bombardment of immature zygotic embryos. Quantitative RT-PCR analysis of the transgenic plants revealed that both genes were expressed in the transformants. *In planta* and *ex planta C. sublineolum* infection assays were carried out with 2 weeks old sorghum seedlings to study the level of disease tolerance by the transgenic and the parent wild type (Wt) lines. The transgenic line, KOSA-1, was found to be more tolerant to anthracnose than the parent Wt. This is the first report of successful co-transformation and genetic enhancement of sorghum after integration of *HarChit* and *HarCho*, two economically important anti-fungal genes. Response to anthracnose was also studied in six Wt sorghum sampled from Kenya: KAT L5, SDSH 513, KAT 412, KAT 487, GBK 0460812, GBK 0460844 and Serena. Qualitative and quantitative rating of the susceptibility and tolerance of different sorghum genotypes showed that the Kenyan cultivar KAT L5 was the most tolerant among the lines studied. Quantitative RT-PCR was used to study the expression of the 2 transgenes, *HarChit* and *HarCho* and 4 endogenous pathogenesis-related (PR) genes: sorghum leucine-rich repeat (*SbLRR*), sorghum chitinase gene (*SbChit*), chalcone-like synthase gene 2 (*SbCHS2*) and gene 8 (*SbCHS8*) after infection with *C. sublineolum*. The fold change (FC) in the expression of *SbLRR*, *SbChit* and SbCHS8 gene were found to be significantly low in the tolerant KAT L5 but high in the susceptible SDSH 513 after infection with *C. sublineolum*. There was a significant difference in expression pattern of the 4 PR-genes in the disease susceptible and resistant cultivars.

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ABBREVIATIONS

aa	amino acids
Avr-Gen	avirulence gene
BAP	6-benzylaminopurine
cDNA	complementary DNA
CaMV	cauliflower mosaic virus
CSPD	3-(4-methoxyspiro{1,2-dioxetan-3,2'-(5'chloro) tricyclo [3.3.1.13,7]decan} -4-yl) phenylphosphate, disodium salt
dATP	2'-deoxyadenosine 5´-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2´-deoxyribonucleoside 5´-triphosphates
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
2,4-D	2,4-dichlorophenoxyacetic acid
DIG	digoxigenin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E. coli	*Escherichia coli*
EtBr	ethidium bromide
FAO	Food and Agriculture Organization of the United Nations
GUS	β-Glucuronidase
HPI	hours post infection
HR	hypersensitive response
ISR	induced systemic resistance
JA	jasmonic acid
LRR	leucine rich repeat

MAR	matrix attachment regions
mRNA	messenger RNA
NBS	nucleotide binding site
nos	nopaline synthase
pat-gene	phosphinotricin-acetyltransferase gene
PCR	polymerase chain reaction
PR-Proteins	pathogenesis related proteins
PR-Genes	pathogenesis related genes
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNAse	ribonuclease
R-Gen	resistance gene
RIP	ribosome inactivating proteins
ROS	reactive oxygen species
RT	reverse transcription
SA	salicylic acid
SAR	systemic acquired resistance
SSC	sodiumchloride-sodiumcitrate
SD	standard deviation
SE	standard error
T_0	direct transgenes regenerant
T_1	first generation from to self crossing
T_2	second generation from T_1 self crossing
T_3	third generation from T_2 self crossing
TBE	Tris/Borate/EDTA electrophoresis buffer
*ubi*1-Promotor	ubiquitin1-promotor
*uid*A, *gus*-gene	β-glucuronidase gene
Wt	wild type
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glukuronsäure

bp	base pair
℃	degree Celsius
cm	centimeter
g	gram
h	hour
kb	kilo basepair
kDa	kilo dalton
m	meter
M	molar
Min	minute
mJ	millijoule
ml	millilitre
mm	millimeter
mM	millimolar
mmol	millimole
ng	nanogram
nm	nanometer
nM	nanomolar
Pa	Pascal
p.A.	per analyse
pg	picogram
psi	pound per square inch
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
rpm	rounds per minute
sec	second

temp.	temperature
Tm	melting temperature
U	unit (restriction enzyme)
UV	ultraviolet light

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1 INTRODUCTION

1.1 SORGHUM AND FOOD SECURITY

According to a UN estimate the world population will likely rise to 9.2 billion by 2050 (UN, 2007). The challenge of feeding the world through the next decades brings into focus the importance of sustainable food supply. This challenge is exemplified by the chronic incidences of food shortages around the world (FAO, 2005). Food insecurity is exacerbated by rapid rise in food prices together with challenges such as climate change, greater demand for food products in emerging economies, agricultural production used for biofuels, rapid population growth, urbanization and animal and plant diseases (FAO, 2008). Poor agricultural practises such as over-tillage, dependence on unreliable precipitation, use of poor seeds, over/under soil fertilization, among others has further compounded this problem (FAO, 2008). Sub-Saharan Africa could have a shortfall of nearly 90 Mt of cereals by the year 2025 if the current agricultural practices are maintained (Thomson, 2008). The unprecedented hike in food prices, which rose by 52% between 2007 and 2008, potents severe economic, social and political consequences (FAO, 2008).

A multi-faceted approach to boost and sustain food production into the next decades will have to integrate both classical and innovative technologies in crop husbandry. Biotic and abiotic factors that limit food production will have to be tackled. It is also inevitable that food production will have to be expanded into hitherto underutilised and marginal arid and semi-arid lands (ASAL). Crops and cultivars that exhibit biotic and abiotic stress tolerance prevalent in ASALs would form the frontline of concerted greening of such austere marginal areas. Sorghum (*Sorghum bicolor* (L.) Moench) is one of the crops that are traditionally produced in marginal areas, where low soil moisture and high ambient temperatures are the main limiting abiotic factors (Wenzel and Van Rooyen 2001; Machado et al., 2002; Gebeyehu et al., 2004; ICRISAT, 2008). The productivity of sorghum in such areas outstrips those of the main staples such as rice, wheat, barley and maize. Sorghum is a primary staple in the semiarid tropics of Africa and Asia for over 300 million people (ICRISAT, 2008).

Sorghum is a tropical plant belonging to the poaceae family and it is believed to have originated in northeast Africa, where it was domesticated about 3000-5000 years ago (Pederson *et al*., 2003). The main races of cultivated sorghum are *bicolor*, *vulgare*, *caudatum, kafir, guinea* and *durra* (Deu *et al*., 1994). Sorghum ranks fifth among cereals in world production, which was 57 million tonnes in 2006, with the developing world accounting for 84% of this total (FAO, 2008). More than 35% of the sorghum produced is directly used for human consumption (Awika and Rooney, 2004). The rest is used primarily for animal feed, production of alcohol and industrial products (Awika and Rooney, 2004). This crop is even of more critical importance in Africa and Asia where farmers have limited farm inputs, a plight worsened by insufficient precipitation. In Kenya sorghum provides better food security in areas where rainfall is limited (KARI, 1996). For example, in Machakos District, which was hit by a drought in 1996, those who planted sorghum fared better than those who planted maize (*Zea mays*) (KARI, 1996). Any intervention that can drastically increase the productivity of this crop would contribute immensely to food security.

1.2 ANTHRACNOSE IN SORGHUM

Plant diseases and pests limit sorghum productivity. Sorghum plants are attacked by fungal, bacterial, and viral pathogens causing root, stalk, foliar, panicle, and caryopsis diseases (Sutton, 1980; Bueso et al., 2000; Waniska, 2001; Waniska *et al.,* 2001; Prom et al., 2005). Fungi cause many severe diseases, such as root and stalk rot caused by *Fusarium moniliforme, Fusarium thapsinum*, or *Colletotrichum* spp, seedling diseases induced by *Pythium* sp., foliar disease such as leaf blight, caused by *Exserohilum turcicum*, zonate leaf spot by *Glococercospora sorghi*, sooty stripe by *Ramulispora sorghi*, rust by *Puccinia purpurea*, Ergot by *Claviceps sorghi* and head smut by *Sporisorium reilianum,* respectively (Waniska *et al.,* 2001; Prom et al., 2005).

One of the diseases of great economic significance is anthracnose caused by *Colletotrichum sublineolum* (Sutton, 1980; Hammer *et al*., 1988; Nicholson and Epstein, 1991).

Anthracnose is associated with black, sunken lenticular symptoms on the infected organs of host species (Nicholson and Epstein, 1991). Sorghum anthracnose was first reported in 1902 in Togo, West Africa (Sutton, 1980), and has since been observed in most regions worldwide, where sorghum is grown. This disease is coupled with great loses in tropical belts where high humidity and temperature is conducive for *C. sublineolum* growth, propagules dispersal and sporulation (Tarr, 1962; Pastor-Corrales and Frederiksen, 1980). Severe sorghum infection retards plant growth or causes death prior to maturity. Ali and Warren (1987) reported sorghum grain yield losses due to foliar infection of 30% for a susceptible cultivar when inoculated with a virulent isolate of the pathogen. Reduction in yields of up to 50% has also been reported in warm and humid areas where grain yields from susceptible cultivars are greatly reduced during severe epidemics (Tarr, 1962; Harris and Cunfer, 1976; Pastor-Corrales and Frederiksen, 1980). Grain yield losses from 41-67% for a susceptible cultivar from natural infection of foliar tissue were reported in Mali (Thomas *et al*., 1996). Apart from anthracnose, *Colletotrichum* species also cause damping-off, blight and spot disease on infected plants (Nicholson and Epstein, 1991).

C. sublineolum is a hemibiotroph whose pathogenesis strategy commences from a transient post-penetrative asymptomatic biotrophy that is rapidly succeeded by a phase of destructive necrotrophy culminating in the appearance of symptoms of disease and production of conidiomata of the pathogen (Pastor-Corrales and Frederiksen, 1980). The pathogen is capable of infecting all above ground tissues of the sorghum plant. Anthracnose in sorghum is commonly divided into three categories based on where symptoms develop on the plant: leaf, stalk, panicle and grain anthracnose (Frederiksen, 1984). Primary infection occurs when conidia are windblown or splashed from debris. Conidia germinate and infection occurs directly through the epidermis or stomata (Hamer *et al.*, 1988; Nicholson and Epstein, 1991). Characteristic disease symptoms on susceptible cultivars include circular to elliptical spots or elongated lesions and as the disease progresses, lesions coalesce covering most of the infected tissues (Hamer *et al.*, 1988; Nicholson and Epstein, 1991) (Figure 1.1). As the

fungus sporulates, fruiting bodies (acervuli) appear as black spots in the centre of the lesions (Hamer *et al.*, 1988; Nicholson and Epstein, 1991). Under favourable environmental conditions, coalescence of lesions will occur on susceptible cultivars resulting in, for example, leaf senescence and premature defoliation or damping off of seedlings (Hamer *et al.*, 1988; Nicholson and Epstein, 1991).

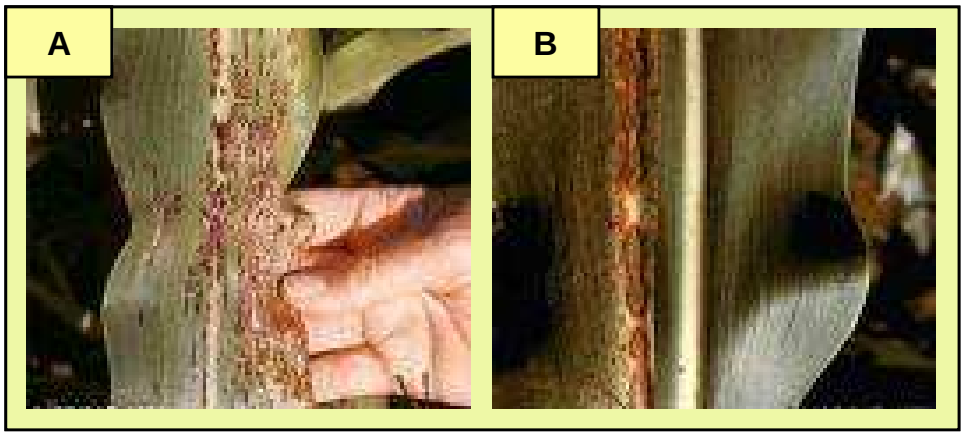


Figure 1.1: Sorghum leaf anthracnose. Typical symptoms are small, circular, elliptical or elongated spots (A). Under conditions of high humidity or high rainfall, the spots increase in number and join together to cover and later kill large portions of the leaf (B).

The first essential feature for successful pathogenesis is the attachment of dispersed fungal conidia to the host plant surface (Hamer *et al.*, 1988; Nicholson and Epstein, 1991). Studies have shown that *Colletotrichum* conidia will adhere rapidly to a wide range of plant and artificial surfaces, including cellophane, polystyrene, polycarbonate and glass (Young and Kauss, 1984; Mercure *et al.*, 1995; Sela-Buurlage *et al.,* 1991), suggesting that adhesion is non-specific. Melanisation of the appressorial cell wall has been shown to be necessary for mechanical penetration of the host cuticle and underlying cell wall (Bonnen and Hammerschmidt, 1989; Rasmussen and Hanau, 1989). Firm anchorage is essential for appresoria to exert the mechanical force required for penetration (Bailey *et al.*, 1992). Three mechanisms have been proposed for cuticle penetration: a) mechanical force alone; b) the secretion of cutin degrading enzymes alone; and c) a combination of both processes (Bailey *et al.*, 1992).

To resist *C. sublineolum* and other fungal pathogen infection, sorghum possesses physical and physiological mechanisms that limit conidia attachment, penetration and spread into surrounding tissues.

1.3 DISEASE RESISTANCE MECHANISMS IN PLANTS

Plants carry a surveillance system to recognize attacking microorganisms and to induce effective defence mechanisms (Dangl and Jones, 2001; Jones and Dangl, 2006). Resistance is often controlled by a gene-for-gene interaction between plant resistance (*R*) and pathogen avirulence (*avr*) genes (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001; Jones and Dangl, 2006). Recognition of pathogen *Avr*-genes products by the plants *R*-genes coded surveillance proteins leads to activation of the hypersensitive response (HR), a type of programmed cell death (PCD) that occurs at or near the site of pathogen entry (Morel and Dangl, 1999; Heath, 2000). The HR is thought to confine the pathogen by stopping its spread from the site of attempted infection, and is likely to involve active plant metabolism (Levine *et al*., 1996). *R*-gene mediated resistance is also associated with the activation of a salicylic acid (SA)-dependent signalling pathway that leads to the expression of certain pathogenesis related (PR) proteins, which are thought to contribute to establishing resistance (Saskia et al., 2000). Genetic analysis of the HR has led to the cloning of *R*-genes, many of which encode receptor-like proteins (Bent, 1996).

1.3.1 Sorghum Leucine-Rich Repeat Gene

Many *R*-genes-encoded R-proteins are composed of three major structural features: a nucleotide binding site (NBS), a leucine-rich repeat (LRR) domain and either a coiled-coil (CC) or a Toll-interleukin receptor (TIR) domain at their N-termini (Dangl and Jones, 2001). The CC, TIR, and NBS domains are known to play roles in protein-protein interactions and signal transduction (Srinivasula *et al*., 1998; Kopp and Medzhitov, 1999; Burkhard *et al*., 2001). LRR domains in R-proteins mediate direct or indirect interaction with pathogen

molecules (Jia *et al*., 2000; Dangl and Jones, 2001). Individual LRR form repeats of β-strand-loop and α-helix-loop units and compose a binding surface predicted to be involved in protein recognition (Kobe and Kajava, 2001). The β-sheets may interact with pathogen ligands and hence determine specificity for pathogen elicitors (Thomas *et al*., 1997; Ellis and Jones 1999; Ellis *et al*., 2000). Regulation of genes encoding putative LRR proteins has been studied in sorghum (Salzman *et al*., 2005). A sorghum LRR strongly induced by SA, among other signalling compounds has been described (Salzman *et al*., 2005). The *NBS-LRR* gene family is involved in response to fungal infection (Salzman *et al*., 2005). Expression of sorghum *LRR* (*SbLRR*) is strongly induced by SA and jasmonic acid (JA), the key signals in the induction of the systemic acquired resistance (SAR) (Salzman *et al*., 2005). It is therefore evident that evaluation of the expression pattern of *SbLRR* in transgenic, resistant and susceptible lines could be insightful in understanding the mechanisms of response of sorghum to pathogen invasion. Understanding the timing of *SbLRR* activity in response to pathogen attack could be important in elucidating tolerance.

1.3.2 Chitinases and other PR Proteins in Disease Response

Sorghum has other innate response mechanisms against pathogens that involve PR-proteins. These involve the inducible plant defences that restrict the spread of the pathogen in incompatible interactions and allow the establishment of SAR (Cao *et al.,* 1998; Saskia *et al.,* 2000). Many members of this group of proteins have *in vitro* antifungal activity and selectively target cellular components of the pathogen. Included in this group are chitinases and β-1,3-glucanases, which attack the cell walls of fungi, and thaumatin-like proteins (TLP) that affect the permeability of fungal membranes (Linthorst, 1991; Cao et al., 1998; Waniska *et al.,* 2001). Recent studies have shown that PR-proteins such as sormatin, chitinases, glucanases, and ribosome-inhibiting protein may play a role in disease resistance in sorghum (Rodriguez-Herrera, 1999; Rodriguez-Herrera *et al*., 1999; Bueso *et al*., 2000). Similar activitiy have been noted in other cereals. For example, a coordinated induction of the

expression of 3 chitinase isoforms was observed in maize seeds in response to infection by the fungus *Fusarium moniliforme* (Cordero *et al*., 1994). Several chitinases (three in the 21-24 kDa range; 28 kDa 30 kDa) have been reported in sorghum (Darnetty, 1993). The level of these chitinases were noted to rise during caryopsis development, however their antifungal activities was not confirmed (Darnetty, 1993). However, chitinase is known to be involved in resistance to plant diseases (Rodriguez-Herrera, 1999; Rodriguez-Herrera *et al*., 1999; Bueso *et al*., 2000)

1.3.3 Chalcone Synthase Gene Family

Sorghum synthesizes a unique class of flavonoid phytoalexins, the 3-deoxyanthocyanidins, as an essential component of defence mechanism against pathogen infection (Lo *et al.*, 2002). In sorghum, accumulation of 3-deoxyanthocyanidins is preceded by accumulation of transcripts encoding chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis (Lo *et al*., 2002). CHS, or naringenin CHS, is a plant specific polyketide synthase that catalyzes the condensation of three units of malonyl-CoA with *p*-coumaroyl-CoA to form naringenin chalcone (Lo *et al*., 2002). This reaction is generally regarded as the committed step leading to the synthesis of different flavonoid compounds (Lo *et al*., 2002). In higher plants, CHS is encoded by a family of genes. A family of 8 *CHS* genes, *SbCHS1* to *SbCHS8*, has been described in sorghum (Lo *et al*., 2002). *SbCHS1* to *SbCHS7* are highly conserved and closely related to the maize *C2* and *Whip* genes encoding CHS enzymes (Lo *et al*., 2002). It has been shown that *SbCHS2*, a member of the *SbCHS1-7* family, encodes a typical CHS that synthesizes naringenin chalcone, which is necessary for the formation of different flavonoid metabolites (Lo *et al*., 2002). On the other hand, *SbCHS8*, re-termed *SbSTS1*, encoded an enzyme with stilbene synthase activity, suggesting that sorghum accumulate stilbene-derived defence metabolites in addition to the well-characterized 3-deoxyanthocyanin phytoalexins. *SbCHS8* is only 81-82% identical to *SbCHS1* to *SbCHS7* at the amino acid level and appears to be more distantly related as revealed by phylogenic analysis (Lo *et al*., 2002). It was later demonstrated that *SbCHS8* , retermed *SbSTS1,* is not

involved in flavonoid biosynthesis *in planta*, instead synthesized pinosylvin and resveratrol as major products *in vitro* using cinnamoyl and p-coumaroyl-CoA as starter molecules, respectively (Yu *et al.,* 2005). *SbCHS8* is considered not to be constitutively expressed but inducible following fungal inoculation. Differential expression of *SbSTS1* in resistant and susceptible sorghum lines suggested that the gene plays a key role in the expression of resistance against *C. sublineolum* (Yu *et al*., 2005). Therefore, *SbCHS1-7* and *SbCHS8* represent a chalcone synthase gene family that are expressed differently during infection (Yu *et al*., 2005). *SbCHS1-7*, such as *SbCHS2* are generally expressed upon non-specific elicitation while *SbCHS8* seems to be active in response to fungal attack (Yu *et al*., 2005). It is therefore expected that a variation exists in the expression of these genes in sorghum cultivars that are susceptible and tolerant to anthracnose.

1.3.4 Anthracnose-Resistance in Sorghum

Resistance of plants to pathogen is based upon physical factors such as mechanical strength of the cuticle, epidermal wall and the resistance of their structural polymers to enzymatic degradation, surface features (e.g. topography, leaf hairs, and epicuticular waxes) that impede the formation of infection structures; structural barriers such as papillae that delay penetration; and secondary metabolites that are toxic or otherwise inhibitory to fungal growth (Heath, 1981). These physical barriers represent the first line of defence to fungal pathogens and some may contribute to tolerance to *Colletotrichum* spp (Mercer *et al.*, 1975). Juvenile sorghum plants rarely exhibit visible symptoms of infection when challenged with fungal pathogens such as *C. sublineolum* (Nicholson, 1988). This apparent expression of resistance has been attributed to the presence of substantial levels of the preformed cyanogenic glycoside, dhurrin, in juvenile sorghum leaves (Ferreira and Warren, 1982). However, studies have shown that fungal pathogens, including *C. sublineolum*, can detoxify hydrogen cyanide, which is the toxic breakdown product of dhurrin (Fry and Munch, 1975; Fry and Evans, 1977; Myers and Fry, 1978).

It has been shown that young sorghum leaves accumulate a complex of phenols in response to invasion by both pathogenic and non-pathogenic fungi, and the five major components of this complex are the 3-deoxyanthocyanidin flavonoids: apigeninidin, luteolinidin, arabinosyl-5-O-apigeninidin, 7-methylapigeninidin, and 5-methoxyluteolinidin (Nicholson *et al.*, 1988; Hipskind *et al.*, 1990; Lo *et al.*, 1996). All these compounds have exhibited fungitoxic activity towards *C. sublineolum*, and are considered to be phytoalexins (Nicholson *et al.*, 1988). In leaf tissue, these phenolics first appear in the cell being invaded, accumulating in inclusions in the cytoplasm (Snyder & Nicholson, 1990; Snyder *et al.*, 1991). These cytoplasmic inclusions migrate to the site of penetration, become pigmented, lose their spherical shape and ultimately release their contents into the cytoplasm, killing the cell and restricting further pathogen development (Snyder & Nicholson, 1990; Snyder *et al.*, 1991).

Unfortunately, the action of the innate defence machinery does not give sorghum total protection and fungal diseases continue to reduce its global productivity. Genetic engineering offers an opportunity for enhancing disease tolerance by introducing traits that limit pathogens ingression.

1.4 GENETIC ENGINEERING FOR DISEASES RESISTANCE

There are a number of options that could be exploited to control plant diseases. These include: use of fungicides, cultural methods (good crop husbandry, e.g. crop rotation), cultivation of resistant/tolerant cultivars and bio-control. The toxicity, environmental harm and expense that come with fungicides, decry their use. Breeding of resistant/tolerant cultivars is a sustainable approach to tackling disease menace. Genetic engineering offers an alternative avenue of increasing the array of traits that could infer anthracnose tolerance. Genes encoding fungitoxic and fungistatic protein could be cloned from a variety of sources and introduced into susceptible crops.

1.4.1 Targeting Fungal Cell Wall Polymers

One of the principles of action of fungitoxic proteins that could be harnessed in genetic engineering is their lytic action on chitin and chitosan. Chitin and chitosan are structural polymers found in the cell wall of various organisms, especially fungi and arthropods (Hendrix and Stewart, 2002; Steyaert et al., 2004). Chitin is generally distributed in fungi, occurring in basidiomycetes, ascomycetes and phycomycetes, where it is a major component of the cell walls and structural membranes of mycelia, stalks and spores (Hendrix and Stewart, 2002; Steyaert et al., 2004) (Figure 1.2). Chitinases endolytically hydrolyse the β-1,4-linkages of chitin (Cabib, 1987; Hendrix and Stewart, 2002; Steyaert et al., 2004). These enzymes are widely distributed in nature including plants (Chen *et al*., 1982; Jones *et al*., 1986). Chitinases play a defensive role against fungal pathogens in plants (Bartnicki-Garcia, 1968; Collinge *et al*., 1993). In fungi, chitinases seem to play a morphogenetic role during apical growth, cell division and differentiation, as well as a nutritional role related to those species saprophytic and mycoparasitic in fungi (Papavizas, 1985; Cabib, 1987; Kuranda and Robin, 1991).

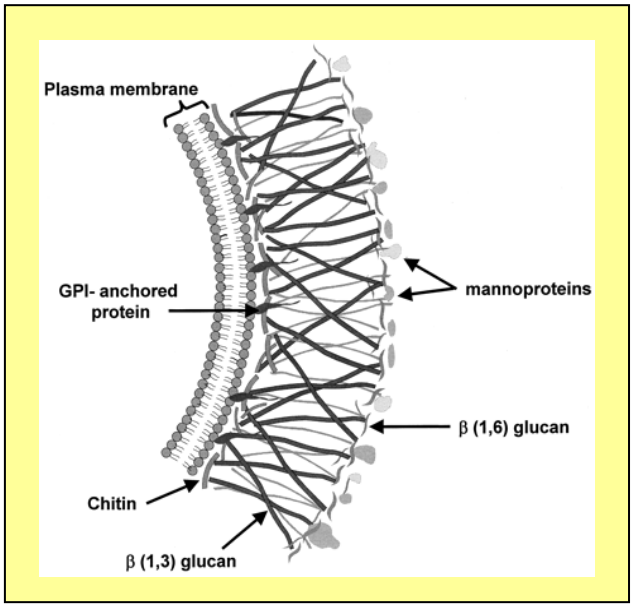


Figure 1.2: Structural component of fungal cell wall. Potential targets of action by cell wall-lysis enzymes are indicated (GPI – glycophosphatidylinositol).

Due to the interest generated from their lytic action against fungal cell wall, a number of genes encoding chitinases have been isolated, sequenced and cloned. Genes encoding chitinases have been cloned from bacteria (Jones *et al*., 1986; Watanabe *et al.,* 1990; 1992), yeast (Kuranda and Robbins, 1991), plants (Collinge *et al*., 1993) and filamentous fungi, such as *Rhizopus oligosporus* (Yanai *et al*., 1992) and *Aphanocladium album* (Blaisean and Lafay, 1992). A cloned *chitinase* gene from *Serratia marcescence,* when introduced into the *Trichoderma harzianum* genome, gave rise to transformants having remarkable advantage in controlling *Sclerotium rolfsii* compared to wild type (Chet *et al*., 1993). Cosmids which carry *chitinase* genes from *S. marcescence* were mobilised into *Pseudomonas* strains which inhibited growth of *Rhizoctonia solani* and *Magnaporte grisea* and reduced disease induced by *Fusarium oxysporium* (Sundheim, 1992).

1.4.2 *Trichoderma Harzianum* Chitinases and Disease Resistance

Nature offers a great opportunity for fungal diseases control through the existence of saprophytic fungi, group of fungi that parasitizes others as a source of nutrition. *Trichoderma harzianum,* a soil-borne fungus known to be a control agent of fungal plant pathogens (Papavizas, 1985), is one of these fungi that produce degrading enzymes which destroy key cell wall structural polymers of fungal pathogens (Hendrix and Stewart, 2002). The degradation and further assimilation of phytopathogenic fungi, namely mycoparasitism, has been proposed as the mechanism accounting for the antagonistic action of *Trichoderma spp* (Sundheim, 1992; Garcia *et al.,* 1994; Steyaert *et al*, 2004). *Trichoderma* spp are biocontrol agents of many economically important pathogens, such as species of *Botrytis*, *Rhizoctonia* and *Sclerotina* (Sundheim, 1992; Lorito, 1998; Steyaert *et al,* 2004). A majority of the biocontrol agents currently used are isolates of *T. harzianum* or *T. atroviride*, from which 16 genes implicated in mycoparasitism have been sequenced (Kubicek and Penttila, 1998; Lorito, 1998; Cohen-Kupiec *et al*., 1999; Donzelli *et al*., 2001). Biocontrol activity of *Trichoderma* spp is attributed to 5-7 distinct enzymes (Haran *et al*., 1995). In the best characterized *Trichoderma spp.* isolate (isolate TM), the system is apparently composed of 2

β-(1,4)-N-acetylglucosaminedases (102 and 73 kDa) and 4 endochitinases (52, 42, 33 and 31 kDa) (Haran *et al*., 1995). The most interesting individual enzyme of the complex is the 42 kDa endochitinase, which can hydrolyze *Botrytis cinerea* cell wall *in vitro* and inhibit spores germination and germ tube elongation of various fungi (Lorito *et al*., 1998; Lorito *et al*., 1994, Schirmböck *et al*., 1994). Chitinases represent therefore one of the key groups of enzymes involved in mycoparasitism (Chérif and Benhaman, 1990; Ridout *et al*., 1986).

The purified enzymes from *T. harzianum* are strong inhibitors of many important plant pathogens and are also able to lyse not only the ´soft´ structures of the hyphal tip but also the ´hard´ chitin wall of mature hyphae, conidia, chlamydospores and sclerotia (Lorito *et al*., 1998). They are substantially more chitinolytic and glucanolytic than the enzymes from other known sources (i.e. up to 100 times more active than the corresponding plant enzymes and effective on a much wider range of pathogens) (Lorito *et al*., 1994; Lorito *et al*., 1996). They are also non toxic to plants even at high concentration (Carsolio *et al*., 1998). Furthermore, the anti-fungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes are used together or in combination with plant PR-proteins, commercial fungicides, cell membrane-affecting toxins or biocontrol bacteria (Lorito *et al*., 1998; Steyaert *et al*., 2004).

Chitinase genes have been cloned and their products used in bioassays for their effectiveness against a number of fungi (Lorito *et al*., 1993; Lorito *et al*., 1994; Schirmböck *et al*., 1994). *In planta* studies have demonstrated that chitinases are effective against many economically important plant pathogens. A chitinase from *T. harzianum* was cloned in *E. coli* and a significant suppression of disease caused by *S. rolfsii* was detected when irrigating with engineered *E. coli* (Chet *et al*., 1993).

The interest in the *chitinase* genes is not only based on their potential application as antifungal agents but also because *chitinase* genes of mycoparasitic fungi are excellent

candidates for reinforcing plant defences through genetic engineering. Subsequently a cDNA (named *Chit42*) of *T. harzianum*, coding for an endochitinases of 42 kDa was cloned (Garcia *et al*., 1994). The cDNA codes for a protein of 423 amino acids. Disease resistance in transgenic tobacco and potato plants was improved by the insertion of the 42 kDa endochitinase from *T. harzianum* (Lorito *et al*., 1998). Selected transgenic lines were highly tolerant or completely resistant to the foliar pathogen *Altenaria alternate*, *A. solani*, *Botrytis cinerea*, and the soil-borne pathogen *Rhizoctonia solani* (Lorito *et al*., 1998). It was also found that introduction of multiple copies of the 33 kDa endochitinase in *T. harzianum* resulted in increased biocontrol ability (Dana *et al*., 2001). Based on these proofs of activity, 42 kDa endochitinase from T. harzianum (*HarChit*) was cloned and used in the transformation of sorghum in this research.

1.4.3 Chitosanase in Defence Response

Chitosanases have the potential of slowing or preventing fungal infection by degrading the structural chitosan found in the cell wall of many fungi (Hendrix and Stewart, 2002). Chitosanase is an enzyme similar to chitinase, capable of hydrolyzing the β-1,4-linkages between N-acetyl-D-glucosamine and D-glucosamine residues in a partially acetylated fungal cell wall polymer (Hendrix and Stewart, 2002).

Glucosamine oligomers, released from fungal cell walls after hydrolysis with chitinase or chitosanase, are elicitors of plant defence response such as stomata closure (Lee *et al*., 1999) and cell wall lignifications (Vander *et al*., 1998). The response elicited by these molecules depends on the length and degree of acetylation of the oligomers released (Vander *et al*., 1998). Long oligomers or intact fungal cell walls will cause little or no reactions whereas oligomers that are relatively short (e.g. products of chitosanase hydrolysis) are active elicitors of plant defence systems (Vander *et al*., 1998).

Many plants have been transformed with *chitinase* genes in an effort to improve plant fungal resistance (Punja, 2001). However, fewer efforts have been made towards transformation of plants with *chitosanase* genes (Hendrix and Stewart). A *chitosanase* gene from *Paenibacillus* sp 61724 was cloned and modified for plant expression and used for tobacco leaf disk transformation (Hendrix *et al*., 2001; Hendrix and Stewart, 2002). It was demonstrated that the *chitosanase* gene expressing transgenic tobacco lines were able to activate general innate defence mechanisms faster than the wild type plants (Hendrix and Stewart, 2002).

1.4.4 Co-expression of *HarChit* and *HarCho* for Enhanced Defence

Plant defence system against microbial pathogens could be bolstered through introduction and constitutive expression of genes coding for anti-microbial compounds such as chitinases and chitosanases. It is also plausible that chitinases and chitosanases could independently and synergistically boost plant defences against fungal pathogens. Co-expression of chitinase and chitosanase genes from *T. harzianum* could therefore result in a synergistic enhancement of anti-fungal activity. In the presented investigation sorghum was genetically transformed with two genes isolated from *T. harzianum:* the *chitinase (HarChit)* and the *chitosanase (HarCho)* genes, as a means of developing disease tolerant transgenic sorghum lines.

1.4.5 Genetic Transformation through Microprojectile Bombardment

Two methods of genetic transformation, microprojectile bombardment and *Agrobacterium* mediated transformation are commonly used to deliver exogenous nucleic acid into plants cells to produce transgenic plants. Particle bombardment involves the use of a mechanical devise to deliver genes into plants. Micron-sized metal particles are coated with DNA and accelerated into target cells at velocities sufficient to penetrate the cell wall but below that which will cause lethal damage. In this manner, desired DNA can be transported into the

cell's interior where it becomes detached from the microprojectile and integrates into the genome.

Normally, the genes are cloned into appropriate plasmids, loaded onto a macrocarrier and accelerated into embryogenic tissue that is thereafter cultured to generate transgenic plants. Several of the major cereal crops including rice (Hies *et al*., 1994), maize (Ishida *et al*., 1996), barley (Tingay *et al*., 1997), wheat (Cheng *et al*., 1997) and creeping beatgrass (Yu *et al*., 2001) have been genetically transformed (Repellin *et al*., 2001). However, fewer successes have been achieved in sorghum transformation. There are fewer reports of successful transformation of this crop than other cereals (Battraw and Hall, 1991; Casas *et al*., 1993, 1997; Goodwin and Chiwamba, 1994; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001). Most of these studies have involved only marker genes and did not include genes of agronomical importance. No commercial transgenic sorghum product has so far reached the consumer market.

1.4.6 Transformation with *Agrobacterium tumefaciens*

About a century after its discovery as the causative agent of crown gall disease (Smith and Townsend, 1907), the Gram-negative soil borne phytopathogen *Agrobacterium tumefaciens* is still central in plant genetic engineering (Tzfira and Citovsky, 2002; Gelvin, 2000). The molecular basis of genetic transformation of plant cells by *Agrobacterium* is the transfer and integration of a region of a large tumour-inducing (Ti) or rhizogenic (Ri) plasmid resident in this bacterium into the plant nuclear genome. The size of the Ti plasmid ranges from 200 to 800 kbp (De Vos *et al*., 1981; Fortin *et al*., 1993; Gerard *et al*., 1992; Unger *et al*., 1985; Wood *et al*., 2001). The transferred DNA (T-DNA) is approximately 10-30 kbp in size (Baker *et al*., 1983; Byrne *et al*., 1983; Lemmers *et al*., 1980; Suzuki *et al*., 2000; Zambryski *et al*., 1980).

The molecular machinery needed for T-DNA production and transport into the host cell comprises proteins that are encoded by a set of bacterial chromosomal (*chv*) and Ti-plasmid virulence (*vir*) genes and a type IV secretion system (T4SS) (Garfinkel *et al*., 1980; Hooykaas *et al*., 1984; Horsch *et al*., 1990; Lundquist *et al*., 1984; Stam *et al*., 1997), a process that resembles DNA transfer between bacteria during conjugation. In addition, various host proteins have been reported to participate in *Agrobacterium* mediated transformation (Tzfira and Citovsky, 2002; Gelvin, 2003), mostly during the later stages of the process (i.e. T-DNA intracellular transport, nuclear import and integration).

In nature, the T-DNA carries a set of oncogenes (Gandin *et al*., 1994) and opine-catabolism genes whose expression in the plant cells leads to neoplastic growth of the transformed tissue and the production of opines, amino acid derivatives used almost exclusively by the bacteria as a carbon and nitrogen source. Recombinant *Agrobacterium* strains in which the native T-DNA has been replaced with genes of interest, are the most efficient vehicles used for the introduction of foreign genes for the production of transgenic plants (Draper *et al*., 1988). Many crops have been successfully transformed with this bacterium. The first transgenic sorghum plant produced by *Agrobacterium* mediated transformation was reported by Zhao *et al*. (2000).

1.4.7 Tissue Culture and Transformation of Sorghum

Identification of appropriate explants and development of a prolific tissue culture system for transformation and generation of transgenic plants from transformed tissues is crucial in genetic engineering. This involves careful choice of totipotent tissues and organs to serve as explant. A number of explants have been used in sorghum tissue culture. These include mesophyll protoplast (Sairam *et al*., 1999), immature zygotic embryos (Gamborg *et al.,* 1977; Thomas *et al*., 1977; Dunstan *et al*., 1978; 1979; Brar *et al*., 1979; Cai *et al*., 1987; Ma and Liang, 1987; Zhang *et al*., 1998; Oldach *et al*., 2001; Takashi *et al*., 2002; Girijashankar *et al*., 2005), immature inflorescence (Brettel *et al*., 1980; Boyes and Vasil, 1984; Cai and

Butler, 1990; Keppler and Pederson, 1997), cell suspensions from shoot tip meristem (Mythili *et al.,* 1999), leaf tips of seedlings (Masteller and Holden, 1970; Brar *et al*., 1979; Davis and Kidd, 1980; Smith *et al*., 1983), leaf fragments (Wernicke and Brettel, 1980), mature embryos (Thomas *et al*., 1977; Cai *et al*., 1987) and anthers (Rose *et al*., 1986). Immature zygotic embryos (IZE) have been the explant of choice for the production of transgenic plant (Casas *et al*., 1993; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002; Tadesse *et al*., 2003; Gao *et al*., 2005). This investigation used IZE as the explant in the tissue culture.

Sorghum is considered one of the recalcitrant crops to tissue culture, plant regeneration, and genetic transformation. Recalcitrance in sorghum tissue culture is reportedly due to the release of phenolics, lack of regeneration in long term *in vitro* cultures, and a high degree of genotype dependence. Sorghum tissues are problematic to culture mainly because of the copious amount of phenolic substances secreted into the culture by its explants (Carvalho *et al*., 2004; Casas *et al*., 1993). Cultured sorghum cells release phenolics into the culture media whose oxidized products inhibit morphogenesis and growth (Casas *et al*., 1993). This problem is even more limiting in the highly pigmented and phenolics-rich sorghum lines. Sorghum explants are also not responsive to most culture media systems routinely used in the transformation of crops such as maize, wheat, barley, and tobacco. The release of phenolics into the culturing medium can be overcome by frequent subculture and by the addition of polyvinyl pyrrolidone phosphate (PVPP) in the medium. White, cream, brown and red sorghum with varying reponse in tissue culture were used.

1.4.8 Transformation for Disease Resistance

Sorghum has been previously transformed with transgenes in an attempt to boost disease resistance and to introduce other traits of economic importance. Girijashankar *et al*. (2005) produced transgenic sorghum plants carrying the synthetic gene "BT *cry*1Ac" under the control of a wound inducible promoter from a maize protease inhibitor gene (*mpi*). Tadesse

and Jacob (2003) introduced the *dhdps-raec1* mutated gene, which encodes an insensitive form of dihydropicolinate synthase, the key regulatory enzyme of the lysine pathway. Overexpression of the gene produced sorghum lines with elevated lysine content. A plant gene, *chiII*, encoding rice chitinase under control of the constitutive CaMV 35S promoter, was transferred to sorghum for resistance to stalk rot (*Fusarium thapsinum*) by Zhu *et al*. (1998) and Krishnaveni *et al*. (2001). Few reports exist of plant transformation with chitosanase gene and fewer studies describing its *in planta* anti-fungal potential (El Quakfaoui *et al*., 1995; Hendrix and Stewart, 2002). Tobacco plants were transformed with the *Paenibacillus spp* 61724 chitosanase and it was found that the chitosanase expressing lines were able to activate defence mechanisms faster than was possible in wild type plants (Hendrix and Stewart, 2002). It was therefore concluded that the enhanced response along with direct attack on fungus through cell wall degradation would translate into increased plant fungal resistance (Hendrix and Stewart, 2002). Based on these, this work was designed to transform sorghum with two genes encoding anti-fungal proteins for disease resistance.

1.5 RESEARCH GOAL AND OBJECTIVES

The goal of this research was to genetically transform sorghum with chitinase *(HarChit)* and a chitosanse *(HarCho)* genes towards development of fungal diseases resistant lines.

To meet this goal, the following objectives were pursued:

1. Optimise sorghum tissue culture protocol and establish a regeneration system for particle bombardment and *Agrobacterium*-mediated transformation.
2. Isolate and clone the chitinase (*HarChit*) and chitosanase (*HarCho*) genes into appropriate vectors for genetic transformation.
3. Carry out genetic transformation of selected sorghum cultivars.
4. Undertake *ex planta* and *in planta* assays to determine the responses of the transformed and wild type sorghum to *Colletotrichum sublineolum* infection.
5. Profile the expression of selected PR-genes in the transgenic and Wt sorghum lines.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Laboratory Consumables

Purity grade “p.A.” chemicals and deionised water (Milli-Q Plus Water system, Millipore, Bedford, U.S.A.) were used for preparation of all solutions and media. As dictated by different requirements, solutions and media were autoclaved (20 min, 120°C, 2 x 105 Pa) or filter sterilised (0.22 µm Millex® GP or SteritopTM, Millipore, Bedford, U.S.A.). General laboratory chemicals were purchased from: AgrEvo (Düsseldorf), Applichem (Darmstadt), Biomol (Hamburg), Brand (Wertheim/Main), Duchefa (Harlem, The Netherlands), Fluka (Buchs), Merck-Schuchard (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma-Aldrich (München). Restriction endonucleases, dNTPs, DNA polymerases, DNA/RNA modifying enzymes and other molecular biology reagents were from Invitrogen (Karlsruhe), Fermentas Life Sciences (St. Leon-Rot) and New England BioLabs (NEB, Frankfurt/Main). Gel-blotting papers were from Schleicher & Schüll (Dassel) and photographic papers for agarose gels were from Mitsubishi (Kyoto, Japan).

2.1.2 Kenyan Sorghum Lines

Clean seeds of indigenous Kenyan sorghum lines were provided by Kenya Agricultural Research Institute (KARI), Katumani and KARI Plant Gene Bank, Muguga. Aralba seeds were obtained from sorghum seed collection at the Department of Applied Molecular Plant Biology, University of Hamburg. Plants were cultivated in the greenhouse under the recommended conditions as reported by Oldach *et al*. (2001) (16h/8h day/night photoperiod of 220 ∓20 µE/m^2/s at changing day/night temperature of 24^{o}C/20^{o}C).

2.1.3 Agrobacterium tumefaciens Strain

A binary vector system was used in this study. A tetracycline resistant *Agrobacterium* strain, LBA4404 (pSB1) (Japan Tobacco Inc) containing a disarmed pSB1 plasmid was transformed with vector constructs containing the *gus*, *chitosanase (HarCho)* and *chitinase (HarChit)* genes and used for sorghum transformation.

2.1.4 Colletotrichum sublineolum

Isolates of *Colletotrichum sublineolum* cultured on half-strength potato dextrose (1/2 PDA) agar were acquired from USDA-ARS, College Station, Texas. The isolates were periodically sub-cultured onto ½ PDA and grown in darkness at 26℃ to maintain actively growing colonies used to induce sporulation for further experiments. Spores of *C. sublineolum* were used in infection experiments. Sporulation was induced by growing ½ PDA derived colonies on 7.25% oatmeal agar (OMA) at 26℃ with backlight illumination for at least 2 weeks.

2.2 METHODS

2.2.1 Experimental Scope, Design and Statistical Analyses

This research was designed to genetically transform sorghum and carry out a comparative study of the morphological and genetic responses to infection with C. *sublineolum* of the transgenic (T) and wild type (Wt) sorghum lines. This study was divided into 4 parts:

1. Genetic transformation through particle bombardment
2. *Agrobacterium* mediated transformation
3. *Ex planta* and *in planta C. sublineolum* infection studies
4. qRT-PCR expression studies of pathogenesis-related genes

Ten sorghum cultivars, including white, brown and red lines, were used for transformation and infection studies. *In planta* and *ex planta* assays were employed to determine the

response to *C. sublineolum* infection. Gene expression studies were done in transgenic lines, respective parent Wt line, susceptible and tolerant sorghum cultivars sampled from Kenya.

Three independent experiments were carried out to study the variables whose effects were under investigation. The independent experiments were done in triplicates, unless otherwise stated. The minimum sample size used in infection and transformation experiments was 30. Data figures were composed from averages from independent experiments and triplicates. ANOVA and Chi-test were used to determine significance of observed differences. Statistical significance was determined with 95% level of confidence of P = 0.05 at respective degree of freedom (df) - $P_{0.05, df}$ Statistica 6.0 statistical computer software was used for analysis. Data were presented as values (standard error – in brackets).

2.2.2 Cloning of Vectors for Particle Bombardment Transformation

2.2.2.1 pUbiHarChit

An inducible 1.275 kb *chitinase* (*HarChit*) gene was isolated from mycelia of *Trichoderma harzianum.* The gene was polymerized through PCR with primers containing *Hinc*II restriction sites-overhangs. The *Hinc*II fragment was cloned into the pUbiCas vector (Dr. D. Becker, University of Hamburg, Germany, unpublished) with the constitutive ubiquitin1 promoter (*ubi*1 promoter) from maize (Christensen *et al*., 1992) and the nopaline synthase terminator (T*nos*) from *Agrobacterium tumefaciens* (Figure 2.1). The constructed pUbiHarChit vector of 5.766 kbp also carried a *bla* gene coding the *beta lactamase* gene conferring ampicilin resistance as a selection marker (Appendix 1.1).

Figure 2.1: pUbi*HarChit* vector showing *ubi*1 promoter, *HarChit* and T*nos* terminator gene regions and restriction sites used in cloning and Southern blot analysis.

2.2.2.2 pUbiCho

A 5.184 kb plasmid containing the chitosanase gene was constructed by cloning the *BamH*I/*Sac*I 708bp *HarCho* gene region from *T. harzianum* into the *pUbiCas* vector containing *ubi*1 promoter, *nos* terminator and the *bla* gene (Figure 2.2 and Appendix 1.2).

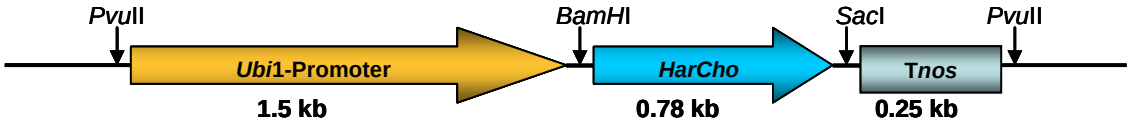


Figure 2.2: pUbiCho vector showing *ubi*1 promoter, *HarCho* and T*nos* terminator gene regions and restriction sites used in cloning and Southern blot analysis.

2.2.2.3 pUbiGus Vector

This 6.3 kb vector (Dr. R. Brettschneider, University of Hamburg, Germany) contains the constitutive *ubi*1 promoter, the β-glucuronidase *uid*A (Jefferson *et al*., 1987) gene and T*nos* terminator from *Agrobacterium tumefaciens* (Figure 2.3).

Figure 2.3: pUbiGus vector showing *ubi*1 promoter, β-glucuronidase (*uid*A) and T*nos* terminator gene regions and restriction sites used in cloning and Southern blot analysis.

The *gus* expression was used to test key parameters in particle bombardment transformation technique.

2.2.2.4 p35SAcS

The herbicide resistance-conferring plasmid construct p35SAcS (AgrEvo, Frankfurt) carries the 35S promoter from *Cauliflower Mosaic Virus* (*CaMV*), the phosphinothricin acetyltransferase gene (*pat gene*) from *Streptomyces viridochromogenes* (Strauch *et al*., 1988) and CaMV 35S terminator (Figure 2.4).

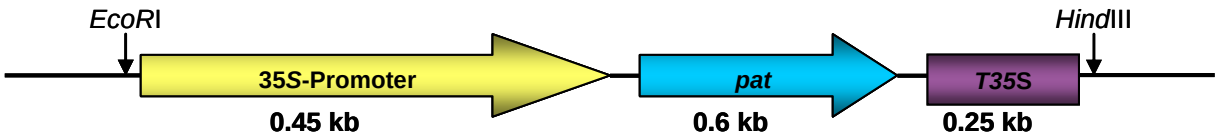


Figure 2.4: p35SAcS vector showing *ubi*1 promoter, *pat* and T*nos* terminator gene regions and restriction sites used in cloning.

2.2.3 Cloning *Agrobacterium* Transformation Vectors

2.2.3.1 p7intCho

A p7int vector (DNA-Cloning-Service, Hamburg) was restricted with *Sfi*I, dephosphorylated and ligated with the 1.8 kb plasmid fragment containing the *ubi*1 promoter and the *nos* terminator restricted from the pBlue-LNU plasmid with the same enzyme. The ligation product, p7int.ubi.nos, was then digested with *BamH*I and *Sac*I (restricting between the promoter and the terminator region), dephosphorylated and used for further cloning. The 770 bp chitosanase gene, cloned from *T. harzianum,* was excised with *BamH*I and *Sac*I from the pUbiCho and ligated with the dephosphorylated p7int.ubi.nos. The ligation product, p7intCho vector (Figure 2.5) was cloned in *E. coli* and later used to transform *Agrobacterium*.

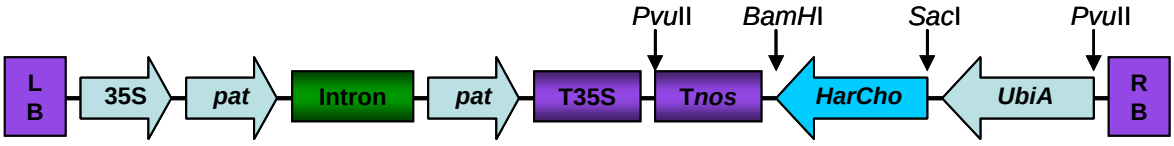


Figure 2.5: p7intCho *Agrobacterium* vector showing the RB and LB delimited genes, *ubi*1 promoter, *pat* and T*nos* terminator and *HarCho* gene regions and restriction sites used in cloning.

2.2.3.2 p7intChit

The p7int and pUbi*HarChit* were double digested with *Hind*III and *EcoR*I and the products ligated to form a 12.225 kb p7int.chit plasmid (Appendix 2.6), which was cloned in *E. coli* and used to transform *Agrobacterium*.

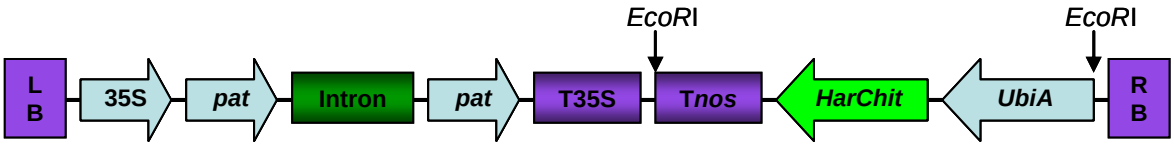


Figure 2.6: p7intHarChit *Agrobacterium* vector showing the RB and LB delimited genes *ubi*1 promoter, *pat* and T*nos* terminator and *HarChit* gene regions and restriction sites used in cloning.

2.2.3.3 p7intChoChit

A blunt-end ligation strategy was used to construct the vector p7inChoChit. The 12.225 kb p7int.chit was linearized with *EcoR*I, dephosphorylated and the protruding termini blunted using the T4 DNA polymerase according to manufacturer's instruction (Fermentas Life Sciences). The 5.184 kb pUbiCho was restricted with *Pvu*II and the resulting *ubi*1-cho-*nos* fragment was isolated and purified after gel electrophoresis. The two fragments were ligated. Two ligation schemes were produced: p7int-nos-cho-ubi-nos-chit-ubi (Cho-Chit: head-tail) and p7int-ubi-cho-nos-nos-chit-ubi (Cho-Chit: tail-tail). Both were used to transform *Agrobacterium tumefaciens*.

2.2.3.4 p7intgus

A spectinomycin and tetracycline resistant LBA4404 (pSB131) strains containing a T-DNA consisting of T-borders, 35S-bar-nos and intron-GUS (Japan Tobacco Inc.) were used.

2.2.4 Primers

2.2.4.1 Chitinase Cloning Primer

HarChit42-HincII (5´-Primer): 5´-CGC GCG TCG ACA TGT TGA GCT TCC TCG GAA A-3´

HarChit42-HincII (3´-Primer): 5´-CGC GCG TCG ACT TAG TTC AGA CCA TTC TTG A-3´

The forward and reverse primers attached at start and stop codons, respectively.

2.2.4.4 Southern Blot HarChit Primers

- HarChitDig (5´-Primer): 5´-TCT CTG GCG ATA CCT ACG CT-3´(Primer attachment at position 244-264)
- HarChitDig (3´-Primer): 5´-AGA TCG GAC TTC CTT CAG CA-3´(Primer attachment at position 556-576). Product length: 332 bp

2.2.4.5 Southern Blot HarCho Primers

- HarChoDig (5´-Primer): 5´-TAG TGG CGG AGC TAC T-3´ (Primer attachment at position 135-151)
- HarChoDig (3´-Primer): 5´- GCT TGC TTT GGG TAA G-3´ (Primer attachment at position 500- 516). Product length: 381 bp

2.2.4.6 qRT-PCR Primers

Primers producing fragments of 167-237 bp were designed from gene sequences of the respective genes under study. The primers were designed to have an optimum annealing temperature of about 60℃ to enable concurrent qRT- PCR runs (Table 2.1).

Table 2.1: qRT-PCR Primers. Table showing target genes, their respective forward and reverse primers, optimal annealing temperatures, polymerization fragment length, primers efficiencies and NCBI accession numbers.

	Gene	Primer Identity	Forward Primer	Reverse Primer	Annealing Temperature	Fragment Length (bp)	Average Primer Efficiency	Accession Numbers
1	Sorghum Leucine-rich Repeat (*SbLRR*)	RT1-SbLRR	GAC AGG TAT ACC ATC ATC GTT	TCA GTG CCA TCC AGG TTG TTT CTA	55.6℃	237	96.75%	BG356045
2	Sorghum Actin (SbAct)	RT4-Actin	AGG CGC AGT CCA AGA GGG GTA	ATG GCG GGG GGT GTT GAA GGT	59.7℃	226	93%	X79378
3	Sorghum Chalcone Synthase-like Gene 2 (*SbCHS2*)	RT3-CHS2	GAC GTG CCC GGG CTC ATC TCC	CGC ATT CGC TCC TTC TCC AG	59.6℃	167	96.67%	AF152549
4	Sorghum Chalcone Synthase-like Gene 8 (SbCHS8)	RT2-CHS8	GTG GTG GGA ACG TGG GTA GC	GGG TGA CAT GGG CGA AGA AA	59.5℃	195	93.87%	AY069951
5	Sorghum Chitinase (*SbChit*)	RT-SbChit	TAC TGC GGC ACG GAC GGA CGA CTA C	TTG GCG GCG CTC AGG AAC G	62.3℃	200	93.37%	AY047608
6	*Trichoderma harzianum* Chitinase (*HarChit*)	RT-HarChit	ACC CCA ATG CCA CCC CCTTCA A	TAC CGG CCT CCC AGC TTC CAC TT	58.4℃	174	99.37%	AB041751
7	*Trichoderma harzianum* Chitosanase	RT2-Cho	GTG GCC AGA GCG AGA CT	TCA CCC CAG ATA CCA TAG AA	53.4℃	199	79.83%	AY571342

2.2.5 Tissue Culture Media

2.2.5.1 Microprojectile Bombardment Media

Isolated IZE were cultured in a regime of MS-based (Murashige and Skoog, 1962) callus induction (CIM), regeneration (REM) and rooting media (ROM), with requisite macroelements, microelements, vitamins, amino acids, recommended additives and plant hormones (Appendix 1.3). The media were solidified with 0.3% gelrite.

2.2.5.2 Agrobacterium mediated Transformation Media

Four transformation media systems were tested for their effectiveness in an *Agrobacterium*-meidated transformation tisusue culture regime. These media were of 4 categories: *Agrobacterium* Inoculation Medium (AIM), Co-Cultivation Medium (ACCM), Resting Medium (ARM), Regeneration Medium (AREM), and Rooting Medium (AROM) (Appendix 1.4).

2.2.6 Microparticle Bombardment

Immature seeds were harvest 14-20 days after flowering, washed 3 times with distilled water, sterilized with 2% sodium hypochlorite containing 0.1% Mucasol for 25 minutes at room temperature on a shaker set at 200 rpm. The seeds were then rinsed 3 times with sterile double distilled water under a sterile hood. Immature embryos were thereafter aseptically isolated from the immature seeds under a dissecting microscope under standard sterile tissue culture conditions.

Gene delivery into the IZE was carried out by particle bombardment using the BIORAD PDS-1000/He Particle Gun (BioRad, München). Isolated IZEs were cultured overnight on CIM and incubated on osmotic medium for 4 hours prior to bombardment. IZE were bombarded with DNA-coated gold particle. DNA coating of gold particles and bombardment of scutellar tissue of sorghum were based on the procedure described by Oldach *et al*. (2001). 2 mg

gold particles with diameters of 0.4-0.8 µm were coated with 2 µg of plasmids DNA of each of the pUbiCho, pUbiHarChit and p35SAcS plasmids and suspended in 250 µl of absolute ethanol, from which 3-10 µl were loaded on a macro-carrier and used to transform an average of 30 IZE (80-267 ng plasmid DNA/embryo) (Appendix 1.5). For *gus* transformation, 5 µg of the pUbiGus was used. Particle bombardment was carried out with helium gas at 900-1550 psi under a partial vacuum of 27 mmHg according to Brettschneider *et al*. (1997). Immature embryos were co-bombarded with gold particles coated with the three plasmids containing *HarChit*, *HarCho* and the herbicide resistance, *pat* genes. The bombarded IZE remained on the osmotic media for 18-24 hours before being transferred to CIM.

2.2.7 Transformation of *Agrobacterium*

2.2.7.1 Preparation of Competent Cells

A single colony of *Agrobacterium* strain LBA4404 (p7int) was grown overnight with 10 µg/ml tetracycline in 5 ml LB medium (Appendix 1.6) at 28℃. 2 ml of this culture was added to 50 ml LB medium and incubated at 28℃ until the culture grew to an OD_{600} of 0.5-1.0, chilled on ice and centrifuged at 3000 xg for 5 min at 4℃. The pelleted cells were resuspended in ice-cold 20 mM $CaCl_2$ solution, dispensed in 0.1 ml aliquots, shock-frozen in liquid nitrogen and stored at -70℃ until used.

2.2.7.2 Plasmid transformation of Agrobacterium tumefaciens

Agrobacterium was transformed with the appropriate plasmid through heat shock treatment. About 0.1-1 µg of the respective plasmid was added to the 0.1 ml of frozen *Agrobacterium* competent cells. The cells were incubated with the plasmids at 37℃ in a water bath for 5 min and thereafter placed on ice for 30 min. The cells were then spread on pre-warmed LB plates (28℃) containing 10 µg/ml tetracycline and 100 µg/ml spectinomycin and incubated for 2 days at 28℃. Cells successfully transformed formed colonies under selection.

2.2.7.3 Culture of transformed Agrobacterium tumefaciens

A single colony of *Agrobacterium* carrying the appropriate vector was taken from solid LB media plate and cultured in 10 ml liquid YEB medium (Appendix 1.7) containing the antibiotics for selection. The culture was incubated at 28℃ at 250 rpm until an OD_{600} of 0.4 was attained. Equal volume of 500 µl *Agrobacterium* culture and 30% glycerine were shock frozen in liquid nitrogen and stored at -70℃ until used in the transformation of sorghum IZE.

2.2.8 Transformation of IZE with *Agrobacterium tumefaciens*

Frozen 1 ml aliquots were cultured to an OD_{500} 0.2-0.8 in 30 ml AB medium (Appendix 1.8) at 28℃ with appropriate antibiotics and 100 µM acetosyringone on rotary shaker. Culture was then centrifuged at 5,000 rpm at 4℃ for 10 min. The bacterial pellet was resuspended and washed with 15 ml of 10 mM $MgSO_4$ and pelleted as described above. The pellet was resuspended in appropriate inoculation medium with 100-200 µM acetosyringone, diluted to the right OD_{600} (0.2-0.8) and used in the inoculation of sorghum IZE. Infection was done in liquid or solid medium and involved incubating IZE with *Agrobacterium tumefaciens* at the specific OD_{600} and duration.

2.2.9 Tissue Culture of Immature Zygotic Embryos

2.2.9.1 Bombarded IZE

Immature sorghum embryos (IZE) were used as the explant for somatic embryogenesis. Sorghum tissue culture was carried out according to the protocol described by Oldach *et al*., (2001). Immature seeds were harvested from sorghum plants produced under standard conditions in the greenhouse. IZE were microscopically isolated from developing seeds under sterile condition, cultured in a series of defined callus induction, regeneration and rooting media. Regenerants were transferred to the greenhouse, hardened off and cultivated under normal conditions.

For induction of callus formation, sorghum IZE were cultured in darkness for 2 weeks at 26℃ on CIM containing 2.5 mg/l and 0.1 mg/ml 2,4-D (2,4-dichlorophenoxyacetic acid) and BAP (6-benzylaminopurine), respectively. Calli were thereafter transferred onto a regeneration medium (REM) with 1 mg/l of BAP and cultured under 16/8Hrs light/darkness cycle at 26℃ for organogenesis. The calli were cultured on REM for 2 weeks without selection and thereafter sub-cultured onto SEL-REM plates with 2 mg/l BASTA for 4-10 weeks, i.e., until ready for transfer to rooting medium. Calli and young regenerants were sub-cultured every 2-3 weeks onto fresh media. Organogenic regenerants with well formed shoots were cultured onto ½ MS rooting-induction media with 2 mg/l BASTA selection (SEL-ROM) without hormones. When regenerants have formed leaves and roots and reached a height of 7-10 cm they were transferred to the Greenhouse, hardened-off (covered with a translucent plastic for 7 days) and finally cultivated under standard light and temperature conditions.

2.2.9.2 Agrobacterium transformed IZE

Transformed IZE were co-cultivated with *Agrobacterium* for 3-7 days in a co-cultivation containing 100-300 µM acetosyringone, 2.5 mg/l 2,4-D and 0.1 mg/l BAP at 21, 26 or 28℃. The calli were thereafter transferred to *Agrobacterium* selection media containing 250 µg/ml Cefotaxime, 2.5 mg/l 2,4-D and 0.1 mg/l BAP and cultured 14 days at 26℃. Calli were then transferred to regeneration and rooting media as described for bombarded embryos.

2.2.10 Analysis of Genes Integration and Expression

2.2.10.1 Histochemical GUS Assay

β-Glucuronidase (GUS) activity in transformed tissues was analysed histochemically (Jefferson *et al*., 1987). Tissues were incubated for 12-16 hours at 37℃ in staining buffer containing X-Gluc {(5-Bromo-4-chloro-3-ndolyl-β-D-glucuronic acid); 100 mM NaH_2PO_4 (pH

7.0); 10 mM EDTA (pH 7.0); 0.5% Triton X-100)} as a substrate (Appendix 1.9). GUS signals were visually enumerated under a dissecting microscope.

2.2.10.2 DNA Isolation

Genomic DNA from sorghum was isolated as described by Dellaporta *et al*. (1983). Approximately 300 g of sorghum leaves were cut and frozen in 2 ml tubes in liquid nitrogen and stored at -70℃ until used. The frozen leaves were ground with ball-bearings through vigorous shaking for 2 minutes at 90 rpm in a Retsch MM2000 Shaker (with a variable amplitude and timer). The milled powder was sequentially extracted with 1 ml extraction buffer (Appendix 1.10), 1 ml phenol/chloroform/isoamylalcohol (25:24:1) and centrifuged at 5000 rpm. The DNA was precipitated from the aqueous phase was with 1/10 volume 3 M sodium acetate of pH 4.8 and 1 volume isopropanol, centrifuged at 1300 rpm, pellet washed twice with 70% ethanol, air dried and dissolved in 400 µl R40 buffer (40 µg/ml RNAse A in 1x TE). The purity, integrity and concentration of the isolated DNA were checked by absorbance spectrophotometry and gel electrophoresis. DNA was stored at 4℃ until analysed.

2.2.10.3 RNA Isolation

Total RNA was extracted from the samples using the peqGOLD TriFast extraction protocol according to the manufacturer's instruction (PeqLab Biotechnologie, Erlangen) (Appendix 1.11). DNA-free RNA was generated by using an endonuclease, DNAse, which digests single and double stranded DNA. The extracted 10 µg total-RNA was treated with 10 U of RNA-free DNAse, appropriate buffer and RNAse inhibitor as described by reagents manufacturer (Fermentas Life Sciences). The purity and integrity of the RNA was checked through gel electrophoresis and absorbance spectrophotometry. The DNA-free RNA was frozen in liquid nitrogen and stored at -70℃ until used in reverse transcription for the generation of cDNA.

2.2.10.4 cDNA Synthesis

cDNA was synthesized from the total RNA using 18-mer oligonucleotide (Oligo$(dT)_{18}$) primer, dNTPs, RNAse inhibitor and the Moloney murine leukaemia virus reverse transcriptase (M-MuLV) as recommended by the reagents manufacturer (Fermentas Life Science) (Appendix 1.12).

2.2.10.5 PCR

PCR analysis was done using a Biometra TGradient cycler (Goettingen, Germany). Parameters and concentrations of primers, requisite buffers, dNTPs, polymerase, template DNA and salts were as described by the reagents manufacturer (Fermentas Life Sciences) (Appendix 1.13)

2.2.10.6 Digoxigenin-Labelling of DNA Probes

Digoxigenin-11-dUTP (DIG-dUTP) from Roche Diagnostics was used to mark DNA probes used in molecular analysis of gene integration through Southern blotting. DIG-marking was done through PCR of plasmid DNA with specific DIG-primers. The PCR reaction mix used for DIG-marking included 0.3 µM of each DIG Primers; 0.3 mM of each dATP, dGTP, dCTP; 0.2 mM dTTP; 35 µM DIG-dUTP; 2 mM $MgCl_2$, 1x PCR buffer; 1 U Taq-Polymerase and 50 ng template DNA.

2.2.10.7 Southern Blot Analysis

Southern blotting was carried out as described by Sambrook *et al*., (1989). 10-25 µg genomic DNA were restricted with endonucleases and separated in 0.8% agarose gels. Gel treatments for Southern blots were performed as described in Sambrook *et al*. (1989). DNA was transferred onto HybondTM NX nylon membranes by capillary transfer (20 x SSC) and fixed to membranes with 120 mJ using StratalinkerTM 1800 UV crosslinker (Stratagene, La

Jolla, U.S.A.). For detection hybridisation with DIG-labelled with *HarChit* and *HarCho* DNA probes (20-25 ng/ml hybridisation solution) was performed at 42℃ using DIG Easy Hyb solution (Roche, Mannheim). Chemiluminescence detection was done with CSPD® substrate according to the manufacturer's prescriptions (Roche, Mannheim).

2.2.10.8 Progeny Segregation

Segregation of the transgenes in sorghum progeny was evaluated from the expression of the herbicide resistance gene. T_1 seedlings were sprayed twice with 200 and 300 mg/l BASTA herbicide 7 and 14 days, respectively, after germination. Surviving and dead plants were then enumerated and computed to establish the segregation ratio.

2.2.11 *Colletotrichum sublineolum* Infection

Sorghum lines studied were: KAT 412 Wt, KOSA1 T_1, KAT L5, KAT 487, GBK 046812, GBK 046820, GBK 046844, Serena and SDSH 513. Sorghum seeds were grown in autoclaved soil in a RUMED growth chamber (Jencons Scientific Ltd, West Sussex, UK). Germination time and rate were recorded to standardize the age of the seedlings used for infection.

In planta and *ex planta* infection experiments were used to study the response of the transgenic, wild type KAT 412 and selected sorghum cultivars to infection with *C. sublineolum*. Preliminary evaluation of anthracnose tolerance was done on 8 sorghum lines. Three cultivars, a tolerant and two susceptible lines, were thereafter chosen for further studies. *In planta* infection involved foliar spray of 1 week old seedlings with conidia while *ex planta* infection entailed infection of leaf segments excised from 2-weeks old seedlings with *C. sublineolum.* Symptoms development was evaluated at specific intervals between 0-144 hours post infection (HPI).

2.2.11.1 Sporulation, Conidia Harvesting and Counting

Conidia of *C. sublineolum* were used in infection experiments. To induce conidia formation, samples of fast growing mycelia maintained on ½ PDA were cultured on 7.25% Oatmeal agar (OMA) medium at 26℃ with backlight illuminati on for at least 2 weeks.

C. sublineolum conidia-laden 7.25% OMA plate was flooded with 20 ml of sterile double distilled water containing 0.01% Tween 20 and gently scrapped with a sterile plastic brush to free conidia from the setae. The suspension was filtered through a 50 nm nylon filter. The conidia-containing stock filtrate was immediately set on ice until used for infection. Serial dilutions of the stock filtrate were pipetted onto a counting chamber and used to estimate the conidia count under a light microscope. Inoculums suspension of 1 x 10^6 conidia/ml was used in all infection experiments.

2.2.11.2 Ex Planta Infection

The second leaf of 2 weeks old sorghum seedling was cut in three equal parts (triplicate) of approximately 3x6 cm using a cutting mould, plated on 0.8% neutral Agar plates with 40 mg/l of anti-senescence compound, benzimidazole, and incubated at 26℃ under 16/8 h light/dark conditions. Two infection methods were used:

1) Point infection: 10 µl aliquot of the conidial suspension was pipetted onto the excised leaf segments.
2) Floral dip infection: leaf segments were briefly dipped into a bath of conidial suspension for 10-15 seconds. Infection chambers were sealed and the leaf response was visually analyzed under natural light every 24 hours post infection (HPI).

2.2.11.3 In Planta Infection

Sorghum seedlings grown on trays in the growth chamber were used. Two leaves-stage seedlings were sprayed with 10^6 conidia/ml suspension until run-off. Seedlings were grown

in 80% RH, 8/16 h light/dark cycle at 28℃ for 48 h and thereafter 12/12 h cycle for the rest of the experimental period. Observations were made at specific time interval between 0-144 HPI. Observations made included: timing of the appearance of infection symptoms, number of leaves showing symptoms, degree and distribution of symptoms, differences in symptoms development and severity in the transgenic, parent Wt KAT 412 and other Kenyan sorghum lines were studied.

2.2.12 Real Time Quantification of Gene Expression

Spatial expression of the introduced genes and selected innate sorghum genes were quantified at the specific time intervals after *C sublineolum* infection. Real time genes expression was computed from levels of cDNA as a measure of abundance of respective template mRNA. Total RNA was extracted at specific intervals from *ex planta* and *in planta* samples and used in the synthesis of cDNA which was used in qRT-PCR analysis.

2.2.12.1 Analysed Genes

The sorghum actin gene (NCBI Accession no. X79378*)* was used as the internal standard for gene expression studies. Expression of the transgenes, *HarCho* and *HarChit* was quantified. The effect of *C. sublineolum* infection on 3 genes: sorghum chalcone synthase like gene 2 and 8 (*SbCSH2* and *SbCHS8*), sorghum leucine rich repeat (*SbLRR*) and sorghum chitinase 5 (*SbChit*), was studied. Homologous sequences in NCBI public database were used in the design of the primers.

2.2.12.2 qRT-PCR Primers, Standard Curves and Primer Efficiencies

The cDNA was used as the template in qRT-PCR gene expression study using specific primers of the genes of interest. Primers were designed by PrimerSelect (DNASTAR) to anneal under the same conditions. Concurrent runs across genes and sorghum lines were

performed (Table 4.1). The primers were used to polymerize their respective genes from cDNA synthesised from the total RNA. 1 ng-1 fg of the polymerized gene fragments were used as the templates in qRT-PCR Biorad iCycler® to determine their respective standard curves and primer efficiencies.

2.2.12.3 qRT-PCR Conditions Optimization

SYBR Green 1 with fluorescein was used in qRT-PCR (Eurogentec, Köln, Germany). The reaction mixtures and thermocycler program were as described by the manufacturer. A primer titration matrix was used to standardize the primers concentration. The dilution and concentration of the cDNA was also optimized towards a 20-35 Ct (threshold cycle) range.

3 RESULTS

3.1 PARTICLE BOMBARDMENT

3.1.1 Sorghum Regeneration through IZE Tissues Culture

Sorghum IZE were isolated from immature seeds 14-20 days after flowering and cultured through 3 stages of tissues cultures – calli induction, tissues regeneration and rooting. Regeneration of sorghum from IZE was achieved through tissue culture on callus induction (CIM), regeneration (REM) and rooting media (ROM) (Figure 3.1). Selection of genetic transformants was done by culturing and rooting regenerants in SEL-REM and SEL-ROM media containing 2 mg BASTA/l. The average time taken for each stage was 14 days for each CIM and REM, 30 days in SEL-REM and SEL-ROM. It therefore took 3 months to develop a tissue culture regenerant ready for transfer to the greenhouse.

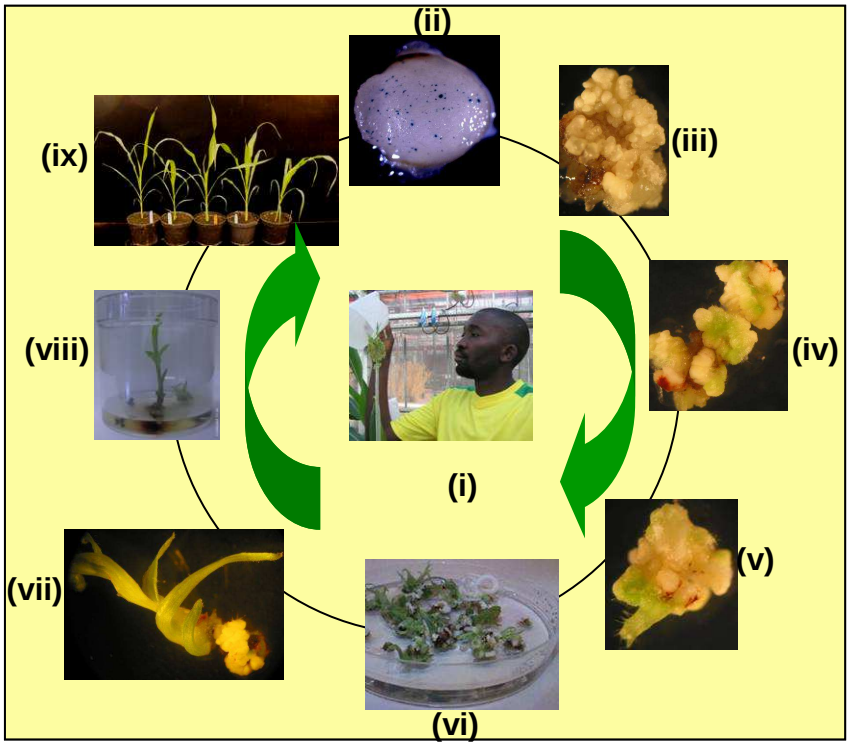


Figure 3.1: Main stages in sorghum tissue culture. Harvesting of immature sorghum seeds 14-20 days after flowering (i), isolated embryo after 2 days in CIM (ii), calli after 2 weeks in CIM (iii), calli after 2 weeks in REM (iv), regenerants in REM (v-viii), regenerant 14 days in SEL-ROM (viii), regenerants in the greenhouse (ix).

3.1.2 Effect of Genotype on Tissue Culture Regeneration

A collection of 11 sorghum cultivars sampled from Kenya was used in tissue culture and genetic transformation. The cultivars were established in the greenhouse and successfully regenerated through tissue culture from IZE. These cultivars were, KAT 412, KAT 369X, KAT 487, SDSH 513, GBK 046820, Serena, KAT L5 (Line 5), ICSV III, GBK 046812, GBK 046842 and GBK 046844. These sorghum lines represented a gamut of seed colour, from white (e.g. KAT 412), brown (e.g. GBK 046820) to red (e.g. Serena) (Figure 3.2). There was a difference in the amount of calli and number of regenerants formed by the various sorghum lines cultured. The difference was correlated to the level of pigments, widely regarded to be phenolic compounds, secreted into the culture media. Genotype dependent variation in the amount of phenolics secreted into the tissue culture media was observed. Genotypes with brown to red seed colour, e.g. KAT L5, GBK 046844, secreted more phenolics than white lines such as KAT 487. Sorghum lines that secreted copious amount of pigments like GBK 046844 also produced significantly less calli and regenerants than KAT 487, which was associated with low level of pigmentation during tissue culture (Figure 3.2).

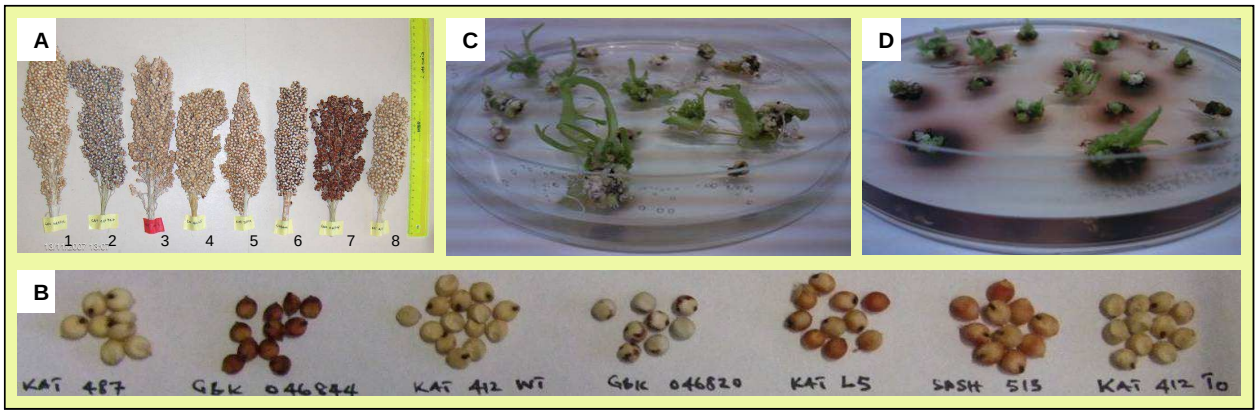


Figure 3.2: Sorghum kernel, seeds colour and phenolics secretion in regeneration media. A: Red to white kernel colour of the different sorghum cultivars 1-GBK 046812, 2-GBK 046820, 3-KOSA-1 T_1, 4-Kat 412 Wt, 5-KAT 369X, 6-Gadam, 7-GBK 046844 and 8-KAT 487; B: red to white seeds of some cultivars studied; C: tissue culture condition in 3 weeks old REM culture of two sorghum lines secreting low KAT 487; D: high levels of pigments in line KAT L5 secreted into tissue culture medium.

This difference in the response of cultured IZE was apparent from the number of regenerants that were produced and survived to the greenhouse stage by the various cultivars (Table

3.1). For example only 0.7% of 608 Serena IZE, a cultivar that secreted high levels of phenolics into culture media, developed regenerants that reached the greenhouse stage as compared to 3% in lower phenolics producing line KAT 412.

Table 3.1: Sorghum cultivars and regenerants formation. Correlation between cultivar's grain colour and pigments secretion into culture media and sorghum regenerants formation after particle bombardment.
Key: +very low, ++ - low, +++ moderate and ++++ - high phenolics production.

Sorghum line	Grain Colour	Amount of Phenol in Culture	Embryos Cultured	Regenerants to Greenhouse	% Embryos forming Regenerants
KAT 412	White	+	3455	105	3.0
Serena	Red	++++	608	4	0.7
KAT 487	White	+	674	8	1.2
SDSH 513	Brown	+++	1746	12	0.7
GBK 046820	Brown	++	600	6	1.0
ICSV	Red	++++	265	2	0.8
KAT L5	Brown	+++	346	3	0.9

3.1.3 Bombardment Pressure and Transient GUS Activity

Microprojectile bombardment conditions were optimized to ensure high survival rate of embryos and high transient *gus* gene expression. Three bombardment pressure levels, namely 1100, 1350 and 1550 psi were evaluated. A triplicate of 30 KAT 412 IZE were bombarded with the plasmid carrying the *gus* gene at 1100, 1350 and 1550 psi, cultured on CIM and transient GUS activity quantified 2 and 14 days after bombardment IZE (Figure.3.3).

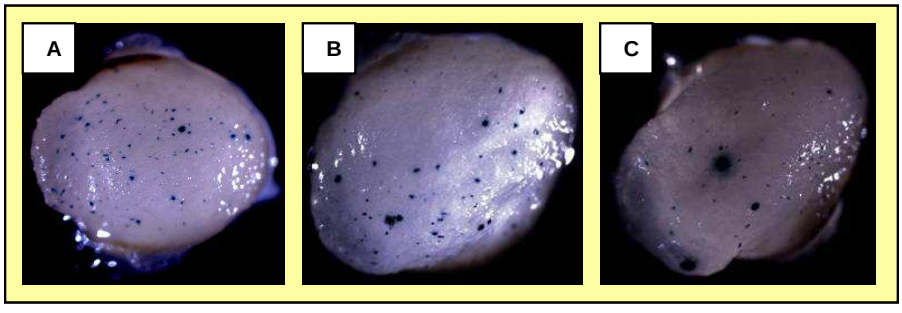


Figure 3.3: Transient *gus* expression in IZE at various bombardment pressure. GUS signal foci in embryos bombarded with 3 µl of suspension containing DNA-coated gold particles at 1100 psi (A), 1350 psi (B) and 1550 psi (C). The average GUS signal/embryo (standard deviation in brackets) from the three bombardment pressures were 276 (18.945), 344 (13.164) and 287 (26.978).

As shown in figure 3.3, it was observed that *gus* expression after 2 days was not significantly different between the 3 pressure levels as all the embryos bombarded dyed blue for GUS gene. All the embryos showed at least 250 GUS signals per scutellum. Expression after 14 days under CIM at 26℃ was pointedly different. The highest level of GUS expression, 58% of bombarded embryos, was recorded with 1350 psi as compared to 37% achieved with 1550 psi (Figure 3.4). Bombardment at 1350 psi was found to result into higher GUS activity and subsequent transformation experiments were carried out at this pressure.

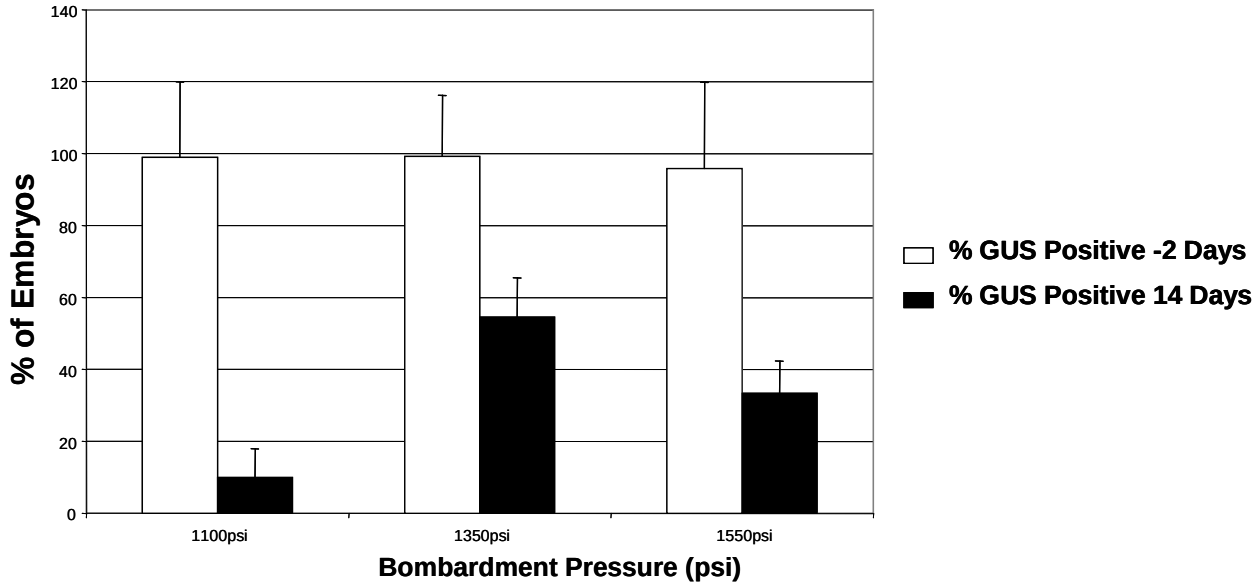


Figure 3.4: Transient *gus* expression in 2 and 14 days IZE and calli at different bombardment pressure. Percentage of KAT 412 IZE bombarded at the different pressures 1100, 1350 and 1550 psi showing GUS activity after 2 and 14 days of culture in CIM. The *gus* expression was higher in at 1350 psi than at 1100 and 1550 psi.

3.1.4 Effect of Pre-culture on Calli and Regenerants Formation

An evaluation was carried out to establish the best pre-culture condition for the formation of regenerants from sorghum IZE. KAT 412 IZEs were pre-cultured on CIM for 1 or 3 days. Average number of regenerants was determined from triplicates consisting of 322 (1-day) and 209 (3-days) pre-cultured embryos that were cultured for 2 weeks in CIM and 2 months in REM (Figure 3.5). Regenerants which had undergone successful organogenesis resulting in developed shoot axis were sub-cultured from REM onto ROM and remaining calli further cultured. Through this, it was possible to carry out 3 and 2 sub-cultures from the 1-day and

3-days pre-cultured embryos, respectively. The 1-day pre-cultured embryos produced more regenerants, with an average (standard deviation in brackets) of 34 (19.09), 29 (7.44), 35 (9.90) as compared to 19 (11.43), 33.25 (9.00) regenerants in 3-days pre-culture. An average of 80.50 (14.89) and 52.25 (7.56) regenerants were produced in 1-day and 3-days pre-cultured plates. Embryos pre-cultured for 1 day were used in all subsequent transformation and tissue culture experiments.

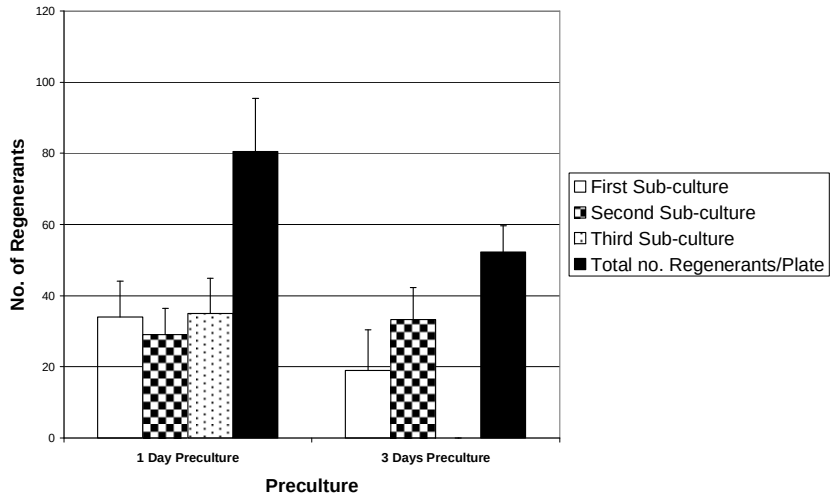


Figure 3.5: Effect of pre-Culture on regenerants formation. 1 day and 3 days pre-cultured IZE were incubated on CIM for 2 weeks and transferred to REM. Regenerants with well developed shoot axis were transferred to ROM and the remaining calli cultured further. 1 day pre-cultured embryos produced more regenerants than 3 days pre-cultured embryos with, respectively, an average of 80 and 55 regenerants from a culture plate containing 30 IZE.

3.1.5 Transformation with Target Genes

Transformation was attempted on 8 sorghum lines: Aralba, KAT 412, KAT 487, KAT L5, GBK 046820, ICSV III, SDSH 513 and Serena. A total of 10,269 embryos from these lines were bombarded in batches of 30 IZE/Petri dish with a plasmid mix containing *HarChit*, *HarCho* and *pat* genes coding for chitinase, chitosanase and herbicide resistance, respectively (Figure 3.5). Bombarded IZE were cultured in CIM, REM, SEL-REM, SEL-ROM and surviving transformants transferred to the greenhouse.

A number of putative transformants that survived selection were regenerated after tissue culture. Of the 10,269 IZE bombarded, 168 (1.157%) survived tissue culture selection with 2 mg/l BASTA in SEL-REM and SEL-ROM. There was genotype influence on the proportion of bombarded IZE forming putative transformants with 0.66% Serena and 3.04% KAT 412 IZE surviving to the greenhouse (Figure 3.6). Only 3 of the 168 putative transformants survived the BASTA-spray test in greenhouse.

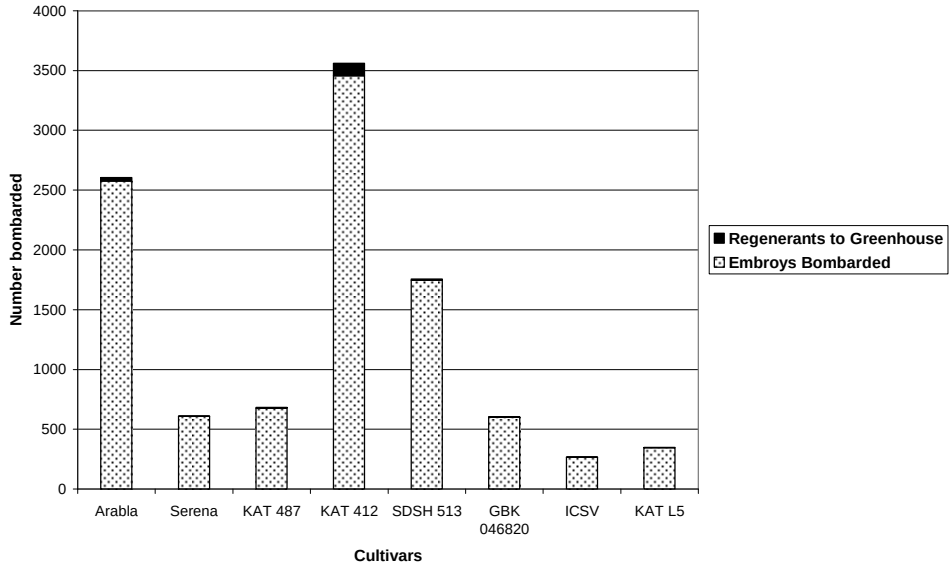


Figure 3.6: Particle bombarded IZE and regenerants formed from various sorghum lines. Sorghum IZE from the 8 cultivars were bombarded with plasmids carrying target genes, tissue cultured and putative transformants transferred to the greenhouse. The putative transformants were sprayed twice with BASTA to select for regenerants with stable transgene integration. Of the 168 putative regenerant transferred to greenhouse, 3 independent transgenic lines from KAT 412 exhibited stable transgene integration.

3.1.5.1 Stable Transformation

Putative transformants that survived SEL-REM and SEL-ROM were transferred to greenhouse and sprayed twice with 200mg/l and 300mg/l BASTA after 7 and 14 days. Three transformants survived the two rounds of BASTA spraying (Figure 3.7). Three BASTA herbicide resistant sorghum plants (KOSA-1, KOSA-2 and KOSA-3) were successfully regenerated after microprojectile bombardment of IZE. Leaf samples were taken for molecular analysis by Southern blot and qRT-PCR to confirm stable integration of *HarChit* and *HarCho* transgenes. The transgenic plants were cultivated under standard greenhouse

condition and the progenies used in subsequent gene integration, segregation and expression studies. The transgenic plants, KOSA1-3, showed normal growth. KOSA-1 produced normal and fertile T_1, T_2 and T_3 progenies.

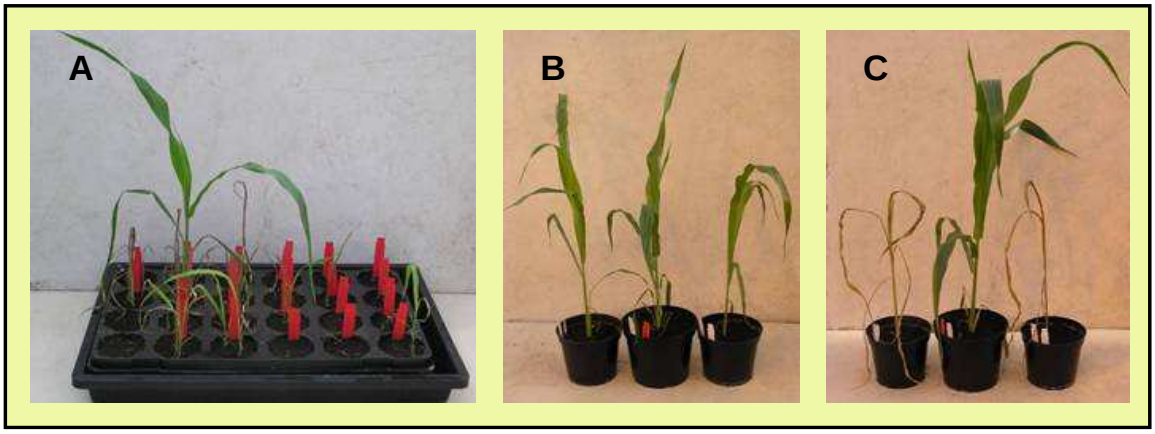


Figure 3.7: KOSA-1 BASTA herbicide resistance test in the greenhouse. Microparticle bombarded sorghum IZE were tissue cultured and putative regenerants transferred to the greenhouse. Putative transgenic KAT 412 – KOSA-1 outgrowing other regenerants (A). Regenerants were sprayed with 200mg/l BASTA after 1 day (B). Resistant KOSA-1 between wilted Wt controls 7 days later (C).

3.1.5.2 Transformation Frequency

Transformation frequency was computed from the total IZE bombarded, putative transformants surviving to the greenhouse and stable transformants confirmed through herbicide spraying. Low transformation frequency was observed (Table 3.2).

Table 3.2: Putative and stable transformation frequency of sorghum cultivars. Number of IZE bombarded, putative (transiently BASTA resistant) and stable (permanently BASTA resistant) transformed regenerants.

Cultivar	Embryos Bombarded	Putative Regenerants	Transgenic Plants	Freq. of Putative Transformation (%)	Freq. of Stable Transformation (%)
Aralba	2575	28	-	1.09	-
Serena	608	4	-	0.66	-
KAT 487	674	8	-	1.19	-
KAT 412	3455	105	3	3.04	0.087
SDSH 513	1746	12	-	0.69	-
GBK 046820	600	6	-	1	-
ICSV	265	2	-	0.75	-
KAT L5	346	3	-	0.87	-
Total	**10269**	**168**	**3**	**9.28**	**0.029**

Evaluation of transformation frequency from the number of cultivars transformed revealed that 1 of 8 cultivars (12.5%) was amenable to transformation. From the total of 10,269 IZE bombarded 3 (0.029%) were herbicide resistant. The 3 (0.087%) of the transformants were part of the 3455 KAT 412 IZE bombarded. Only 2 (105+300) batches of KAT 412 IZE produced the transgenic plants. This reveals a transformation frequency of 0.74%.

3.1.5.3 Progeny Segregation Ratio

A total of 341 seeds of KOSA-1 were planted and sprayed twice with 200 mg/l and 300 mg/l BASTA at 7 and 14 days after germination, respectively. Analysis of BASTA resistant T_1 progeny of KOSA-1 showed that an average of 73.92% were transgenic (Table 3.3). The expected segregation ratio of single locus dominant gene integration is 3:1 (75% transgenic). The segregation ratio showed that the *pat* gene was integrated at a single locus and was inherited in simple Mendelian fashion.

Table 3.3: Segregation of T_1 Progeny of the transgenic sorghum line - KOSA-1. Percentage germination, number of transgenic plants after BASTA spray test and percentage transgenic progenies from 4 experiments with 100 seeds each.

	% Germination	No. Transgenic	No. Non Transgenic	% Transgenic
1	80	55	25	68.75
2	89	70	19	78.65
3	90	72	18	80.00
4	82	56	26	68.29
Total	**341**	**253**	**88**	**74.19**
Average	**85.25**	**63.25**	**22**	

3.1.5.4 Southern Blot Analysis of Gene Integration

Southern blot analysis was carried out using DIG-labelled *HarChit* and *HarCho* probes. DNA of KAT 412 Wt, KOSA-1 T_0 and positive control plasmids: pUbiCho and pUbiChit, were digested with the respective restriction enzymes that linearised and excised out the *Ubi*1-gene-*nos* regions of the *chitinase* and *chitosanase* genes. Southern blot analysis showed a

different integration pattern of *HarChit* and *HarCho*. There were at least a single and 6 integration of *HarChit* and *HarCho*, respectively (Figure 3.8a). Multiple copy integration of *HarChit* in KOSA-1 was apparent.

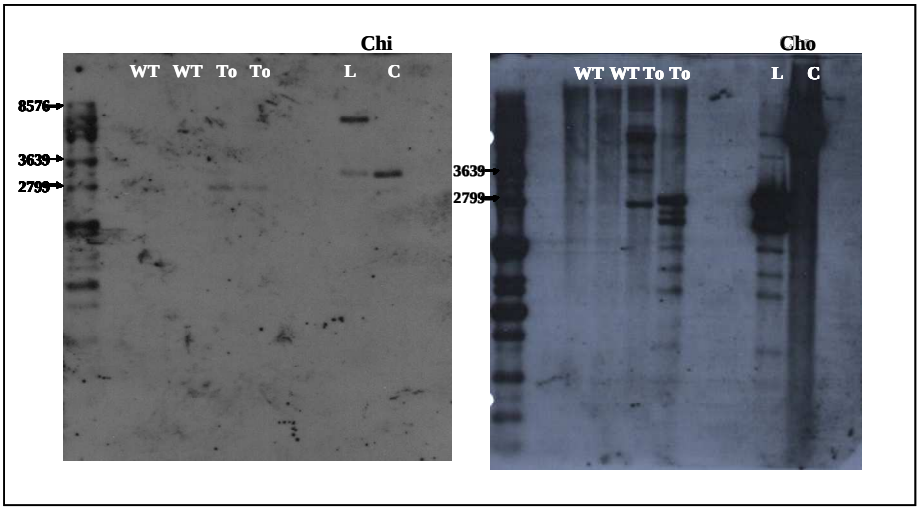


Figure 3.8a: Southern blot analysis of KOSA-1 T_0 with *HarChit* (left) and *Cho* (right) probes, positive control – linearized (L) and cassette cut-out (C) of pUbiHarChit and pUbiCho were used. Cassette out fragment –*ubi*1-Chit-nos- of 4.525 kb lane C (A) and *ubi*1-HarCho-nos of 2.53 kb lane C (A). A single band representing a single integration was noted *HarChit* blot (A). Several bands (at least 5 in Cho) representing multiple integrations can be seen (B).

Southern blot analysis of KOSA-1 T_0 and T_1 was carried out to confirm the inheritance of the transgenes. Results showed that the transgenes were inherited in KOSA-1 T_1 (Figure 3.8b).

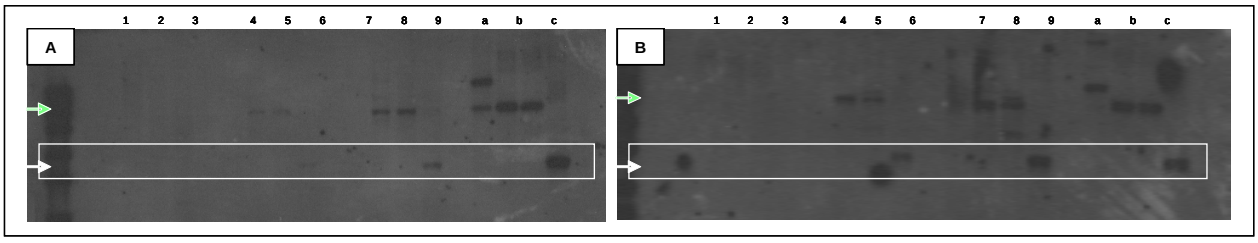


Figure 3.8b: Southern blot analysis for gene integration in KOSA-1 T_0 and KOSA-1 T_1. Southern blot of KAT 412 T_0 and T_1 with DIG-labelled *HarChit* (A) and *HarCho* (B) probes. Linearized band (green arrow), cassette out band (white arrow). 1-3 (KAT 412 Wt), 4-6 (KOSA-1 T_0), 7-9 (KOSA-1 T_1), a-c (plasmid positive control). DNA samples were linearized with *Hind*III and *EcoR*I for *HarChit* and *BamH*I and *Sac*I for *HarCho*. *HarChit* gene cassettes were excised with *EcoR*I and *Hind*III double digestion and *HarCho* with *Pvu*II.

The 3 independent transgenic lines KOSA-1, 2 and 3 were analyzed by Southern blot. DNA was extracted from these primary transformants, restricted with respective enzymes that

linearized and excised the gene cassettes in the original plasmids carrying *HarCho* and *HarChit* used in transformation. Gel electrophoresis was done with undigested, linearized and cassette excised plants DNA and plasmids. This analysis confirmed the integration of the two transgenes in the three primary transformants (Figure 3.9).

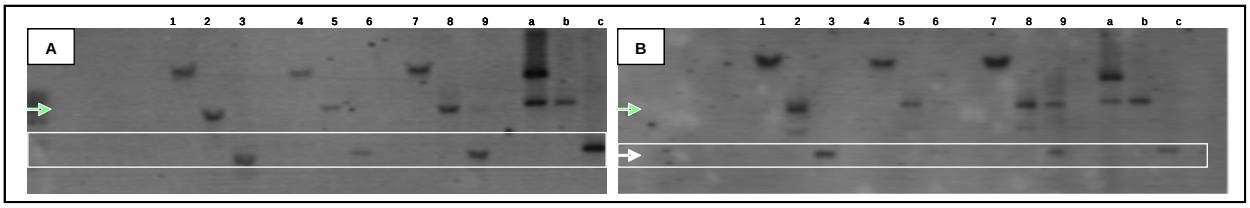


Figure 3.9: Southern blot for gene integration and inheritance in KOSA-1, 2 and 3. Southern Blot of KOSA-1(1-3), KOSA-2 (4-6) and KOSA-3 (7-8) with *HarChit* (A) and *Cho* (B) probes, positive control (a, b, c) were pUbiHarChit (A) and pUbiCho (B). All samples consisted of un-restricted DNA, linearized and cassette out restricted DNA. Linearized band (green arrow), cassette out band (white arrow).

Integration of the two transgenes was also confirmed in 3 successive KOSA-1 generations, i.e., T_0 to T_3 (Figure 3.10). DNA was extracted from the seedlings of primary transformant (T_0), first, second and third generation (T_1, T_2 and T_3, respectively) of KOSA-1 and analysed by Southern blot.

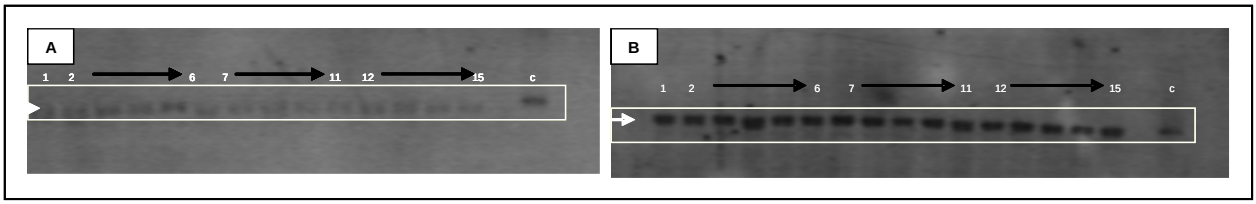


Figure 3.10: Southern blot analysis for gene integration and inheritance in KOSA-1 T_0-T_3. Southern blot of KOSA-1 T_o, (1) T_1, (2-6), T_2 (7-11) and T_3 (12-15) with *HarChit* (A) and *HarCho* (B) probes. The DNA of the KOSA-1 T_0-T_3 seedlings were restricted with enzymes that excised the gene cassettes: *Hind*III and *Eco*RI for *HarChit* and *Pvu*II for *HarChit*. Plasmids pUbiHarChit and pUbiCho (c) were used as positive controls.

3.1.5.5 Expression of HarChit and HarCho in KOSA-1

Expression of the transgenes *HarChit* and *HarCho* in KOSA-1 was confirmed by qRT-PCR. Seedlings of KOSA-1 were sprayed twice with BASTA and the leaves of transgenic survivors harvested at specific intervals for total RNA extraction. Total RNA was reverse transcribed

into cDNA and used for gene expression study in a Biorad qRT-PCR iCycler. Expression of the transgenes was quantified from the reverse transcribed cDNA which was a measure abundance of their respective mRNA. Fold changes were computed in respect to the expression of the sorghum actin gene (*SbAct*), a house-keeping gene which was used as the internal control. It was established that both genes were expressed in all the transgenic seedlings of KOSA-1 analyzed. The expression level varied between 0.5-2 FC (Figure 3.11).

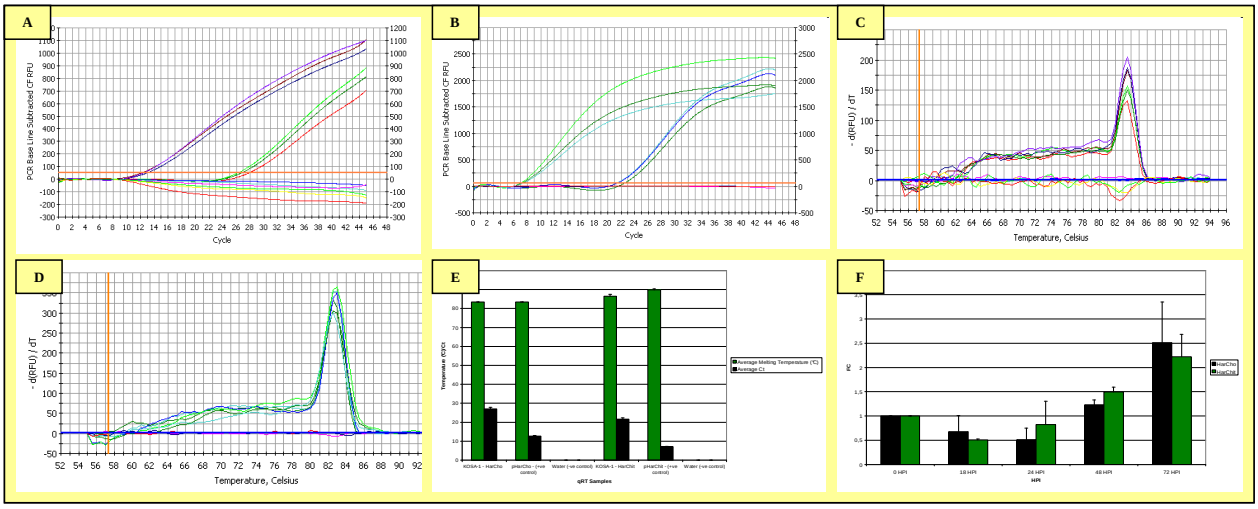


Figure 3.11: qRT-PCR expression of *HarChit* and *HarCho* in KOSA-1 T_0. cDNA of KOSA-1 T_0, pUbiHarChit and pUbiHarCho were analysed by qRT-PCR. *HarChit* (A) and *HarCho* (B) were profiled in KOSA-1 T_1 and the plasmids used as the positive control. Analysis of the melting curves of *HarChit* (C) and *HarCho* (D) in comparison to their respective positive controls (E) confirmed their identities and expression in sorghum. Determination of fold changes (FC) in the activity of the two transgenes in 2 weeks old seedlings of KOSA-1 established their expression in all the samples studied during a 72 hour analysis window (F).

3.2 *AGROBACTERIUM*-MEDIATED TRANSFORMATION

The *Agrobacterium tumefaciens* strain expressing the *gus* marker gene was used to study the optimum conditions for *Agrobacterium*-mediated transformation. The following variables were studied: 1) 4 media sets: inoculation, co-cultivation, resting, selection and regeneration media; 2) Inoculation time (60-240 min); 3) Embryos inoculation technique: liquid vs. solid; 4) Co-cultivation method: scutelli direction (adaxial and abaxial); 5) Co-cultivation temperature: 21, 26 and 28^{o}C; 6) Co-cultivation duration: 2 – 7 days.

3.2.1 Liquid and Solid Phase *Agrobacterium* Infection

The effect of inoculation phase was studied to establish whether liquid immersion negatively affected IZE survival and calli formation after infection with *Agrobacterium*. Two inoculation phases were used: liquid and solid phase. 3 batches, each containing 30 IZE of KAT 412, were infected with *Agrobacterium* either in liquid or solid state. In solid phase-infection, IZE were infected with droplets of inoculums while embedded on co-cultivation medium and in liquid phase-infection embryos were suspended in AIM for 30 minutes (Figure 3.12). The number and proportion of surviving IZE developing calli was quantified 4 and 10 days after *Agrobacterium* infection.

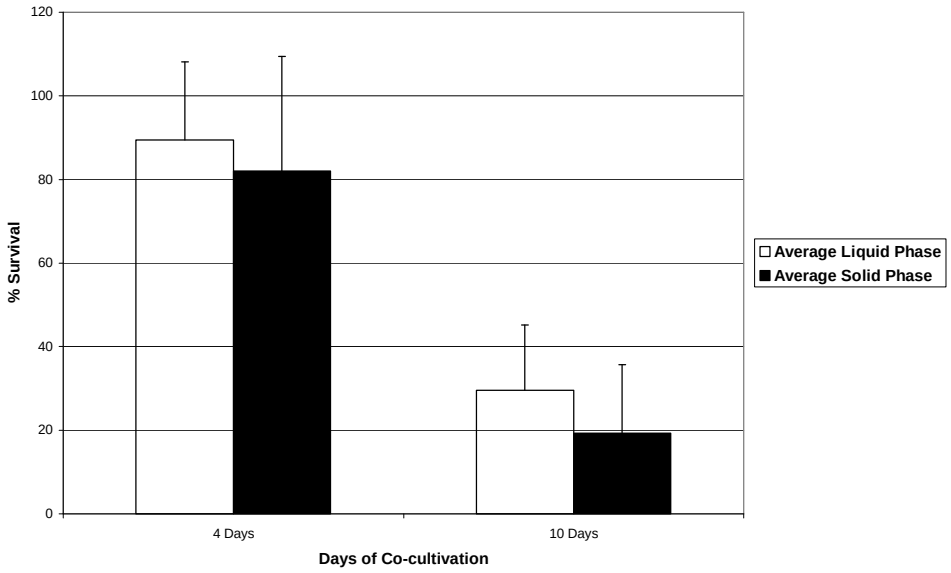


Figure 3.12: Effect of *Agrobacterium* liquid and solid phase inoculation of KAT 412 IZE. KAT 412 IZE were infected with *Agrobacterium* on either liquid or solid phase. Survival of IZE calli was analysed after 4 and 10 days in calli induction media. Liquid phase inoculation resulted in better IZE survival and calli development.

The results showed no significant difference between the liquid and solid media after 4 days in culture. However, survival after 10 days was markedly higher in IZE cultures infected in liquid than solid medium. Liquid suspension positively affected calli formation. The result showed that liquid phase infection was associated with higher calli formation (29.28%) than solid phase (16.13%).

3.2.2 Effect of the Duration of *Agrobacterium* Infection

An experiment was carried out to determine the effect of the duration of *Agrobacterium* infection on the calli development from IZE infected on solid and in liquid media. Triplicates of 30 KAT 412 IZE were incubated with *Agrobacterium* culture for 60-240 minutes under the two phases of inoculation and the number of IZE that developed calli formed enumerated after culturing at 26℃ for 10 days in ARM. Infection duration significantly affected calli development (Figure 3.13). A longer inoculation period did not negatively affect the development of calli. Better calli development was noted in IZE that was incubated for longer duration with *Agrobacterium* in liquid media. Inoculation for 60 minutes and 240 was associated with 1.01% and 53% in liquid medium and 1.5% and 49.67% in solid medium, respectively.

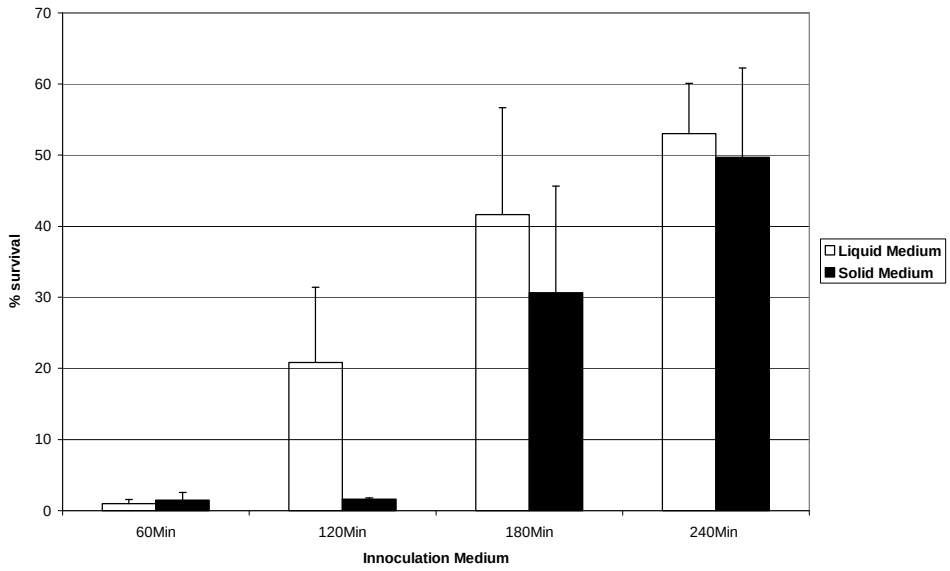


Figure 3.13: Effect of infection duration of KAT 412 IZE with *Agrobacterium* on IZE survival. IZE were inoculated for 50-240 minutes under shaking. The surviving IZE were enumerated 10 Days after Inoculation. Longer periods of inoculation did not have a negative effect on IZE survival.

3.2.3 Sorghum Embryos Orientation and Regeneration

Intimate contact with *Agrobacterium* is prerequisite for any transformation event. Increasing the level of contact of scutelli with culture media containing acetosyringone could be a way of

improving the chances of transformation. A barley transformation protocol (Lütticke, 2006) involved a 24 hours upside down (scutelli on media) culturing of embryos onto co-cultivation media as a means of enhancing *Agrobacterium*-mediated transformation. An experiment was carried out to determine the effectiveness of this culture method on sorghum calli formation. Triplicates of sorghum and barley IZE were infected with *Agrobacterium* on two media MK2 (Lütticke, 2006) and ZIP (Zhao *et al*., 2000), co-cultivated adaxially or abaxially overnight and normally (adaxially) for the remainder of culture period at 21℃ and 26℃. Calli-forming IZE were enumerated after 10 days. It was found that calli formation was severely reduced in sorghum embryos cultured upside down overnight on co-cultivation media (Table 3.4). Barley embryos did not show similar response.

Table 3.4: Effect of IZE abaxial and adaxial co-cultivation with *Agrobacterium*. IZE of sorghum line Aralba and barley were infected with *Agrobacterium* and either abaxially or adaxially cultured overnight in co-cultivation medium, and then transferred to callus induction medium. Calli formation was analysed after 10 days in callus induction medium. Adaxially cultured IZE produced better calli.

Source of Embryos	Culture Medium	Upside Down (Abaxial)		Normal (Adaxial)	
		21℃	26℃	21℃	26℃
Aralba (sorghum)	MK2	+	-	++	+
	ZIP	+	-	++	+++
Golden promise (Barley)	MK2	++	++	++	++
	ZIP	+	+	+	+

3.2.4 Effect of IZE Pre-culture on Mortality and Callus formation

Pre-culture has been reported to improve the survival of sorghum embryos after *Agrobacterium* infection (Zhao *et al.,* 2000). An investigation was carried out to quantify calli formation from fresh, 1 day pre-cultured and 3 days pre-cultured embryos. Sorghum IZEs were isolated from KAT 412, inoculated immediately, after 1 day or after 3 days of pre-culture in ACIM. It was found that fresh embryos and 1 day pre-cultured embryos formed more calli than 2-days and 3-days pre-cultured embryos (Figure 3.14).

Table 3.5: Effect of Pre-culture on calli formation after *Agrobacterium* transformation. IZE were isolated from KAT 412 and either inoculated directly (fresh) or after being pre-cultured for 2, or 3 days. Percentage of IZE forming calli was computed after 10 days. Longer pre-culture resulted in lesser calli formation.

Embryos Pre-culture	% Embryos forming Calli	Calli formation	Comments
Fresh	94,37	+++	Good Calli
1 Day	55,56	++	Fair Calli
2 Days	8,00	+	Poor Calli
3 Days	6,67	*	Poor Calli

3.2.5 Co-cultivation Temperature Effect on Callus Formation

Co-cultivation temperature is an important variable in *Agrobacterium*-mediated transformation of sorghum. Two co-cultivation temperatures were tested. Embryos of 3 cultivars were co-cultured with *Agrobacterium* at 21℃ and 26℃ and the number of embryos forming calli quantified after culturing for 10 days (Figure 3.12).

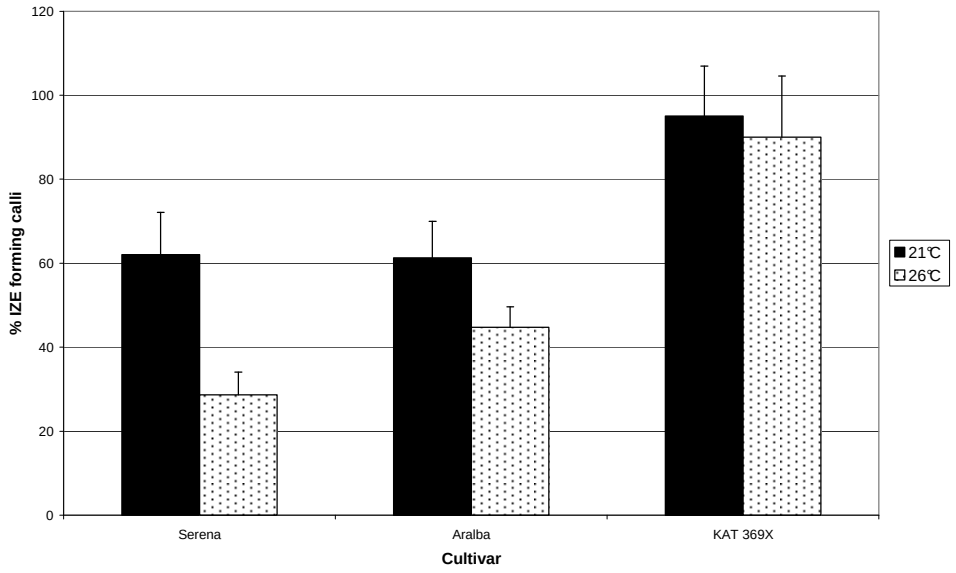


Figure 3.14: Effect of co-cultivation temperature on calli formation after *Agrobacterium* infection. IZE from 3 sorghum cultivars were infected with *Agrobacterium* and co-cultivated either at 21℃ or 26℃ then tran sferred to callus induction medium. The percentage of IZE that formed calli was computed 10 days after infection. Co-cultivation at 21℃ resulted in better calli develo pment than at 26℃.

The results showed that co-cultivation at 21℃ resu lted in more calli formation than at 26℃. This was observed in all the three sorghum lines studied.

3.2.6 $MgSO_4$ Activation and Regeneration Potential

A set of pre-culture treatment and culture conditions were studied to establish the best parameters for IZE survival and calli development (Figure 3.15). Embryos were pre-suspended in inoculation medium, 200 µM acetosyringone and 0.01% Silwet®. These were then inoculated with either *Agrobacterium* culture-pellet that was unwashed or washed (with 10 mM $MgSO_4$). Sorghum IZE that were pre-washed formed more calli and were readily regenerated.

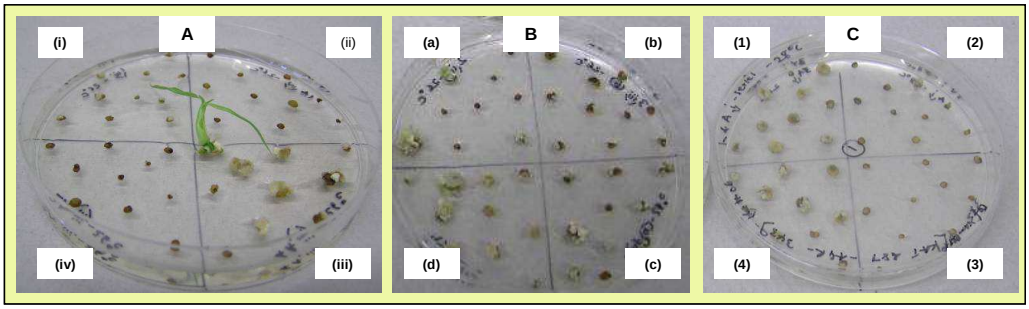


Figure 3.15: Organogenesis and regeneration of sorghum after *Agrobacterium* infection under different pre-culture treatment. IZE were isolated and infected with *Agrobacterium* (OD=0.3) that was either pre-washed with 10 mM $MgSO_4$ (A and B) or unwashed (C). The washed embryos were either cultured at 21℃ (A) or 26℃ (B). IZE infected with washed *Agrobacterium* and cultivated at 21℃ produced the best calli.

3.2.7 Transient and Stable GUS Activity in *Agrobacterium*-

The sorghum line, Aralba, was transformed with *Agrobacterium* containing the *gus* gene. Aralba IZE were isolated, pre-cultured for 1 day and infected with *Agrobacterium* carrying *gus* gene. The IZE and *Agrobacterium* were co-cultivated for 4 days and then transferred to callus induction medium for 2 weeks. GUS activity was histochemically tested after 2 days and 14 days in callus induction media (Figure 3.16).

Transient and stable *gus* expression on sorghum calli were achieved. The transient *gus* expression events were observed after two days in culture did not lead to stable integration except for one event out of the 1,578 transformed IZE.

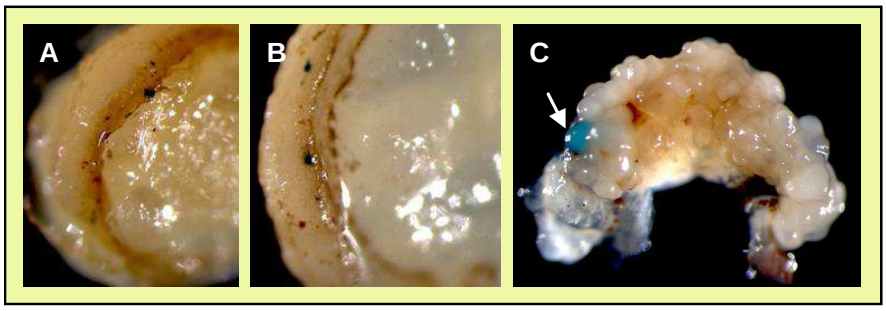


Figure 3.16: Transient and stable *gus* expression in *Agrobacterium*-infected Aralba IZE. Aralba IZE were infected with *Agrobacterium* carrying *gus* gene. The IZE and *Agrobacterium* were co-cultivated for 4 days and then transferred to callus induction medium for 2 weeks. GUS activity was histochemically tested after 2 days (A and B) and 14 days (C) in callus induction media. Transient (A and B) and stable *gus* expression on sorghum calli (C) (arrow) was achieved.

3.3 RESPONSES TO *COLLETOTRICHUM SUBLINEOLUM* INFECTION

3.3.1 *Ex Planta* Infection Responses

Ex Planta (in vitro) infection was carried out on 2 weeks old sorghum leaf segments of 8 sorghum lines. Leaves of 24 seedlings of each cultivar were excised and cut into 3 equal segments and plated on 0.8% neutral agar contain 40 mg/l benzimidazole (Figure 3.17).

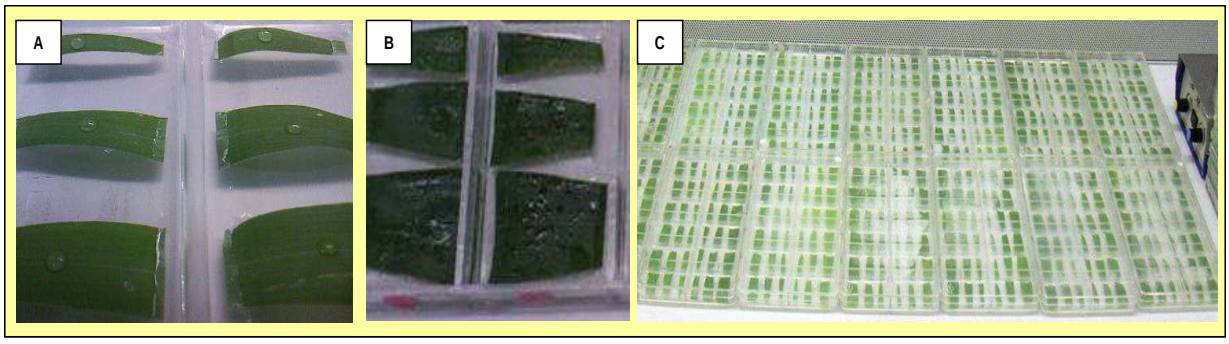


Figure 3.17 Point and spray infection. Sorghum leaf segments on *in vitro* culture chambers after point infection with 10 µl inoculum (A) and spray infection (B). The samples in culture plates were left to briefly dry under a sterile hood (C) and thereafter incubated in a growth chamber under standard light and temperature conditions.

The response of the leaf segments was evaluated for signs of necrosis, referred to as necrotic local lesions (NLL) and discoloration after every 24 h until 144 hours post infection (HPI). The NLL and pigmentation/discoloration of the leaf segments from the sorghum lines were visually compared under normal light. Infection of leaf segments with *C. sublineolum* symptoms was achieved in both inoculation methods applied.

Symptoms development in infected leaf segments was achieved by incubation at 26℃ under 16/8h light/darkness rhythm. It was possible to maintain the segments from uninfected leaves for 8 days in culture without significant senescence. This enabled at least 6 days of studies on infection-induced responses.

3.3.1.1 Wild-type and KOSA-1 T_1 Reponses

Comparison of *in vitro* response of wild type (Wt) KAT 412 and first generation progenies (T_1) of transgenic KAT 412 KOSA-1 leaf segments was carried out to determine the effect of the introduced anti-fungal chitinase and chitosanase genes in a non-host interaction with *C. sublineolum*. Triplicates of leaf segments from sets of 24 transgenic and wild type sorghum seedlings were infected with *C. sublineolum* through floral dip and symptoms of disease development monitored for 6 days. Wt developed *C. sublineolum*-induced pigmentation and necrosis sooner than the T_1 line (Figure 3.18). The Wt leaf segments also showed higher level of necrosis and conidia growth (Figure 3.18 – upper row) than those excised from the T_1 line (Figure 3.18 – lower row). Typical rusty pigmentation and NLL associated with anthracnose response was visually apparent in the majority of leaf segments 48 HPI in Wt as compared to 72 HPI in the transgenic samples. The effect was associated with *C. sublineolum* inoculation because the controls, uninfected leaf segments, remained largely healthy (arrowed columns, Figure 3.18).

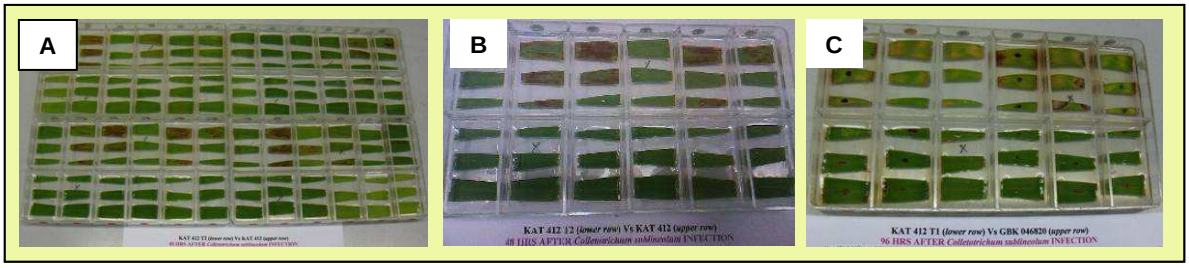


Figure 3.18: Leaf Segment response to infection. Leaf segments were cultured on 0.8% Agar and infected with *C. sublineolum*. Leaf responses were analysed 48 hours after infection. Response of KOSA-1 T1 was compared to KAT 412 Wt and GBK 046820. (A) KOSA-1 leaves (lower rows) developed less typical rusty pigmentation and NLL response than KAT 412 Wt A and B (upper row) and GBK 046820 in (C) (upper row).

The experiments showed that the disease symptoms developed later in the transgenic than in wild type sorghum and therefore the introduced genes delayed the onset and severity of *C. sublineolum* induced NLL and leaf pigmentation.

3.3.1.2 Comparison of Transgenic with Kenyan Sorghum Lines

An experiment was designed to evaluate *in vitro* responses in leaf segments of KAT 412 Wt, KOSA-1 T_1 and other 6 sorghum cultivars sampled from Kenya: KAT L5, KAT 487, SDSH 513, Serena, GBK 046812, GBK 046820 and GBK 046844. Triplicates of leaf segments from 24 seedlings were infected through point inoculation and observed for 6 days. Differences among cultivars were noted on the time required for the appearance of visible responses (pigmentation) and onset of leaf segment necrosis after C. *sublineolum* infection (Figure 3.19).

Figure 3.19: *Ex planta* leaf assay at 48 HPI. Leaf segments were cultured on 0.8% Agar after infection with *C. sublineolum.* Comparison of response leaf segments of (A): KAT 412 T_1 (lower row) vs KAT 412 Wt (upper row) (B) SDSH 513, GBK 046844: the latter showing more leaf pigmentation than former; (C)GBK 046820 (lower) more pigmented than KAT 487 (upper row).

The first visible response was generally noted 48 HPI in KAT 412 Wt, KOSA-1 T_1, SDSH and Serena, but appeared later in KAT L5 and KAT 487. This experiment showed that KAT 487 and GBK 046844 showed the least and most NLL at 48 HPI, respectively. KAT 412 and KOSA-1 T_1 showed signs of NLL at nearly the same time i.e., by 48 HPI.

Another experiment was carried out to quantify the response of the leaf segments. Leaf segments were infected through floral dip and the number showing *C. sublineolum*-induced response enumerated between 48-120 HPI. Symptoms were noted 48 HPI in KAT 412 Wt, SDSH 513 and Serena (Figure 3.20).

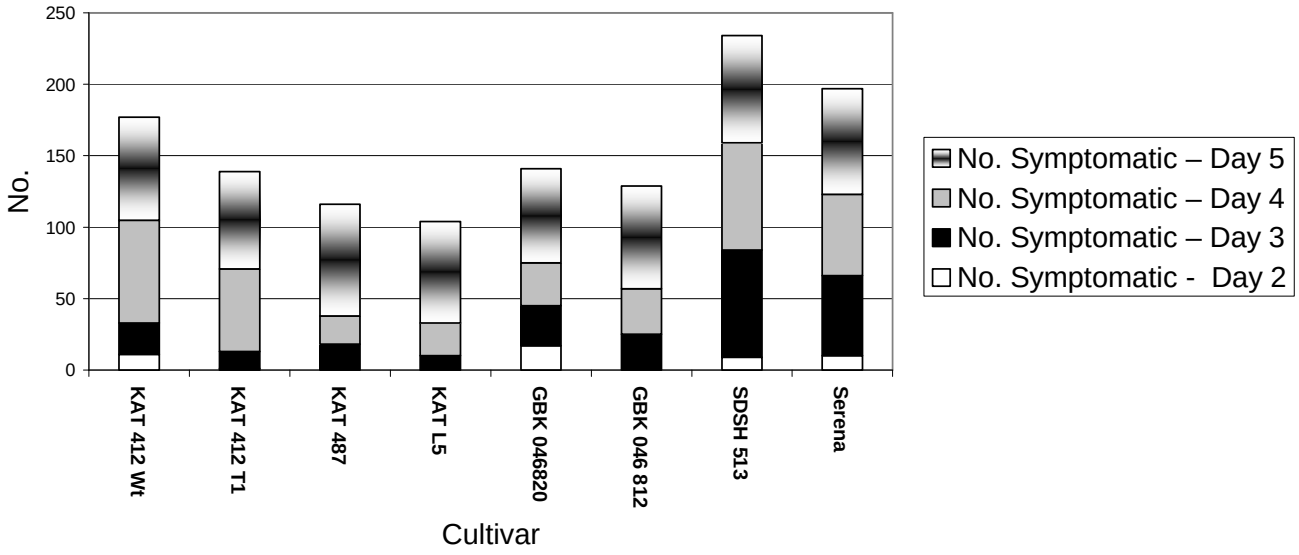


Figure 3.20: Cultivars comparison of *ex planta* response. Number of leaf segment of various sorghum cultivars showing NLL between 2 and 5 days after *C. sublineolum* Infection.

The line having the least number of symptoms 72 HPI was KAT L5. NLL developed later in KAT 412 T_1 than Wt, SDSH 513, Serena, and GBK 046820 but earlier than KAT L5, KAT 487 and KAT 046812. It was therefore concluded that KAT L5 and SDSH 513 were the most and least tolerant lines, respectively. All the leaf segments from all the sorghum lines were symptomatic by day 5 (120 HPI) and were extensively swathed with NLL.

3.3.2 In planta C. sublineolum Infection Assay

Response to *C. sublineolum* in intact tissues was studied by infecting 1 week old seedlings and observing disease development over 7 days. Infected seedlings were grown in a controlled chamber and disease development quantified in terms of the number of seedlings showing symptoms (disease incidence), onset, severity and nature of symptoms, among other parameters studied.

3.3.2.1 Transgenic and Wild Type KAT 412

A *C. sublineolum* infection experiment was done to determine the difference in anthracnose tolerance between KAT 412 Wt and T_1. Seedlings were infected and development of typical of anthracnose symptoms was observed for 7 days. Typically NLL seen a rusty spots developed in both the transgenic and the wild seedlings. However, more Wt than T_1 seedlings were disease-spotted. Infection-induced NLL was first observed in KAT 412 Wt, 48 HPI. By the fourth day (96 HPI) nearly all Wt seedlings had rusty symptoms. The first two leaves were most affected in both lines (Table 3.6).

Table 3.6: Anthracnose Symptoms in the transgenic and wild type KAT 412. Pathology of C. sublineolum infection of KOSA-1 T1 and KAT 412 seedling over a period of 168 hours after infection. Development of necrotic local lesion (NLL), drooping leaves and complete leaf tip necrosis occurred later in transgenic line than Wt seedlings.

	Pathology	**% Seedlings**			
		48 HPI	**96 HPI**	**144 HPI**	**168 HPI**
KAT 412 Wt	Rusty Spots	96%	99	100	100
	Necrosis (NLL)	-	70	100	100
	Drooping of 2nd Leaf	20	40	100	100
	Recovery of 2nd Leaf	-	-*	-	-
	Complete necrosis of 2nd Leaf Tip	-	-	8	10
KAT 412 T_1	Rusty Spots	20	90	100	100
	Necrosis	-	20	40	50
	Drooping of 2nd Leaf	-	-	40	60
	Recovery of 2nd Leaf	-	-	40	50
	Complete necrosis of 2nd Leaf Tip	-	-	30	30

Tips of the third leaf were also symptomatic. Drooping of the second leaves was also pronounced in Wt. The 2nd leaves of T_1 showed resilience and recovery without drooping. These results were clearer by 120 HPI. Leaf rusting, drooping and necrosis were severe in Wt than T_1. There was complete necrosis of the 2nd leaf in all Wt and 3/11 T_1 seedlings 7 days after infection.

Comparison of symptoms development confirmed that the leaves of the transgenic line exhibited lesser necrosis than wild types. For example by 168 HPI nearly all the second leaf of KAT 412 Wt seedlings showed complete necrosis as compared to only 30% of T_1 seedlings. Recovery of leaves after developing symptoms of infections was higher in transgenic line (arrows, Figure 3.21).

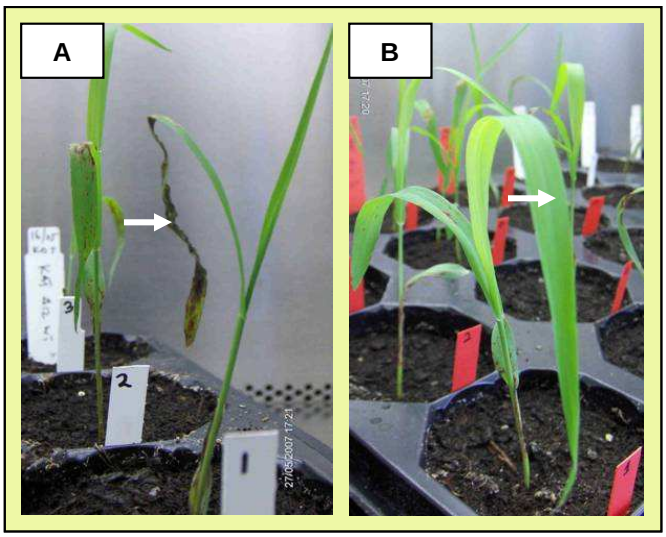


Figure 3.21: *In Planta* sorghum infection experiment. KAT 412 Wt and KOSA-1 T1 seedlings were spray-infected with *C. sublineolum*. Symptoms development was recorded on the 5th day after infection. Shrivelled and necrotic Wt (A) and more tolerant and healthier-looking transgenic KOSA-1 (B) 5 days after infection.

3.3.2.2 Evaluation of C. sublineolum Tolerance in Sorghum Lines

In addition to the above pair-wise evaluation of transgenic and wilt type KAT 412, multiple comparison involving 6 other Kenyan sorghum lines: KAT 487, KAT L5, GBK 046820, GBK 047844, SDSH 513 and Serena was carried out to rank *in planta* responses. 30 seedlings of

one week old sorghum seedlings of each of the cultivars under investigation were infected with *C. sublineolum* conidia and cultivated in a growth chamber under standard conditions. Development of disease symptoms was evaluated for 144 hours. Infection of sorghum seedlings was achieved in all the sorghum lines studied under high humidity and temperature. Seedlings of KAT L5 and SDSH 513 developed the least and most symptoms, respectively (Figure 3.20). Comparison of the Wt and T_1 showed that the seedlings of the former (62.07%) and the latter (28.21%) had developed symptoms by 144 HPI. By the same time, 13.51% and 96.97% of KAT L5 and SDSH seedlings, respectively, showed signs of infection. This experiment established that the transgenic line was more tolerant to anthracnose than the wild type. It was also confirmed that KAT L5 and SDSH 513 were the most tolerant and susceptible lines respectively (Figure 3.22).

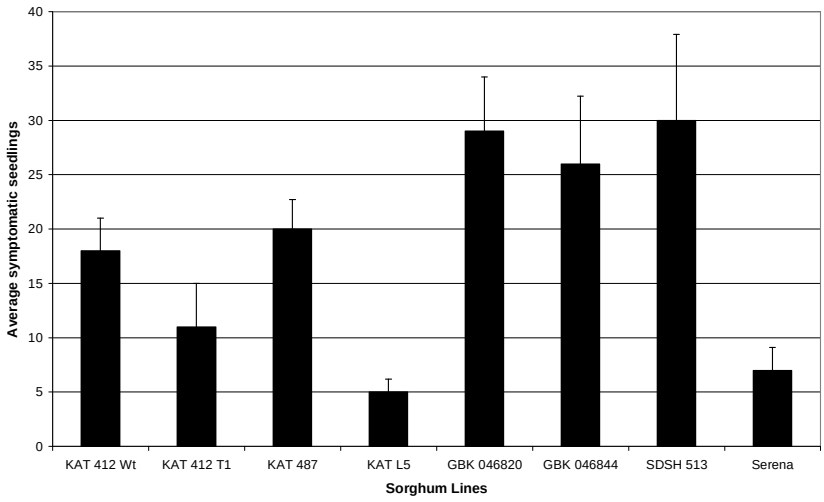


Figure 3.22: *In Planta* infection response in transgenic and Kenyan cultivars. Average number of seedlings of transgenic and various sorghum cultivars showing symptoms at 144 HPI with *C. sublineolum.*

3.3.2.3 Anthracnose in Transgenic, Susceptible and Tolerant Sorghum

Comparison of response to infection was made between the transgenic line and the two cultivars that showed extreme responses under *ex planta* and *in planta* assays: tolerant KAT L5 and susceptible SDSH 513. An experiment was designed by infecting 30 seedlings from each cultivar with conidia of *C. sublineolum.* Development of symptoms was quantified after

24, 48, 72, 96, 120 and 144 HPI. The experiment was repeated 3 times. The number of seedlings showing any signs of *C. sublineolum* infection was enumerated at every observation interval. Results showed that the response varied among the 4 lines studied, with SDSH 513 seedlings suffering the most necrosis and senescence on the first two leaves (Figure 3.23).

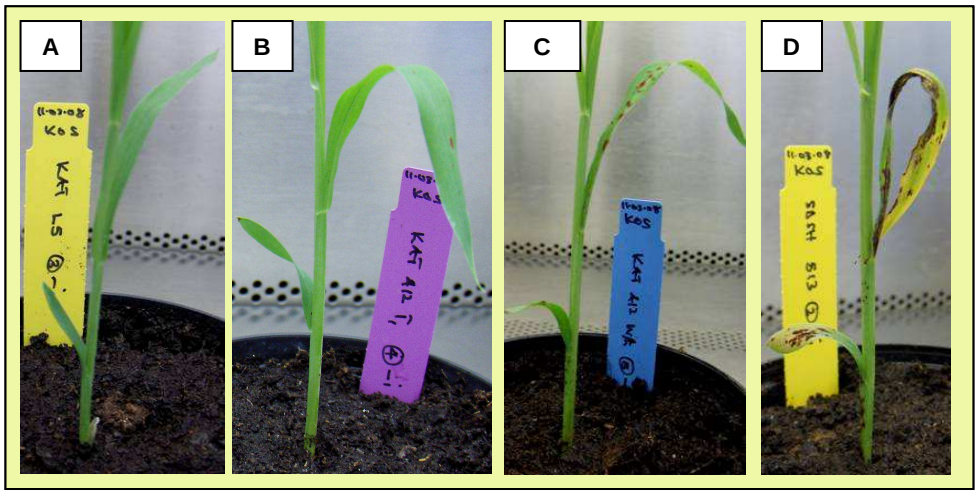


Figure 3.23: Seedlings response to *C. sublineolum* infection. Sorghum seedlings were infected and disease development monitored over a period of 144 HPI. Anthracnose symptoms on the first two leaves at 144 HPI: no symptoms on KAT L5 leaves – resistant (A), few necrotic local lesions ((NLL) on KAT 412 T_1 leaves - tolerant (B), extensive NLL in KAT 412 Wt – less tolerant (C) and extensive NLL on both leaf blades and sheath, chlorosis and discoloration of both leaves of SDSH 513 – susceptible (D).

Seedlings of the transgenic line showed more tolerance than the Wt, while KAT L5 was the most tolerant. A large proportion of KAT L5 seedlings showed no signs of disease during the experimental period. It was also apparent that, unlike KAT L5, infection in KAT 412 Wt, T_1 and SDSH 513 extended to the first and second leaf blades and sheaths by 144 HPI. The timing of the onset, distribution and severity of disease symptoms varied among the cultivars. The first symptoms appeared 48 HPI on the leaves of SDSH 513 and Kat 412 Wt. Disease symptoms were apparent in all the cultivars by 72 HPI. Observation of the symptoms distribution revealed that by 144 HPI, infection symptoms in KAT L5 were restricted to the leaf blades of the second leaves which were spotted with few NLL. Similar pattern was seen on leaves of T_1 seedlings, albeit with higher density. However, infection symptoms in KAT 412 Wt were more severe and extended to the blade and leaf sheath of first and second

leaves. Infection symptoms were most severe in SDSH 513 where the leaf blades and sheaths of the first and second leaves were diseased and showed extensive NLL and necrosis (Figure 3.23).

Quantification of disease symptoms by scoring the number of seedlings exhibiting symptoms showed that 5%, 30%, 45% and 90% seedlings of KAT L5, KAT 412 T_1, KAT 412 Wt and SDSH 513, respectively, were symptomatic at 72 HPI (Figure 3.24).

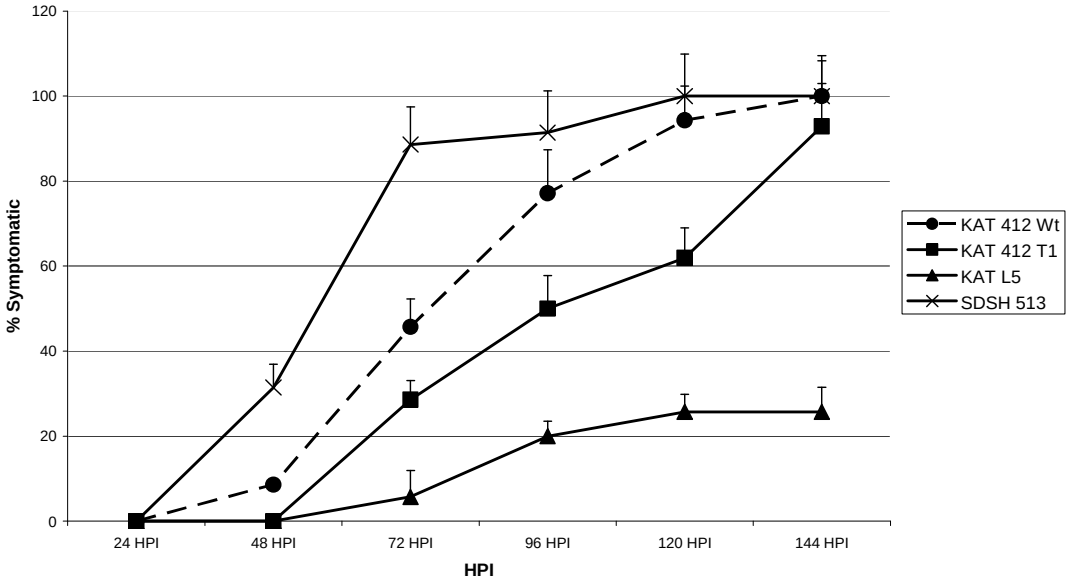


Figure 3.24: Symptoms development after *C. sublineolum* infection. Sorghum seedlings were infected and the proportion of those showing symptoms enumerated between 24-144 HPI. Fewer KAT L5 (25%) developed symptoms later (72 HPI) than SDSH 513 that had more (100%) and sooner (48 HPI) infection. KOSA-1 was symptomatic later (72 HPI) and more tolerant than KAT 412 Wt.

This translated to about 15% more tolerance of the transgenic line than the Wt. It was also noted that less than an average of 30% of KAT L5 seedlings were symptomatic by 144 HPI. On the other hand, nearly all the seedlings of KAT 412 Wt, T_1 and SDSH were symptomatic by 144 HPI. This underscored the tolerance of KAT L5.

3.4 REAL TIME QUANTIFICATION OF GENE EXPRESSION

Expression of the transgenes, chitinase (*HarChit*) and chitosanase (*HarCho*), and sorghum innate genes were profiled by quantitative real time PCR (qRT-PCR). Four innate PR-genes representing the upstream and downstream pathogenesis response system were also selected for analysis. These were sorghum leucine-rich repeat (*SbLRR*), sorghum chalcone synthase-like gene 2 (*SbCHS2*), sorghum chalcone synthase-like gene 8 (*SbCHS8*) and sorghum chitinase (*SbChit*). The expression of *HarChit, HarCho*, *SbChit*, *SbCHS2*, *SbCHS8* and *SbLRR* were studied in leaves of seedlings of one transgenic line, KOSA-1 and 5 other wild sorghum lines. The 5 sorghum lines, KOSA-1, KAT 412 Wt, KAT L5, Serena and SDSH 513 and were planted under controlled temperature and light conditions in a growth chamber. One week old seedlings were infected with 1 x 10^6 conidia/ml of *C. sublineolum*. Triplicate random samples of the infected second leaf were taken at specific time intervals for RNA extraction, first strand cDNA synthesis and real time gene expression quantification. Two types of samples were infected: intact seedlings (*in planta* assay) and detached second leaves (*ex planta* assay). The detached leaves were cultured on 0.8% neutral agar containing 40 mg/l benzimidazole. Leaf samples from intact leaves were taken at specific time interval (hours post infection – HPI) and expression of the PR-genes quantified between 0-144 HPI. Samples of detached leaves were taken only at 48 HPI. Quantification of gene expression in qRT-PCR cycler was done by evaluating the changes in level of cDNA. Paired analyses of infected and non-infected samples were done. Gene expression was denoted as fold changes (FC) and was computed from the difference in the level of cDNA between the infected and control samples. Up- or down-regulation were deduced from the FC, i.e., FC = 1 denoted no change in gene activity; thus up-regulation >FC=1> down-regulation. House-keeping gene, sorghum actin, was used as the reference control. Analysis of variation (ANOVA) and post ANOVA Newman-Keuls test was done to determine the significance of the FC variation noted between the time (HPI), genes and cultivars.

3.4.1 Expression of Chitinase and Chitosanase in Transgenic Sorghum

Transgenes *HarChit* and *HarCho* were quantified in KOSA-1 T_1 to ascertain the activity of these introduced genes in healthy and infected plants. Stable integration and expression of the introduced chitinase and chitosanase were confirmed by qRT-PCR in the leaves of T_1 seedlings. Expression of the two transgenes was also quantified after infection with *C. sublineolum* from 0-72 HPI. Both genes were expressed in all the seedlings analysed as well as in infected and healthy leaves. Expression of *HarChit* and *HarCho* oscillated between 0.5 – 2.5 FC (Figure 3.25).

The average FC (SE – standard error) of *HarChit* and *HarCho* was 0.95 (0.11) and 1.03 (0.16), respectively. Statistical analysis established that there was no significant difference in the expression of the two transgenes ($P_{0.05, 1} = 0.641$). It was also established that there was no significant effect of time ($P_{0.05, 4} = 0.214$) and gene x time ($P_{0.05, 4} = 0.660$) interaction.

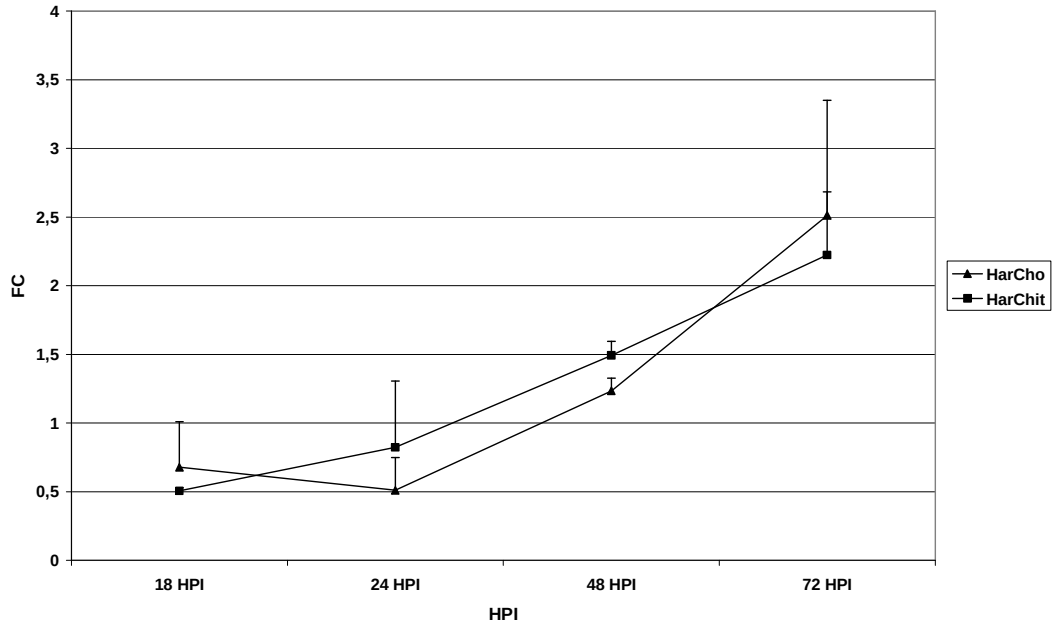


Figure 3.25: Expression of *HarChit* and *HarCho* after *C. sublineolum* infection. KOSA-1 seedlings were infected with conidia of the pathogen and changes in the expression of the transgenes between 0-72 HPI quantified by qRT-PCR. Both genes were expressed in infected and non infected seedlings. Expression oscillated between 0.5-2.5 FC (1 FC = no change in gene activity). No difference was noted in the expression of the two transgenes. Time had no significant effect on the expression of these genes.

3.4.2 Expression of Innate PR-Genes

The expression of sorghum innate *SbCHS2*, *SbChit, SbCHS8* and *SbLRR* was studied in healthy and *C. sublineolum* infected seedlings. Preliminary studies were done on the expression of *SbCHS2* in KAT 412 Wt and KOSA-1 T_1. The other 3 genes were quantified in KAT 412 Wt, KOSA-1 T_1 as well as in *C. sublineolum*-tolerant KAT L5 and susceptible SDSH 513. Evaluation of the expression of the innate genes after infection with *C. sublineolum* revealed that time and genotype had significant effect on the expression of *SbChit*, *SbCHS8* and *SbLRR* but not *SbCHS2*. The three genes were also differentially expressed. Newman-Keuls statistical test showed that the changes in the expression of *SbCHS8* and *SbLRR* were not extensively different ($P_{0.05, 1} = 0.678$). Expression of *SbChit* was found to be significantly different from that of *SbCHS8* and *SbLRR* ($P_{0.05, 1} = 0.00012$ and 0.00014, respectively). Of the 4 genotypes studied, SDSH 513 was found to be notably different from KOSA-1 T_1, KAT 412 and KAT L5. In the 0-144 HPI analysis window, most of the significant changes in genes expression occurred between 48-144 HPI.

3.4.2.1 Expression of SbCHS2

A preliminary study was done to study the expression of *SbCHS2* in seedlings of KOSA-1 and KAT 412 Wt. Seedlings were infected with *C. sublineolum* and changes in the expression of *SbCHS2* analysed at 18, 24, 48 and 72 hours after infection. Paired analysis of the infected and non infected samples showed that *SbCHS2* was expressed in both KOSA-1 T_1 and KAT 412 Wt during the 18-72 HPI under investigation. Changes in the expression of *SbCHS2* after infection with *C. sublineolum* varied between 1.112 – 7.070 FC. Statistical evaluation of the expression of *SbCHS2* showed that there was no significant difference in its expression in KOSA-1 and KAT 412 Wt ($P_{0.05, 1} = 0.532$) (Figure 3.26).

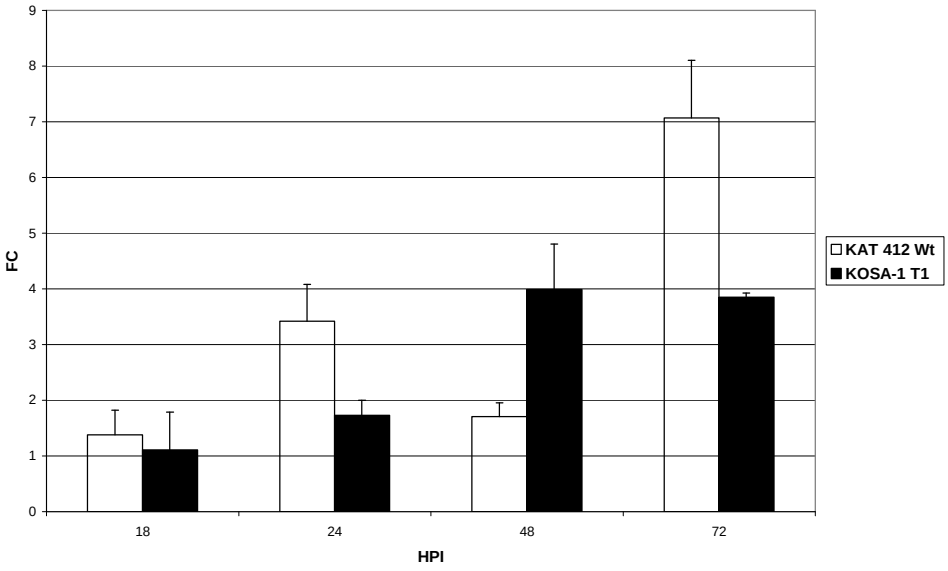


Figure 3.26: Expression of *SbCHS2* in KOSA-1 T_1 and KAT 412 Wt. Transgenic and Wt sorghum were infected with *C. sublineolum* and the activity of *SbCHS2* profiled for 72 HPI. A fold change of a maximum of about 7 FC was noted. No significant difference was observed in the change in gene activity between the transgenic and Wt sorghum ($P_{0.05, 1}$ = 0.532). Time was also found not to affect the changes in expression of this gene ($P_{0.05, 5}$ = 0.207).

The average change in the expression of *SbCHS2* during the first 72 hours after *C. sublineolum* infection was 6.96 (3.994) and 3.78 (1.394) in KAT 412 Wt and KOSA-1 T_1, respectively. Time was found to be inconsequential in the expression of this gene after infection with the pathogen ($P_{0.05, 5}$ = 0.207).

3.4.2.2 Expression of *SbChit*

Expression of innate sorghum *chitinase* gene, *SbChit* was evaluated in the transgenic and wild type sorghum lines viz. KOSA-1 T_1, Wt, KAT L5 and SDSH 513. Differences in the expression of this gene according to time and cultivar were analysed. *SbChit* was expressed in all the seedlings tested. The average fold change in the expression of this gene between 0-144 HPI was 15.891 (4,719). Significant difference was noted in the expression of *SbChit* between the 4 sorghum lines evaluated ($P_{0.05}$ = 0.0006-0.001). The highest expression of *SbChit* was noted in SDSH 513 while the least was in KAT L5 (Figure 3.27).

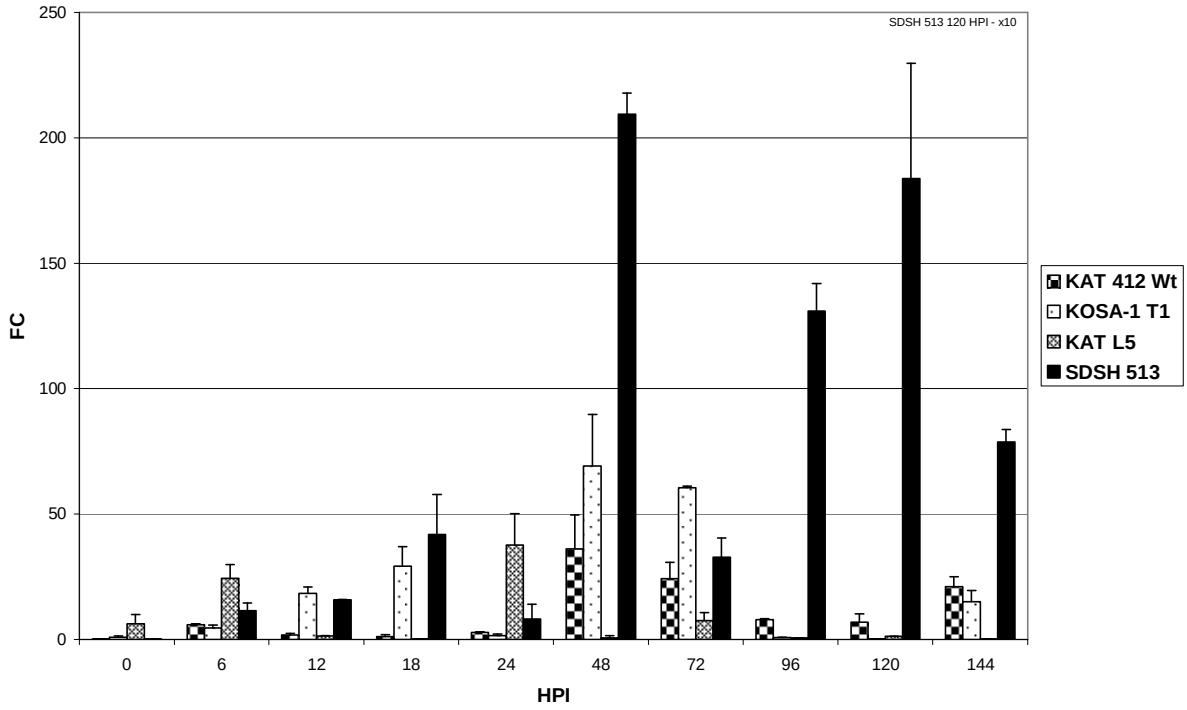


Figure 3.27: Expression of *SbChit* in sorghum 0-144 hours after infection with *C. sublineolum*. Sorghum seedlings were infected with *C. sublineolum* and FC in expression of *SbChit* quantified between 0-144 HPI by qRT-PCR. The highest and the least expression of *SbChit* was in SDSH 513 and KAT L5, respectively. The average FC (standard error) was 2.430 (0.8159), 7.5849 (2.7945), 13.5183 (5.8425) and 45.3681 (19.4574) in KAT L5, KAT 412 Wt, KOSA-1 T_1 and SDSH 513, respectively. Significant effect of time was noted with the highest activity being noted between 48 – 120 HPI.

The highest fold change in the expression of this gene was 209.42 in SDSH 513, while the least was 0.194 in KAT L5. Expression of *SbChit* in KAT 412 Wt and KOSA-1 T_1 oscillated between 0.225 to 69.199 FC between 0-144 HPI. The average FC in *SbChit* expression was 7.5849 and 13.5183 in KAT 412 Wt and KOSA-1 T_1 respectively. No significant differences were noted between Wt and KOSA-1 T_1. High activity and change in expression of *SbChit* was noted in both transgenic and Wt sorghum between 18-72 HPI than the rest of the time under study. Expression of *SbChit* in KAT L5 seedlings showed a different trend. Activity of *SbChit* was lower in KAT L5. The average change in *SbChit* expression in KAT L5 was 2.430. Most of the *SbChit* activity in KAT L5 was seen between 6-24 HPI with the highest increase of 37.678 FC being attained at 24 HPI. Expression of *SbChit* in SDSH 513 was markedly different from the previous 3 lines. *SbChit* was highly up-regulated in this cultivar attaining a maximum of 209 FC at 48 HPI. The high change in expression was extended from 48-144 HPI during which FC of 209, 33, 130, 183, and 73 was recorded.

3.4.2.3 Expression of SbCHS8

Expression of *SbCHS8* was evaluated in KOSA-1, KAT 412, KAT L5 and SDSH 513. The activity of this gene was also monitored over a period of 144 hours after infection with *C. sublineolum*. *SbCHS8* was expressed in both the non- and infected samples of all the seedlings analysed. Expression of *SbCHS8* was different to that *SbCHS2* but similar to *SbLRR*. The average change in the expression of SCHS8 was 46.1083 (19.992). Significant difference in the expression of *SbCHS8* was noted at 120-144 HPI ($P_{0.05}$ = 0.0001-0.016). The main difference in the expression of this gene was observed in SDSH 513, where it was markedly up-regulated (Figure 3.28). A comparison of the transgenic and Wt KAT 412 revealed that the changes in the expression of *SbCHS8* was higher in KOSA-1 than in Wt with an average FC of 20.489 and 16.293 in the former and the latter, respectively.

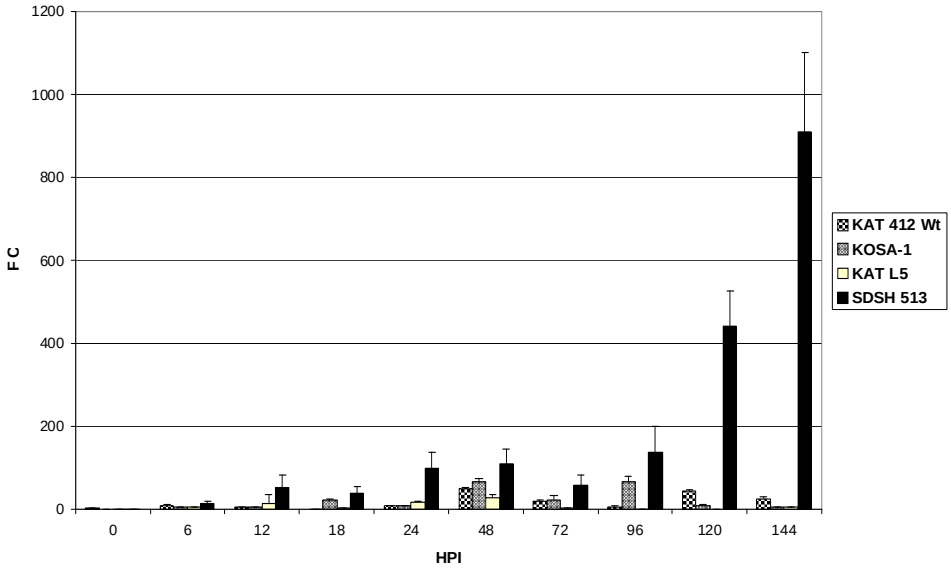


Figure 3.28: Expression of *SbCHS8* in KAT 412, KOSA-1 T_1, KAT L5 and SDSH 513 after infection with *C. sublineolum*. Transgenic and wild type sorghum lines were infected with *C. sublineolum* and thereafter the gene activity quantified for 144 hours. Expression of *SbCHS8* was higher in SDSH 513 than the other 3 cultivars. Activity was lowest in Kat L5 where an average FC of 7.373 was recorded.

Highest activity of *SbCHS8* was at 48 and 96 HPI in KAT 412 Wt and KOSA-1, respectively. A different trend was found in KAT L5 where the average activity of *SbCHS8* was 7.373. Expression of this gene was significantly up-regulated in SDSH 513, in which the average fold change in the activity was 185.728.

3.4.2.4 Expression of SbLRR

SbLRR was quantified in seedling leaves of the 4 cultivars infected with *C. sublineolum*. *SbLRR* was expressed in all the samples analysed. The average change in the expression of this gene was 44.402 (14.3903). Expression of *SbLRR* was highest in SDSH 513. The maximum change in expression of *SbLRR* in SDSH 513 was 483.932 (Figure 3.29). The least expression was in KAT L5 in which the maximum FC was 46.700. The average change in *SbLRR* expression in the 4 cultivars, in ascending order, was 11.390, 18.100, 28.530 and 163.254 in KAT L5, KOSA-1, KAT 412 Wt and SDSH 513, respectively.

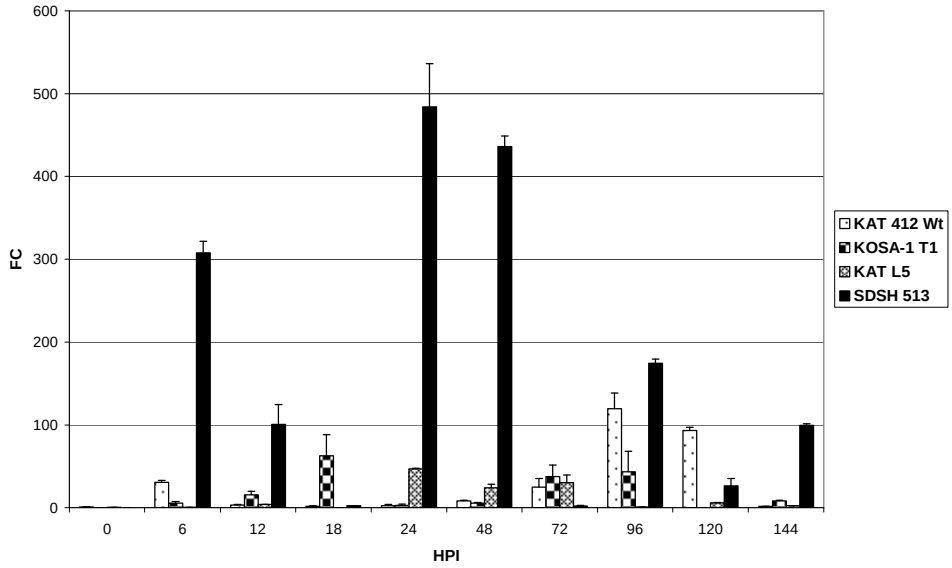


Figure 3.29: Expression *SbLRR* in Sorghum after infection with *C. sublineolum*. Transgenic and wild type sorghum lines were infected with *C. sublineolum* and thereafter the activity *SbLRR* quantified for 144 hours. The highest change in activity was noted in SDSH 513 where a maximum FC of 436 was reached at 48 HPI. KAT L5 showed the least activity of this gene with an average FC of 11.90. The transgenic and Wt sorghum showed an average change in activity of 18.1 and 28.53, respectively.

3.4.3 Comparison of *In Planta* and *Ex Planta* Gene Expression

A comparison was carried out on the activity of the genes discussed above in *ex planta* and *in planta* infected leaves. This comparison was carried out in KOSA-1, KAT 412 Wt, KAT L5, SDSH 513 and Serena. Leaf samples from both *in planta* and *ex planta* assays were taken at 48 HPI and analysed for changes in the expression of the target genes. All the genes

were expressed in the *ex planta* and *in planta* samples (Figure 3.30). Two general trends of responses were observed: higher genes activity in *ex planta* than in *in planta* samples of KOSA-1, KAT 412 Wt and KAT L5 and vice versa in SDSH 513. All the samples, except SDSH 513, showed higher changes in the expression of all the genes studied in *ex planta* than *in planta* samples.

Fold changes in the expression of the two transgenes, *HarCho* and *HarChit*, was quantified. Comparison of the changes in the expression of *HarCho* and *HarChit* in *ex planta* and *in planta* samples showed that there was no significant difference in the activity of the transgenes. The average change in expression of *HarCho* was 1.246 and 1.01, while that of *HarChit* was 1.381 and 1.021 in *in planta* and *ex planta* samples, respectively.

Analysis of KOSA-1 T_1 showed that the changes in the expression of *SbCHS2*, *SbCHS8*, *SbChit* and *SbLRR* was higher in the *ex planta* than in *planta* samples. The average fold change in expression of all these genes was 1.095 and 2.405 in *in planta* and *ex planta* samples respectively. The same trend was found in Wt KAT 412 where the average change in *in planta* and *ex planta* genes expression was 0.936 and 3.693, respectively. No significant difference was noted between the wild type and transgenic KAT 412. Of the 4 genes studied in both KOSA-1 and KAT 412 Wt, *SbCHS2* showed the highest activity, reaching a maximum FC of 10.133.

Analysis of KAT L5 revealed that there was consistent down-regulation of all the genes studied in the *in planta* as compared to *ex planta* samples. The average fold changes in the expression of all genes was 0.163 and 4.491 in *in planta* and *ex planta* samples, respectively. The highest gene activity was noted in *SbCHS2* whose FC was 6.089 in *ex planta* assay. The least active was *SbLRR* that had an FC of 0.072 in *in planta* samples. The trend observed in KAT412 Wt, KOSA-1 and KAT L5 was also seen in the activity of the innate PR-genes in Serena. The activities of these genes were lower in the *in planta* than in

ex planta samples reaching an average of 0.955 and 6.340 in the former and the latter assays, respectively. The main difference was noted in the expression of *SbLRR,* which was relatively lower in KOSA-1, KAT 412 Wt and KAT L5 but was up-regulated by an average of 12.871 FC in Serena.

The activity of the PR-genes was different in SDSH 513 in comparison to KOSA-1, KAT 412 Wt, KAT L5 and Serena. The activity of *SbCHS2*, *SbCHS8*, *SbChit* and *SbLRR* were higher in *in planta* than in *ex planta* infected samples. The average change in the expression of these genes was 4.919 and 0.819 in *in planta* and *ex planta* infected leaves, respectively. The gene showing the highest activity was *SbCHS2,* whose up-regulation reached an average of 15.325 in *in planta* samples.

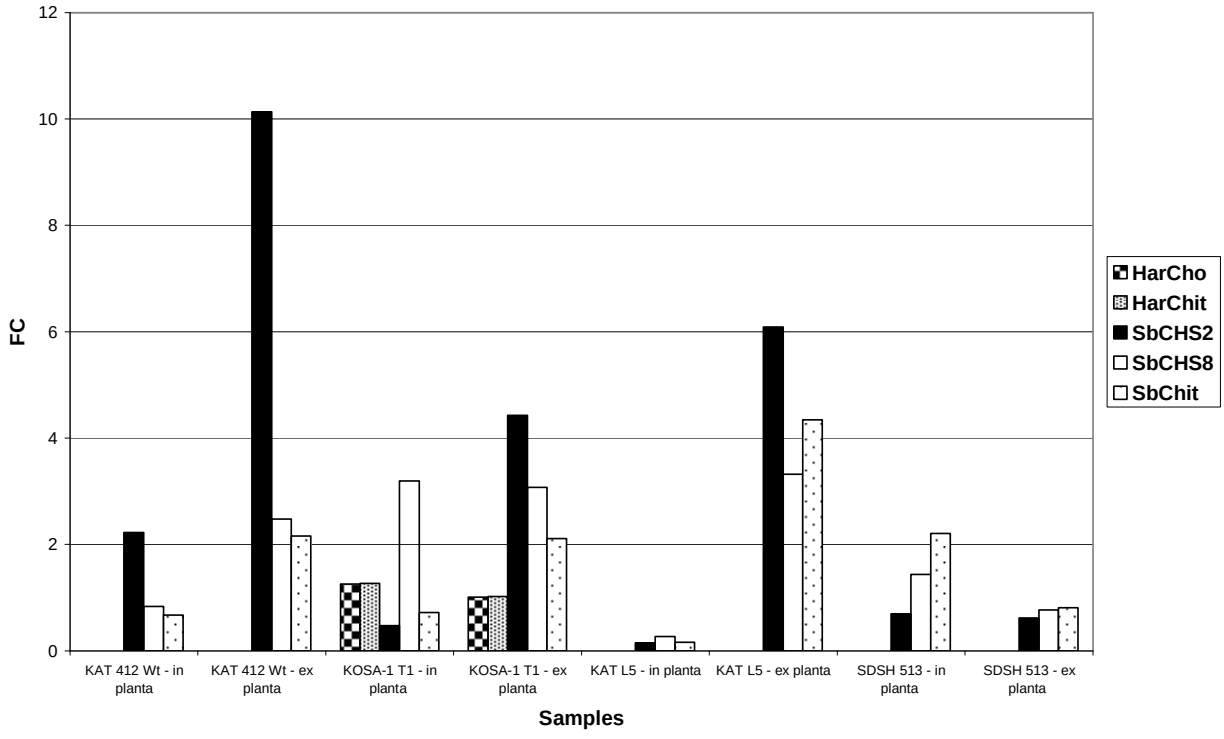


Figure 3.30: Comparison of *in planta* and *ex planta* genes expression assays. Intact (*in planta*) and excised (*ex planta*) sorghum leaves were infected with *C. sublineolum* and sampled 48 HPI for gene expression analysis. The *ex planta* infected leaves of all the cultivars except for SDSH 513 showed higher activity of all the innate sorghum genes than the *in planta* samples. High activity of *SbCHS2* and *SbLRR* was noted, reaching a FC of 10.133 and 15.325, respectively. The expression level of the transgenes *HarCho* and *HarChit* was the same in both assays.

4 DISCUSSION

The goal of this research was to genetically transform sorghum with antifungal genes in order to develop lines that are resistant to fungal diseases. Two antifungal genes, *HarChit* and *HarCho*, encoding antifungal enzymes, chitosanase and chitinase, were cloned from *Trichoderma harzianum* and used in the transformation. To achieve this goal, tissues culture methods were optimised for *in vitro* regeneration of selected sorghum cultivars from immature zygotic embryos (IZE). Particle bombardment and *Agrobacterium*-mediated transformation were used to transform sorghum IZE with the target genes. Transformation was carried out in 8 sorghum lines, from which KAT 412 was successfully transformed with the target genes, *HarChit, HarCho* and the selection marker *pat* gene encoding the herbicide-resistance conferring enzyme phosphinothricin acetyltransferase. Three independent sorghum transformants were generated and stable integration and inheritance of the anti-fungal genes were confirmed by molecular analysis. *Colletotrichum sublineolum* infection assays were carried out to test the response of transgenic line KOSA-1 to pathogen challenge. *In planta* and *ex planta* assays showed that transgenic progenies were more tolerant to anthracnose than the parent Wt. KOSA-1 T_1 was further compared to other sorghum cultivars sampled from Kenya. The comparison revealed the existence of sorghum genotypes that are either more tolerant or susceptible than the transgenic line. Quantitative RT-PCR analysis was done to profile the expression of the transgenes and selected PR-genes. The real time gene expression investigation confirmed the expression of the transgenes and demonstrated a genotype-dependent difference in the activity of PR-genes such as *SbLRR*, *SbCHS8* and *SbChit* in the susceptible and tolerant sorghum lines.

4.1 SOMATIC EMBRYOGENESIS AND MICROPARTICLE BOMBARDMENT

A collection of sorghum cultivars consisting of white, brown and red lines, were sampled from Kenya and used as explants for genetic transformation through microprojectile bombardment. A tissue culture protocol was optimised for successful regeneration of

plantlets from transformed embryonic tissues of IZE. This work achieved somatic embryogenesis of all the Kenyan sorghum cultivars sampled through tissue culture of IZE.

4.1.1 Genotypic Variation in Somatic Embryogenesis

Somatic embryogenesis was achieved with sorghum lines that secreted low (e.g., KAT 487, KAT 412) and high (e.g., Serena, KAT L5) amount of phenolics into the culture medium. A genotype-dependent effect on calli development and plantlets regeneration ability was observed. The cultivars that secreted high levels of phenolics developed less calli and regenerants. This was observed in the number of plantlets regenerated *in vitro* and transferred to the greenhouse. An average of 0.7% of 608 Serena IZE, a cultivar that secreted high levels of phenolics into culture media, developed regenerants that reached the greenhouse stage as compared to 3% in lower phenolics producing KAT 412. The phenolics production was also correlated to the seed colour. Sorghum lines with brown-red seeds secreted more phenolics than the white lines. This observation was further demonstrated by the lower number of regenerants achieved with other brown-red phenolics-rich cultivars such as KAT L5, GBK 046844 and SDSH 513. Such genotype influence on somatic embryogenesis has been reported. It has been observed that sorghum explants, especially from cultivars that are rich in phenolics, are recalcitrant to morphogenesis during tissues culture (Cai and Butler, 1990; Casas *et al*., 1993). Tissue culture of high phenolics producing sorghum is challenging and because such lines are less responsive to the standard media used in routine tissue culture of other grasses such as wheat, maize, and barley (Cai and Butler, 1990). Such genotypic variation in the response of immature embryos to tissue culture has been previously noted (Casas *et al*., 1993; Takashi, 2002). Nevertheless, successful regeneration was obtained in all the sorghum cultivars studied. Similar success is not routinely achieved in the tissue culture that involves a cross section of sorghum lines varying seed colour because some lines have low morphogenesis and rarely produce regenerants, even after months of tissue culture (Casas *et al*., 1993).

Attempts have been made to ameliorate the negative effect of phenolics secretion into culture media. Anti-oxidants and anti-phenolics compounds and formulations such as polivinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP), dithiothreitol, coconut water, silver nitrate and active carbon are been used in sorghum tissue culture with mixed results (Zhao *et al*., 2000; Takashi, 2002). Prolific tissue culture and regenerants formation was achieved in this study without these additives. This was done by focussing on the effect of pre-culture and timing of various tissue culture stages. One day pre-cultured embryos cultured for 14 days in CIM and thereafter in successive regeneration and rooting media produced good results.

The significance of the successful tissues culture of both phenolics-rich and white lines lies in the potential use of such an established and prolific tissue culture regime to tap into the genetic resource present in the Kenyan land races for further transformation work. This is important considering the fact that most of the high tannins and phenolics-rich brown-red sorghum lines are relatively tolerant to biotic and abiotic stress (Jambunathan, 1992; Esele *et al*., 1993; Andilakshmi *et al*., 1999; Waniska *et al*., 2001). These lines are more resistant to pest and diseases as compared to the white lines. On the flipside, copious secretion of phenolics into the culture media also presents a good opportunity for bioengineering of sorghum for *in vitro* biosynthesis and harvesting of economically important secondary metabolites.

4.1.2 Microprojectile Acceleration Pressure

It is important to optimise the microparticle bombardment parameters to minimise injuries to IZE since this is detrimental to their eventual morphogenesis in culture. To establish the most appropriate particle acceleration pressure, one-day pre-cultured IZE were bombarded with the *gus* gene at 1100 to1550 psi and cultured in CIM for 14 days. *Gus* expression was evaluated at day 2, 7 and 14. Expression of the *gus* gene revealed that differences exist between the bombardments pressures studied. However, this difference was not apparent

soon after bombardment but was clear by day 7. Bombardment at higher pressure led to low *gus* counts than at 1100 and 1350 psi. Higher levels of GUS expression, 58% of bombarded embryos, were recorded with 1350 psi as compared to 37% achieved with 1550 psi. Mortality of the bombarded embryos with higher pressure could contribute to the lower transient *gus* expression after bombardment with 1550 psi. Successful transformation through microparticle bombardment is hinged on the acceleration of desired DNA into cells without causing lethal damage. An intricate balance must be struck between the acceleration pressure and associated tissue injury to limit post-bombardment trauma. The negative effects of IZE injury have been noted, whereby injured IZE secreted copious amount of phenolics in culture media which in turn limited morphogenesis (Carvalho *et al*., 2004).

One of the treatments used to limit cell rupture during particle bombardment is osmotic treatment. Explants were pre-cultured in high sucrose (60 g/l) media for 4 hours before bombardment. This treatment reduces cell turgor pressure and tissues rupture at high pressure bombardment. It has also been shown that osmotic pre-treatment increases transient expression and enhances integration of transferred DNA (Vain *et al*., 1992).

Profiling of transient GUS activity in bombarded IZE established that there was no detectable gene expression at the end of calli induction phase, 14 days after bombardment. It is probable that the transgene expression at this point is only possible from genes that are stably integrated into the genome of the cultured tissues. Casas *et al*. (1993) also found that *gus* expression could not be detected in sorghum tissues later than 3 weeks after bombardment. This difference in the duration of expression could be attributed to the effect of genotype. Casas *et al*. (1993) showed that the greatest effect on reporter gene expression was attributed to variation amongst genotypes.

4.1.3 Effect of Pre-Culture

An evaluation of the effect of longer (3 days) pre-culture of sorghum IZE in improving tissue culture output established that this technique was not better than a shorter (overnight) period. Theoretically, longer pre-culture is expected to result into more cells available for transformation and further tissue culture. However, the limited calli development noted after longer pre-culture of IZE suggests a negative effect of bombardment on these tissues.

4.1.4 Putative Transformation

Immature zygotic embryos were isolated from a collection of sorghum cultivars and used in genetic transformation through particle bombardment. Transformation was attempted in 10,269 IZE, from which a number of putative transformants survived BASTA herbicide selection in regeneration medium (SEL-REM) and rooting medium (SEL-ROM) and reached the greenhouse. Of the 10,269 IZE bombarded, 168 (1.157%) survived tissue culture selection with 2 mg/l BASTA in SEL-REM and SEL-ROM and were transferred to the greenhouse. Of the 168 regenerants that expressed resistance to selection under tissue culture, 3 exhibited stable integration of the target genes. A high number of regenerants showed transient resistance under BASTA selection during tissue culture. This phenomenon of transient resistance was noted by Casas *et al*. (1993), who observed the survival of putative transformed calli under herbicide treatment on maintenance medium. However, these putative transformants died when transferred to BASTA-containing regeneration medium under alternating light/dark conditions. Exposure to light accelerates the development of phytotoxic activity of the herbicide and the mortality of the putative transformants could be attributed to this enhanced phytotoxicity (Casas *et al*., 1993). Similar observations were made in the transformation of pearl millet, another plant related to sorghum and belonging to the poaceae family (Girgi, 2003).

Death of regenerants that survived herbicide selection until the greenhouse stage has been ascribed to gene silencing. Gene silencing is considered to be an obstacle in the

transformation of sorghum (O´Kennedy, 2006). In a study using *gus* as a reporter gene, it was observed that GUS activity, which was high in transient assays, could not be detected in calli that had been maintained for prolonged periods on BASTA selection pressure despite of the fact that Southern blot analysis indicated the presence of the *uidA* gene. Lack of *gus* gene expression, in spite of its integration, pointed to gene silencing. It was concluded that DNA methylation occurred in sorghum cells that inactivated the expression of transferred genes (Casas *et al*., 1993). Emani *et al*. (2002) reported methylation-based transgene silencing of the reporter gene *uidA* and the herbicide resistance *bar* gene in transgenic sorghum. Girijashanakar *et al*. (2005) obtained low level of *cry1Ac* transgene expression but not complete silencing. Probable correlation exists between gene silencing and the promoter used. Zhu *et al*. (1998) observed that gene silencing was restricted to the *chitinase* gene driven by the *CaMV 35S* promoter, whereas the selectable marker gene *bar* driven by the *ubi1* promoter was fully expressed in all transgenic progeny of all transformation events. This phenomenon of gene silencing could also be responsible for the overall low transformation achieved in this investigation.

4.1.5 Stable Genetic Transformation

Of the 8 sorghum lines targeted for transformation, one line, KAT 412, was successfully transformed with *HarChit* and *HarCho* genes. Three BASTA herbicide resistant sorghum plants (KAT 412, KOSA-1, KOSA-2 and KOSA-3) were successfully developed after particle bombardment of IZE. Co-integration and expression of the two transgenes were confirmed by Southern blot and qRT-PCR analysis: This integration and expression of the two transgenes of agronomic importance into the genome of sorghum is an significant success in the development of disease tolerant plants. The production of transgenic sorghum plants via particle bombardment of IZEs was reported for the first time by Casas *et al*. (1993) and subsequently by Zhu *et al*. (1998), Able *et al.* (2001), Emani *et al*. (2002), Tadesse *et al.* (2000, 2003), Gao *et al*. (2005) and recently by Howe *et al*. (2006). Transformation of sorghum, like most other plants in the poaceae family, is a rare event. The transformation

frequencies reported in these successful efforts are generally lower than those achieved in other plants such as wheat, barley and tobacco.

Evaluation of transformation frequency belies the lack of standardized method of its calculation. In this investigation, 4 transformation frequencies could be computed: 12.5% (from the number of cultivars transformed), 0.029% (from the total number of IZE transformed), 0.087% (from the total number of KAT 412 IZE transformed) and 0.74% (from the 2 batches of IZE that produced the 3 transgenic plants). Nevertheless, the overall transformation frequency was low. Transformation frequency in microparticle bombardment transformation is notably low. Successful transformation of sorghum has been achieved with low frequencies: 0.008 – 0.33% (Casas *et al*., 1993; 1997), 1.3% (Tadesse *et al*., 2003) and 1.5% (Girijashankar *et al*., 2005). Higher transformation frequency has been achieved with *Agrobacterium*-mediated transformation. Frequencies of 2.1-2.5% were, for example, reported by Zhao *et al*. (2000) and Gao *et al*. (2005). The transformation frequency attained in this research was much lower than previously reported.

The low transformation frequency of sorghum may be attributed to various factors that affect the response of sorghum to morphogenesis as well as innate characteristics that make the transgene integration and transformants regeneration difficult. The possibility of inherent characteristics of sorghum scutellar cells that make them unresponsive to transformation was contemplated (Casas *et al*., 1993). The large amount of phenolics produced by sorghum cultures (Cai and Butler, 1990; Casas *et al.,* 1993) may also be responsible to the low transformation frequency. Studies have been done to optimise sorghum transformation parameters, such as media composition, use of anti-oxidants (e.g. polyvinylpyrrolidone), frequent sub-culture to avoid phenolics accumulation (Zhao *et al*., 2000), distance between the rupture disk and the target tissue in biolistic transformation, helium inlet aperture and pressure of helium gas (Able *et al*., 2001). However, these have not significantly improved the transformation frequency of sorghum to the level noted in other monocots like wheat and

barley or dicots such as tobacco. It was shown that osmotic pre-treatment increase transient expression and enhances integration of transferred DNA (Vain *et al*., 1992). Low transformation was noted in this research regardless of the osmotic pre-treatment.

The low transformation frequency confounds the theoretical basis of gene delivery and integration during tissue culture. It is postulated that DNA is delivered into the primary explant resulting in the transformation of the embryogenically competent cells in the scutellum prior to the initiation of embryogenesis. Regardless of whether the somatic embryos arise directly from competent cells or are derived from callus intermediary, DNA is delivered at a stage when one (or few) progenitor cell(s) of each organized structure exists (Casas *et al*., 1993). These cells are expected to undergo neoplastic multiplication into masses of cells that form the calli, undifferentiated clones of the original transformants. This should result in an appreciable high number of transgenic plants, an eventuality not achieved in sorghum transformation. Studies into the origin and location of transformable/competent IZE cells would be an important step in improving transformation frequency.

Transformation is known to be affected by the physiological condition of the explant. Biotic and abiotic stress affects the transformation success. Artificial growth conditions in greenhouse marked by low illumination affected the state and quality of the explant used for transformation and tissue culture. These factors have more adverse effects on tropical plants, such as sorghum when grown in a greenhouse in a temperate land with winter-summer season fluctuations. Short days, low illumination and overcast skies in winter were sources of abiotic stress to sorghum used in this investigation. Biotic stress, such as greenhouse diseases and pests, further compromised the explant quality.

4.1.6 Transgenes Integration and Progeny Segregation

Stable integration of the transgenes into the genome of the transformed KAT 412 was confirmed by Southern blot which showed multiple and single copy integration of *HarCho* and

HarChit, respectively. The segregation ratio of T_1 progeny was determined by selecting for the *bar* gene through spraying with the BASTA herbicide. Averages of 74.19% of the T_1 progenies from KOSA-1 were transgenic. The segregation conformed to the expected Mendelian inheritance. Single dominant locus integration of transgenes is expected to result in a 3:1 (75% dominant) segregation ratio. Similar segregation was previously proved by the analysis of the T_1 progeny which showed an average segregation ratio of 97/129, 145/182 and 52/69 of the seedlings survived after treatment with the herbicide (Casas *et al*., 1993). Microprojectile bombardment genes delivery and integration into plant genomes is essentially random (Hansen and Wright, 1999; Taylor and Fauquet, 2002), therefore the integrations observed in this investigation were also random events.

4.2 AGROBACTERIUM-MEDIATED TRANSFORMATION

Agrobacterium mediated transformation of sorghum was carried out using a binary system. This method is associated with higher transformation frequency and also less expensive than microparticle bombardment. Hence, a transformation protocol using the reporter gene *gus* was used to optimise *Agrobacterium* transformation protocol. A number of sorghum cultivars were used in the protocol optimization and transformation.

4.2.1 Transformation and Tissues Culture Conditions

Agrobacterium-mediated transformation involves a series of culture media for bacterial growth, inoculation, co-cultivation with IZE, selection, callus induction and regeneration of transformants. Optimization of the transformation stages is critical for the gene delivery and recovery of transformants. Preliminary *Agrobacterium* inoculation and co-cultivation showed that bacterial-induced mortality was a problem. Most IZE did not survive *Agrobacterium* inoculation and co-culture. Survival of IZE and subsequent callus development after their inoculation and co-cultivation with *Agrobacterium* are critical in transformation. The sensitivity of immature embryos to *Agrobacterium* infection is considered to be a limiting step

for high transformation efficiency (Zhao *et al*., 2000; Carvalho *et al*., 2004). Two ways were used to limit the IZE mortality: 1) use of *Agrobacterium* inoculums with low OD (usually below 0.2-0.8) and 2) removal excess *Agrobacterium* after infection by rinsing of IZE with inoculation medium without *Agrobacterium*. Removal of excess *Agrobacterium* have been found to significantly improve the survival rate of sorghum explant (Carvalho *et al*., 2004).

4.2.1.1 Effect Solid and Liquid Phase inoculation

To limit the *Agrobacterium*-induced mortality, two inoculation methods were tested: solid and liquid phase inoculation. The result showed that liquid phase infection was associated with higher calli formation (29.28%) than solid phase (16.13%). This observation could be explained by the microscopy investigations that were done to clarify the *Agrobacterium* induced mortality of IZE. Immature embryos observed by scanning electron microscope during co-cultivation were noted to be coated with a layer of *Agrobacterium* cells embedded and trapped in a crust of material probably produced by *Agrobacterium* (Carvalho *et al*., 2004). This crust could be considered to be involved in the *Agrobacterium* sensitivity of the sorghum embryos. The formation of such matrix and *Agrobacterium* mediated necrosis might have been more enhanced in solid phase than in liquid phase infection. Point inoculation with a toothpick or needle, made without injuring the embryos, showed that only those sectors that received *Agrobacterium* became brown. This indicated a localized necrotic effect.

4.2.1.1 Effect of Pre-culture

Sorghum IZEs have been noted to be sensitive to infection with *Agrobacterium* (Zhao *et al*., 2000). Pre-culture of IZE is regarded as another method that could limit this sensitivity. Sorghum IZE were pre-cultured for 1-3 days and thereafter infected and co-cultivated with *Agrobacterium.* It was found that pre-culture did not lead to better calli development. Previous observation indicated that pre-cultured IZE were less affected by *Agrobacterium*

infection (Zhao *et al*., 2000). Immature embryos cultured for 3-5 days before co-cultivation and callus which was 2-4 weeks old showed a much higher survival rate than fresh IZE (Carvalho *et al*., 2004). This finding was in contrast to the result of this investigation. However, these results should be considered in respect to the effect suspending calli-forming pre-culture IZE in liquid infection media. It is apparent that actively dividing and calli-forming cells may be more sensitive to the *Agrobacterium* inoculation and co-cultivation procedure that intact embryos.

4.2.1.2 Infection Duration

The duration of infection of IZE with *Agrobacterium* can be optimised to reduced post-infection necrosis. To determine the most conducive infection duration, IZE were incubated with *Agrobacterium* for 60-240 minutes. It was found that longer duration of infection did not negatively affect calli formation. Incubating the embryos for longer duration led to higher survival rate of IZE. It is probable that longer duration of incubation under shaking eliminated the formation of the adhesive matrixes over IZE. This demonstrated the importance of limiting the OD of the inoculums for better calli formation. It also augmented the importance of rinsing off of excess *Agrobacterium* from IZE for better calli formation.

4.2.1.3 Effect of Co-Cultivation Temperature

Sorghum IZE and *Agrobacterium* were co-cultivated at 21 and 26℃ to test the effect of temperature on GUS activity and callus formation. Co-culture at 21℃ resulted in more calli formation in the 3 sorghum lines studied. This result confirms the effect *Agrobacterium*-induced necrosis on IZE. Necrosis reduced post-infection survival IZE and calli formation. *Agrobacterium* growth was slower at 21℃ than at 26℃ resulting in lower mortality of IZE. This led to higher numbers of IZE that developed calli. This result is also important towards development of tissue culture protocols that could lead to higher transformation frequencies. The importance of temperature in *Agrobacterium* transformation could be further clarified by

the mechanism of bacterial T-DNA transfer into the plant. Bacterial mature T-DNA is exported into the host cell by VirB/D4 type IV secretion system (Veronst *et al*., 2000; Christie, 1997). VirB proteins either form the membrane channel or serve as ATPase to provide energy for export channel assembly and export process (Gelvin, 2003). Several proteins, including VirB2, VirB5 and VirB7 make up the T-pilus of the type IV secretion system (Sagulenko *et al*., 2001; Lai and Kado, 2000). VirB2, which is processed and cyclised, is the major pilin protein (Eisenbrandt *et al*., 1999; Jones *et al*., 1996; Lai and Kado 1998; Lai and Kado, 2000). Although *vir* genes induction is maximal at approximately 25 – 27℃ (Alt-Moebe *et al*., 1988; Jin *et al*., 1993; Turk *et al*., 1991), the pilus has been found to be more stable at lower temperature (approximately 18-20℃) (Fullner and Nester, 1996; Lai *et al*., 2000; Baron *et al*., 2001). Reviewing co-cultivation temperatures among other transformation parameters, could contribute to a higher transformation success.

4.2.1.4 Physiological and Physical State of Explant

Physiological state of the explants used in tissue culture has been reported to affect calli formation and even transformation frequency. Immature embryos isolated from donor plants growing under sub-optimal conditions, such as water stress and low temperature, did not show any apparent growth during co-cultivation and often died later (Carvalho *et al*., 2004). When cut or injured, IZE produced toxic compounds that inhibited *Agrobacterium* growth during co-cultivation (Carvalho *et al*., 2004). Some embryos also produced black pigments that inhibited growth of *Agrobacterium*. These effects may restrict transformation because of the limited interaction of the explant with *Agrobacterium*. The following key observations were made:

1) adaxially cultured embryos showed poor calli development and regeneration;
2) the potential of sorghum embryos to develop calli after co-cultivation is critical to the success of *Agrobacterium* mediated transformation;

3) up to 3 days of co-cultivation gave better calli development, longer days of co-cultivation were associated with *Agrobacterium* overgrowth and high embryos mortality.

4.2.2 Challenges in *Agrobacterium*-mediated Transformation of Sorghum

Sorghum genetic transformation with *Agrobacterium* remains a challenge. Source and physiological condition of explant, type of transformation vectors, tissue culture condition and innate plant protein (such as H2A, VIP 1, BTI, KU70 and Rad 52) have been cited to influence transformation (Mysore *et al*., 2000; Zhao *et al*., 2000; Carvalho, 2002; Tzfira *et al*., 2002; Van Attikum and Hooykaas, 2003; Hwang and Gelvin, 2004; Gao *et al*., 2005; Tzifira and Citovsky, 2006). It is postulated that *Agrobacterium* adopts cellular processed to transform its host (Tzfira and Citovsky, 2002; Gelvin, 2003). Various host proteins have been reported to participate in key stages of *Agrobacterium*-mediated process, i.e. T-DNA intracellular transport, nuclear import and integration (Tzfira and Citovsky, 2002; Gelvin, 2003). The difficulty in the transformation of sorghum may lie in the inability of *Agrobacterium* to adopt cellular responses critical for eventual T-DNA integration into the genome of this plant. Such cellular responses include DNA and protein transport, targeted proteolysis and DNA repair (Tzfira and Citovsky, 2002; Gelvin, 2003).

Agrobacterium transformation process begins with the bacterium-plant attachment. The two-component sensory signal antenna, which consists of VirA and VirG proteins, plays an important role in *Agrobacterium* transformation. This stage of transformation has been greatly improved by the use of acetosyringone, whose addition mimics wounding of the natural dicotyledonous host plants and induces autophosphorylation of *VirA* and ultimately expression *Vir* genes. Reports have showed that the effect of acetosyringone is concentration sensitive. Higher level has been found to be inhibitory. At the same time, effect of concentration was also found to be related to the tissue used as the explant. A working concentration of 100-300 µM has been found to be effective in most sorghum

transformation. A similar concentration range was used in this investigation. However, this did not lead to successful transformation, pointing towards a different causative mechanism or agent.

The export of T-DNA into the host cell is mediated by VirB/D4 type IV secretion system (T4SS) (Chritie, 2004). This step is known to require the interaction of the bacterial T-pilus with at least one-host specific protein (Hwang and Gelvin, 2004). Several proteins, including VirB2, VirB5 and VirB7 make up the T-pilus (Schmidt-Eisenlohr *et al.*, 1999). VirB2 which is processed and cyclised, is the major pilin protein. The pilus may function as a conduit or hook. Two critical factors that relate to pilus docking are relevant for the success of T-complex export into the host cell. These are culture temperature and phenolics secretion response of sorghum explant. Although *vir* gene induction is maximal at approximately 25-27℃ (Alt-Moebe *et al*., 1988; Turk *et al*., 1991; Jin *et al*., 1993), the pilus of some *Agrobacterium* strains is most stable at lower temperatures (approximately 18-21℃) (Fullner and Nester, 1996; Lai *et al*., 2000; Baron *et al*.,. 2001). To test the significance of temperature on transformation, IZE and *Agrobacterium* were co-cultivated at 21, 26, and 28℃. However, none of these temperature levels re sulted into stable transformation of the sorghum lines studied.

Sorghum IZE secrete copious amount of phenolics into culture media. It has been noted that red and brown sorghum lines, which are associated with higher amount of these compounds than white lines, respond poorly to morphogenesis and transformation (Zhao *et al*., 2000; Gao *et al*., 2005). It is therefore interesting to investigate the probable role of these compounds in the process of the *Agrobacterium* pilus docking, its initial interaction with plant cell membrane and eventual export of the T-complex into the sorghum IZE cells. The dense structure of the cytoplasm is another huddle that separates T-complex from its final destination, the nucleus. Cytoplasmic transit is another stage of the transformation process that may limit transformation. The dense structure of the cytoplasm composed of a mesh of

microtubules, actin and intermediate filament network, greatly restricts the Brownian diffusion of large macromolecules (Luby-Phelps, 2000). It is very likely that the T-complex, similar to many DNA viruses (Dohner and Sodeik, 2005), is delivered to the cell nucleus with the assistance of the host intracellular transport machinery (Tzfira and Citovsky, 2006). Biophysical particle tracking and fluorescently labelled VirE2-ssDNA complexes, suggests that dynein motors are required for the directed movement of the T-complex towards the nucleus (Salzman *et al*., 2005).

The large size (~15.7 nm outer diameter) of the mature T-complex (Abu-Arish *et al*., 2004) suggests an active mechanism for its nuclear import, most likely by the nuclear-import machinery of the host cell. It has been suggested that VirD2 and VirE2 interact with host proteins for their nuclear import (Tzfira and Citovsky, 2006). VirD2 interacts with AtKAPα, a member of the *Arabidopsis* karyopherin α-family (Ballas and Citovsky, 1997) while VirE2 interacts with the plant VirE2-interacting protein 1 (VIP1) (Tzfira *et al*., 2001) and its functional homolog, the bacterial VirE3 protein (Lacroix *et al*., 2005). Both act as molecular adaptors between VirE2 and the host cell karyopherin-α, enabling VirE2 to be ´piggy-backed´ into the host cell nucleus (Tzfira *et al.*, 2002; Lacroix *et al*., 2005). The ability of VIP1 to interact with the chromosomal protein H2A-1 histone, known to function during the T-DNA integration step, and its interaction with VirE2 suggests that *Agrobacterium* uses VIP1´s intranuclear mobility to deliver the T-complex to the point of integration in the host chromatin. This understanding of the mechanism of trans-cellular T-DNA transport and its integration opens the possibility of developing easily transformable sorghum lines by expression (preferably, transiently) of the genes known to be involved in transformation.

4.3 RESPONSE OF SORGHUM TO *C. SUBLINEOLUM INFECTION*

4.3.1 *Ex Planta* Infection Assay

In this study leaf segments of sorghum seedlings were used to test the response of sorghum lines to *Colletotrichum sublineolum* infection. *Ex planta* assays compared the response of transgenic KOSA-1, KAT 412 Wt, KAL L5, SDSH 513 and 4 other sorghum cultivars. Infection of leaf segments allows a fast and high throughput method for scoring the response of large numbers of lines under the same conditions. Similar methods were used for *in vitro* infection assays as described by Girgi *et al*. (2006) in pearl millet and by Oldach *et al*. (2001) in wheat. Leaf segments studies are made possible through the use of anti-senescence compounds such as benzimidazole. In this study, excised leaves were in 0.8% neutral agar containing 40 mg/l benzimidazole. It was therefore possible to carry out *ex planta* assay of the responses of excised leaf segments to *C. sublineolum* infection over a period of at least 7 days without undue senescence.

A comparison was made between the response of the transgenic KOSA-1 T_1 and wild type KAT 412 sorghum lines. Leaf segments from the transgenic KOSA-1 were found to be more tolerant to *C. sublineolum* and exhibited delayed and lower levels of necrosis and pigmentation after infection than those excised from the wild type plants. This assay showed that introduction of the two genes into sorghum caused a significant protection against *C. sublineolum* under the conditions of this investigation.

Further comparison was made between the transgenic and selected sorghum lines from Kenya. This study revealed the existence of genotypic variation in the response to *C. sublineolum* infection. Tolerant and susceptible lines were represented in the lines studied. KAT L5 and SDSH 513 were found to be the most tolerant and susceptible of all the lines tested. This demonstrated the existence of genetic diversity in Kenya that could be exploited for further transformation for anthracnose resistance.

Symptom development in the excised leaves after *C. sublineolum* infection varied. There was difference in the time taken for the onset of necrosis of the leaf segments among the tested lines. The localised distribution of the infection, especially in point inoculated samples was a demonstration of localised plant response to infection that is targeted at the point of pathogen entry. The lines that took longer to develop symptoms like KAT L5 could be considered to possess innate localised protection mechanisms that do not require systemic resistance machinery. However, the importance of systemic protection was shown by the development of symptoms in all the excised leaf segments in all the sorghum lines. All the excised leaves eventually developed symptom and were totally colonised with fungal fruiting structures by the end of the experiment. Cooperation of systemic and local defence machinery can function only in intact leaves and therefore all the excised leaves succumbed to anthracnose by the end of experiment (8 days).

4.3.2 *In planta* Infection Assay

Response to infection by intact sorghum was studied by infecting 1 week old seedlings and observing disease development over a period of 7 days. Sorghum seedlings were infected through spraying with 10^6 conidia/ml of *C. sublineolum.* Infected seedlings were grown in a chamber under controlled conditions and disease development quantified in terms of the number of seedlings showing symptoms (disease incidence), onset, severity and distribution of the symptoms. Studies with intact leaves and seedlings confirmed the observation made with leaf segments. KOSA-1 was found to be more tolerant than Wt seedlings, in which higher incidences of anthracnose was seen. Further examination revealed that seedlings from Wt did not show recovery after infection. The transgenic lines were however more resilient and showed recovery and the infected leaves continued to grow beyond the experimental period. This observation highlighted the importance of intact systemic defence machinery in disease response. In excised leaves, necrosis and death of entire segments was observed in both transgenic and wild type lines by the end of the 7-day experimental period.

The *in planta* infection study was extended to compare the transgenic with other Kenyan sorghum lines. Results from disease incidence analysis showed a largely similar trend with the *ex planta* experiment. KAT L5 and SDSH 513 were found to be the most and the least tolerant sorghum lines, respectively. There was however differences in the order of tolerance among the cultivars that fell between the SDSH 513-KAT L5 susceptible-tolerant extremes. The order of tolerance from the *ex planta* assays, from the least to most tolerant, was: SDSH 513, Serena, KAT 412 Wt, GBK 046820, KAT 412 Wt, GBK 046812, KAT 487 and KAT L5; while the order with *in planta* study was: SDSH 513, GBK 046820, Serena, GBK 046844, KAT 487, KAT 412 Wt, KAT 412 T_1 and KATL5. This difference may be an exemplification of the importance of systemic defence system.

Further experiments were done to profile the response of sorghum seedlings of KAT 412 Wt, KOSA-1 T_1 and the two lines that were most susceptible and tolerant, i.e., SDSH 513 and KAT L5. The results showed that symptoms appeared within 48 HPI in SDSH and KAT 412 Wt. By 72 HPI, all the sorghum lines showed some signs of the disease, in which approximately 5%, 30%, 45% and 90% of KAT L5, KAT 412 T_1, KAT 412 Wt and SDSH 513 were symptomatic. This confirmed the difference in response to anthracnose in the sorghum lines studied. It was also confirmed that the transgenic line was more tolerant than the wild type KAT 412.

The temporal development of symptoms noted above bears a correlation to similar studies previously done to profile accumulation of pathogenesis-related secondary metabolites. It has been shown that young sorghum leaves accumulate a complex of phenols in response to invasion by both pathogenic and non-pathogenic fungi, and the five major components of this complex are the 3-deoxyanthocyanidin flavonoids: apigeninidin, luteolinidin, arabinosyl-5-O-apigeninidin, 7-methylapigeninidin, and 5-methoxyluteolinidin (Nicholson *et al.*, 1988; Hipskind *et al.*, 1990; Lo *et al.*, 1996). All five of these compounds have exhibited fungitoxic

activity towards *C. sublineolum*, and are considered to be phytoalexins (Nicholson *et al.*, 1988). In leaf tissue, these phenolics first appear in the cell being invaded, accumulating in inclusions in the cytoplasm (Snyder & Nicholson, 1990; Snyder *et al.*, 1991). These cytoplasmic inclusions migrate to the site of penetration, become pigmented, lose their spherical shape and ultimately release their contents into the cytoplasm, killing the cell and restricting further pathogen development. Studies to document responses of sorghum leaves after infection with *C. sublineolum*, noted that by 24 HPI colourless inclusions accumulate in the cytoplasm of cells beneath fungal appresoria (Nicholson *et al*., 1988) and by 30 HPI inclusions have increased in size and become pigmented orange and start to coalesce and are intensely red pigmented by 36 HPI. The inclusions release their contents into the cell and pigmented inclusions start to accumulate in surrounding cells after 42 HPI. It was found that by 54 HPI the cells have released their contents into the cells and the cell walls are intensely red. These responses form part of the visible manifestation of the pathogen-plant interaction normally seen as rusty/red pigmentation on infected leaves.

Sorghum seedling leaves exhibit few, if any, symptoms when inoculated with various fungi. It has been reported that anthracnose resistant and susceptible cultivars are asymptomatic in the seedling stage and plants must be about 5 weeks old before they will exhibit symptoms in response to inoculation (Ferreira and Waren, 1982). The resistance has been attributed to dhurrin, sorgoleone and other secondary metabolites. It was however, possible to infect sorghum seedlings and induce pathogenesis through growth in high humidity and temperature for the first 48 HPI. These conditions were favourable to *C. sublineolum* conidia germination and infection.

4.4 REAL TIME EXPRESSION OF *PR-GENES*

Expression of the transgenes, the chitinase and chitosanase, and 4 sorghum innate genes representing the upstream and downstream pathogenesis response system were profiled by qRT-PCR after infection of seedlings with *C. sublineolum*. The four innate PR-genes

selected for analysis were sorghum leucine rich repeat (*SbLRR*), sorghum chalcone synthase-like gene 2 (*SbCHS2*), sorghum chalcone synthase-like gene 8 (*SbCHS8*) and sorghum chitinase (*SbChit*). Study of the real time expression of the PR-genes was divided into two parts: *in planta* and *ex planta* analysis. *In planta* analysis entailed expression studies in intact seedlings over a period of 144 hours after infection. Leaf samples were taken for analysis at specific intervals. *Ex planta* studies was carried out from samples of excised leaves 48 hours after infection. *In planta* expression of these genes was studied in the leaves of seedlings of one transgenic line, KOSA-1, KAT 412 Wt, KAT L5, and SDSH 513. One week old seedlings were infected with 1 x 10^6 conidia/ml of *C. sublineolum*. Triplicate random samples of the infected second leaf were taken at specific time interval for RNA extraction, first strand cDNA synthesis through reverse transcription and real time gene expression quantification in a qRT-PCR cycler.

4.4.1 Expression of Chitosanase and Chitinase

Transgenes *HarChit* and *HarCho* were quantified in KOSA-1 T_1 to ascertain the activity of these introduced genes after infection with the pathogen. Expression of *HarCho* and *HarChit* were confirmed in KOSA-1 T_0, and T_1. This is the first report of co-expression of two transgenes of agronomic relevance in sorghum. Co-expression of *HarChit* and *HarCho* were profiled in sorghum leaves over a period of 72 hours after infection with *C. sublineolum.* Expression of *HarChit* and *HarCho* oscillated between 0.5–2.5 FC and the average fold change of these genes were 0.95 and 1.03, respectively. Statistical analysis established that there was no significant difference in the expression of the two transgenes ($P_{0.05,\ 1}$ = 0.641). It was also established that there was no significant effect of time ($P_{0.05,\ 4}$ = 0.214) and gene x time ($P_{0.05,\ 4}$ = 0.660) interaction. Expression of both genes was under the control of the constitutively expressed *ubi*1 promoter from maize. Both genes were therefore expected to be constitutively expressed regardless of biotic or abiotic stress or time. The observed insignificant change in expression was a proof of the expected expression pattern.

4.4.2 Expression of Innate PR-Genes

The expression of *SbCHS2*, *SbChit, SbCHS8* and *SbLRR* was studied in *C. sublineolum* infected and healthy seedlings. Preliminary studies were done on the expression of *SbCHS2* in KAT 412 Wt and KOSA-1 T_1. *SbChit, SbCHS8* and *SbLRR* were quantified in KAT 412 Wt, KOSA-1 T_1 as well as in *C. sublineolum*-tolerant KAT L5 and susceptible SDSH 513. Evaluation of the expression of the innate genes after infection with *C. sublineolum* established that time and genotype had significant effect on the expression of *SbChit*, *SbCHS8* and *SbLRR* but not *SbCHS2*. The three genes were also differentially expressed. Newman-Keuls statistical test of variance showed that the changes in the expression of *SbCHS8* and *SbLRR* were not significantly different ($P_{0.05, 1} = 0.678$). Expression of *SbChit* was found to be significantly different from that of *SbCHS8* and *SbLRR* ($P_{0.05, 1} = 0.00012$ and 0.00014, respectively). Of the 4 genotypes studied, SDSH 513 was found to be significantly different from KOSA-1 T_1, KAT 412 and KAT L5. In the 0-144 HPI analysis window, most of the significant changes in genes expression occurred between 48–144 HPI.

4.4.3 Expression of *SbCHS2 in KOSA-1 and KAT 412 Wt*

A preliminary study was done to study the expression of *SbCHS2* in seedlings of KOSA-1 and KAT 412 Wt. Paired analysis of the infected and non infected samples showed that *SbCHS2* was expressed in both KOSA-1 T_1 and KAT 412 Wt during the 18-72 HPI under investigation. Statistical evaluation of the expression of *SbCHS2* showed that there was no significant difference in its expression in KOSA-1 and KAT 412 Wt ($P_{0.05, 1} = 0.532$). Time after infection with the pathogen was also found to have no significant effect in the expression of this gene ($P_{0.05, 5} = 0.207$). The insignificance in the change of expression of SCHS2 could be explained by role of this gene secondary metabolism and stress-associated response system.

Chalcone synthase (CHS) catalyzes the first committed step in flavonoid biosynthesis, in the same pathway that is involved in biosynthesis of phytoalexins and an array other flavonoid-

derived phytochemicals (Austin and Noel, 2003). The enzyme is the prototype of the plant type III polyketide synthase (PKS) family, including the closely related stilbene synthases (STSs), pyrone synthases, acridone synthases, valerophenone synthases, and benzalacetone synthases (Springob *et al*., 2003), giving rise to the diversity of type III PKS-derived phytochemicals throughout the plant kingdom (Austin and Noel, 2003). CHS has been described to consist of a family of 8 CHS genes, *SbCHS1* to *SbCHS8*, in sorghum (Lo *et al*., 2002). *SbCHS1* to *SbCHS7* (AF152548–AF152554) are highly conserved (at least 97.5% sequence identity at amino acid level) and closely related to the maize *C2* and *Whp* genes encoding CHS enzymes. Expression of *SbCHS2* like other members of this gene family (*SbCHS1 to SbCHS7)* has been suggested to be not specific to fungal infection (Lo *et al*., 2002). Growing seedlings have been noted to accumulate certain flavonoids for which the involvements and upregulation of *SbCHS2* is a prerequisite (Lo *et al*., 2002). The observed expression of *SbCHS2* could therefore be explained to be part of general cellular basal metabolism that are not directly influenced by biotic stress exerted by *C. sublineolum* infection. Previous studies have noted that this gene was not specifically expressed in response to pathogen infection (Lo *et al.,* 2002). This study confirmed this observation in KOSA-1 and Wt sorghum. This is a demonstration that the introduction of the transgenes did not have a negative effect on the expression of the polyketide family of genes.

4.4.4 Expression of *SbCHS8*

Expression of *SbCHS8* was evaluated in KOSA-1, KAT 412 Wt, KAT L5 and SDSH 513. *SbCHS8* was expressed in both the non- and infected seedlings of sorghum. Expression of *SbCHS8* was different to that *SbCHS2* but similar to *SbLRR*. The average change in the expression of SCHS8 was 46.1083 (19.992). A comparison of the transgenic and Wt KAT 412 revealed that the changes in the expression of *SbCHS8* in KOSA-1 and KAT 412 Wt were not significantly different. The average FC were 20.489 and 16.293 in the former and latter, respectively. Highest activity of *SbCHS8* was at 48 and 96 HPI in KAT 412 Wt and KOSA-1, respectively. A different trend was found in anthracnose tolerant KAT L5 where the

activity of this gene was lower with an average change in expression being 7.373. Much higher activity was recorded in SDSH 513. The activity of this gene was significantly up-regulated in the anthracnose susceptible SDSH 513, in which the average fold change in the activity was 185.728. The above results were different from the previous reports in 2 aspects. Previous reports contended that *SbCHS8* was only expressed after infection (Lo *et al*., 2002). The reports also postulated that higher expression of this gene is related to disease tolerance (Lo *et al*., 2002).

The expression of this gene was first noted in sorghum seedlings that infected with *C. sublineolum* (Lo *et al*., 2002). *SbCHS8* (AY069951) was isolated as one of the genes in the chalcone synthase gene family. It was found that it is only 81% to 82% identical to the other members of the family, viz., SbCHS1 to SbCHS7, at the amino acid level and appeared to be more distantly related as revealed by phylogenetic analysis (Lo *et al*., 2002). These findings suggested that *SbCHS8* was duplicated from the ancestral form of *SbCHS1* to *SbCHS7* and diverged in protein coding sequence. It was later demonstrated that *SbCHS8* encodes a stilbene synthase (STS) enzyme and gene expression was activated during host and non-host defence responses (Yu *et al*., 2005). The high and low activity of this gene in susceptible and tolerant sorghum cultivars, respectively, raises questions on its role in the defence response.

Similarity of response in Wt and T_1 established that the expression *SbCHS8* was not significantly affected by the introduction of the two transgenes in sorghum. Gene responses are multi-channelled and interlinked (Ryals, 1996; Cao *et al*., 1998). A deletion or addition of one gene product in a complex pathway such as in chalcone biosynthesis, may not affect the expression of other genes involved in the synthesis of other intermediates. Complex compensatory, alternative and feedback mechanisms exist that modulate the responses. Insignificant effect of the introduced *HarChit* and *HarCho* expression on the action of *SbCHS8* is a demonstration of such interplay in complex pathways regulation.

The different trend in the expression of *SbCHS8* observed in KAT L5 and SDSH 513 could be clarified by correlating its activity and the state of the leaf samples in which it was up-regulated. It was noted that *SbCHS8* was up-regulated in all leaf samples that exhibited disease symptoms. Statistical analysis of *SbCHS8* showed that most of the up-regulations occurred between 96-144 HPI when the seedlings were showing clear symptoms. In addition, leaves of SDSH 513 on the other hand, showed signs of infection as early as 48 HPI, which corresponded to a fold change of 108.080. It should be therefore concluded that up-regulation of *SbCHS8* in sorghum is associated with symptoms development. Asymptomatic stages of infection are associated with low activity of this gene. A previous report indicated that *SbCHS8* was preferentially up-regulated in sorghum lines that showed tolerance to anthracnose (Yu *et al*., 2005). *SbCHS8* was expressed in anthracnose tolerant KAT L5, but in its activity was much higher in susceptible SDSH 513.

It is also relevant to point out that *SbCHS8* was considered to be expressed exclusively upon *C. sublineolum* infection (Yu *et al*., 2005). This study, however, established that *SbCHS8* was also expressed in non-infected leaves. This critical observation could be interpreted from results obtained from other plant species where it was found that the expression of STS genes was induced by a variety of abiotic and biotic stresses, such as elicitor treatment, pathogen inoculation, wounding, UV irradiation, and post-harvest wilting procedures (Preisig-Müller *et al*., 1999; Versari *et al*., 2001). Constitutive expression of CHS8-related STS genes was described in young seedlings of grapes (Sparvoli *et al*., 1994). It was concluded that this presumably representing a pre-existing defence mechanism (Sparvoli *et al*., 1994). It was demonstrated in this study that constitutive expression of *SbCHS8* occurs in sorghum.

4.4.5 Endogenous Sorghum Chitinase Gene

Expression of endogenous sorghum *chitinase* gene, *SbChit* was evaluated in KOSA-1 T_1, Wt, KAT L5 and SDSH 513. *SbChit* was expressed in all infected and healthy seedlings.

The average fold change in the expression of this gene between 0-144 HPI was 15.891 (4,719). Significant difference was noted in the expression of *SbChit* between the 4 sorghum lines evaluated ($P_{0.05}$ = 0.0006-0.001). The highest expression of *SbChit* was noted in SDSH 513 while the least was in KAT L5 (Figure 3.27). The fold change in the expression of this gene was 209.42 in SDSH 513, while the least was 0.194 in KAT L5.

Involvement of sorghum innate chitinase in disease resistance has been studied in kernels (Castor, 1981; Seetharaman *et al*., 1996). Diseases resistance in sorghum involves several mechanisms that include hardness of the kernel, kernels with red pericarp, endosperm textures, high tannins, high concentration of anthocyanidins and plants with pericarp intensifier genes (Castor, 1981; Forbes *et al*., 1992; Waniska *et al*., 2001). It was found that antifungal proteins such as sormatin, chitinases, glucanases and ribosomal inhibiting protein (RIP) may play a role in grain mold resistance (Bueso *et al*., 2000; Waniska *et al*., 2001). Chitinase and sormatin contents in sorghum kernel increased from the period after anthesis to physiological maturity when some resistant sorghum lines were inoculated with a mixture of *Fusarium moniliforme* and *Curvularia lunata* (Bueso *et al*., 2000). These studies did not clearly demonstrate a strong association between resistance to grain mold and the accumulation of sormatin and chitinase. It was concluded that moderately resistant cultivars could employ other strategies to eschew or restrict fungal invasion either before or after physiological maturity (Prom *et al*., 2005). Rodriguez-Herrera *et al*. (1999) compared levels of 4 AFP using mold resistant and susceptible lines. Infection with grain mold resulted in the induction and/or retention of more AFP in the resistant lines suggesting that coexpression of AFP may be required to confer resistance (Rodriguez-Herrera *et al*., 1999). It was also found that chitinase, sormatin and RIP concentration were 1.5-14 fold higher in the resistant lines compared to susceptible lines and were associated with grain mold resistance (Rodriguez-Herrera *et al*., 1999). Further *in vitro* studies have established that plant chitinases readily attack and degrade chitin (Boller, 1985). A coordinated induction of expression of 3 chitinase isoforms was observed in maize seeds in response to infection by

the fungus *F. moniliforme* (Cordero *et al*., 1994). Form the above studies it is consistent to associate the expression of innate sorghum *chitinase* (*SbChit*) to disease response.

No significant difference was noted between the expression *SbChit* in Wt and T_1 samples. In contrast *SbChit* in KAT L5 showed a consistent low activity. However, it should be noted that was expressed in both healthy and infected KAT L5 leaves. This could serve as baseline deterrence to pathogens ingression and infection. It was also eminent that samples with clear signs of infection, contained higher levels of *SbChit* than control samples. This gene could therefore be considered to be intricately involved in disease response and its level could be a good indicator of the degree of tolerance to anthracnose.

The involvement of *chitinases* in plant defence against invading fungi was demonstrated in development of a transgenic sorghum carrying a gene coding for a rice chitinase (Zhu *et al*., 1998). It was found that the development of stalk rot symptoms was significantly reduced in transgenic sorghum plants with higher expression of rice chitinase, whereas plants with low or no expression of the transgene showed normal disease progression (Zhu *et al*., 1998). The baseline high expression of chitinase in the more tolerant KAT L5 could therefore be associated to the tolerance to anthracnose in this cultivar.

4.4.6 Sorghum Leucine-Rich Repeat

SbLRR was quantified in seedling leaves of the 4 cultivars infected with *C. sublineolum*. *SbLRR* was expressed in all the samples analysed. The average change in the expression of this gene was 44.402 (14.390). Expression of *SbLRR* was highest in SDSH 513. The maximum change in expression of *SbLRR* in SDSH 513 was 483.932. The least expression was in KAT L5 in which the maximum FC was 46.700. The average change in *SbLRR* expression in the 4 cultivars, in ascending order, was 11.390, 18.100, 28.530 and 163.254 in KAT L5, KOSA-1, KAT 412 Wt and SDSH 513, respectively.

SbLRR is involved in direct and indirect interaction with pathogen molecules during infection (Jia *et al*., 2000; Dangl and Jones, 2001). *SbLRR* is known to be involved in pathogen-signal transduction. Higher activity of this gene therefore is an indication of defence response system induction through its interaction with the pathogen. The low level of activity noted in KAT L5 is reminiscent of the limited activity of most of the genes studied in this cultivar. However, the low response in KAT L5 did not lead to a higher disease infestation. On the contrary, KAT L5 remained asymptomatic longer than all the sorghum lines studied. This observation points to another mechanism of pathology and response.

It has been elucidated that *Botrytis cinerea*, a necrotrophic fungus, caused disease in *Nicotiana benthamiana* through the activation of two plant signalling genes, *EDS1* and *SGT1*, which have been shown to be essential for resistance against biotrophic pathogens (Oirdi and Bouarab, 2007). Interestingly, virus-induced gene silencing of these two plant signalling components enhanced *N. benthamiana* resistance to *B. cinerea*. This mechanism, whereby pathogens counteract plant immune response of the host to infect and spread into surrounding tissues has also been seen in several pathogens (Boaurab *et al*., 2002; Abrahamovitch *et al*., 2003; 2006a; 2006b). Hypersentive reaction to pathogen ingression does not therefore necessarily lead to disease resistance. The low response and disease tolerance in KAT L5 could point to a different mechanism of tolerance that renders induction pathogenesis-related genes irrelevant. A number of mechanisms of tolerance are known in sorghum. The first line of defence is usually the cuticle. *C. sublineolum* is known to infect sorghum through stomata and directly through the cuticle. Plant mechanisms that make it difficult for *C. sublineolum* to attach onto and penetrate the plant cuticle offer a strategy of tolerance. *C. sublineolum* is known to exert extreme invasive pressure through the infection peg that makes infection upon attachment and germination of conidia a largely physical process. The force exerted by the infection peg was computed to amount to 16.8±3.2 µN (Bechinger *et al*., 1999). According to an analogy, a force of 17 µN exerted over the palm of a human hand would enable it to support an 8 tonne school bus or an adult killer whale

(Money, 1999). The magnitude of this force convinced researchers to analyse the nature of attachment of conidia onto leaf surfaces. This penetrative force of the infection peg would be little significance if the conidia are not firmly attached onto the leaf surface. It has been therefore postulated that conidia attachment may be a critical stage in the infection process. It is therefore plausible that KAT L5 posses leaf surface agents that make it difficult for the conidia of *C. sublineolum* to attach onto its leaves.

4.4.6 Comparison of *in planta* and *ex planta* Gene Expression

A comparison was carried out on the activity of the PR-genes in *ex planta* and *in planta* infected leaves of KOSA-1, KAT 412 Wt, KAT L5, SDSH 513 and Serena. Leaf samples from both *in planta* and *ex planta* assays were taken at 48 HPI and analysed for changes in the expression of the target genes. All the genes were expressed in the *ex planta* and *in planta* samples. All the samples, except SDSH 513, showed higher changes in the expression of all the genes studied in *ex planta* than *in planta* samples. Analysis of KOSA-1 T_1 showed that the changes in the expression of *SbCHS2*, *SbCHS8*, *SbChit* and *SbLRR* was higher in the *ex planta* than in *in planta* samples. The average expression of all these genes was 1.095 and 2.405 in *in planta* and *ex planta* samples, respectively. The same trend was found in Wt KAT 412 where the average change in *in planta* and *ex planta* genes expression was 0.936 and 3.693, respectively. No significant difference was noted between the wild type and transgenic KAT 412.

SbCHS2 showed higher activity than *SbCHS8*, *SbChit* and *SbLRR* in KOSA-1 and KAT 412 Wt, reaching a maximum FC of 10.133. There was consistent lower expression of all the PR-genes in KAT L5´s *in planta* as compared to *ex planta* samples. The average fold changes in the expression of all genes was 0.163 and 4.491 in *in planta* and *ex planta* samples, respectively. The least active was *SbLRR* that had an FC of 0.072 in KAT L5 in *planta* samples. The trend observed in KAT412 Wt, KOSA-1 and KAT L5 was also seen in the activity of the innate PR-genes in Serena. The activities of these genes were lower in the

in planta than in *ex planta* samples reaching an average of 0.955 and 6.340 in the former and the latter assays, respectively. The activity of the PR-genes was different in SDSH 513 in comparison to KOSA-1, KAT 412 Wt, KAT L5 and Serena. The activity of *SbCHS2*, *SbCHS8*, *SbChit* and *SbLRR* were higher in *in planta* than in *ex planta* infected samples.

These results could be interpreted in terms of the visually observed response of the *ex planta* and *in planta* samples. *Ex planta* samples showed clear symptoms consisting of necrotic local lesions. As noted previously in this discussion, development of symptoms and leaf pigmentation after infection with *C. sublineolum* is correlated to accumulation of pathogenesis-related secondary metabolites. It has been shown that young sorghum leaves accumulate a complex of phenols in response to invasion by both pathogenic and non-pathogenic fungi, and the five major components of this complex are the 3-deoxyanthocyanidin flavonoids: apigeninidin, luteolinidin, arabinosyl-5-O-apigeninidin, 7-methylapigeninidin, and 5-methoxyluteolinidin (Nicholson *et al.*, 1988; Hipskind *et al.*, 1990; Lo *et al.*, 1996). The higher fold changes in the expression of *SbCHS8*, *SbLRR* and *SbChit* in *ex planta* than *in planta* samples is correlated to the more symptoms development in the former. Infection and complete colonization of *ex planta* excised leaf samples by *C. sublineolum* occurred by 144 HPI. Associated higher activity of *SbCHS8*, *SbLRR* and *SbChit* in KOSA-1, KAT 412 and KAT T5 is a proof that these genes are involved in pathogenesis response. The low activity of these genes in *ex planta* samples of SDSH 513, a cultivar which was also found to be susceptible, demonstrates the intricate relationship in the expression of these genes,

5 CONCLUSION AND OUTLOOK

The goal of this research was to transform sorghum with two antifungal genes, *HarChit* and *HarCho,* in order to develop transgenic plants resistant to fungal diseases. This research produced 3 independent, transgenic sorghum lines co-expressing the two target genes, through particle bombardment. The transformation frequency achieved in this study was 0.087%. This augmented the challenges in sorghum transformation and the necessity for further work on the optimization of the transformation process and investigations into the molecular and cellular basis of non-homologous transformation and transgenes integration.

Transient *gus* expression in sorghum calli was achieved with *Agrobacterium*-mediated transformation. The difficulty in achieving stable transformation with *Agrobacterium* indicates that there is still work to be done for efficient modification of sorghum for successful genetic engineering. Optimization of parameters that are considered to be crucial for transformation, such as the screening of highly regenerative genotypes and the development of an efficient plant tissue culture system should be pursued. Research into plant proteins involved in T-complex plant docking, cytoplasmic transport, nuclear pore import and genome integration will definitely open new possibilities of how to make *Agrobacterium*-mediated transformation widely applicable in grasses such as sorghum.

Ex and *in planta Colletotrichum sublineolum* assays demonstrated the tolerance of transgenic plants towards fungal infection compared to wild type plants. Transgenic sorghum developed infection symptoms later than the wild type lines. Transgenic lines also showed resilience and recovery after infection. Comparison of *ex planta* and *in planta* infection assays revealed the importance of intact tissues in disease response. This demonstrated the importance of innate systemic response to *C. sublineolum* tolerance. Tolerant and susceptible lines were found in the collection of sorghum sampled from Kenya. The transgenic sorghum was found to be less tolerant to *C. sublineolum* than KAT L5, which was found to be the most resistant of the lines studied. This demonstrated the existence of a

genetic diversity among the Kenyan land races and cultivars that could be used for further transformation work for disease resistance.

The transgenes *HarChit* and *HarCho* were constitutively expressed in healthy and infected transgenic plants. Genotypic variation existed in the expression of parthenogenesis-related genes: *SbChit*, *SbCHS8* and *SbLRR*. This research established that *SbChit, SbSTS* and *SbLRR* could be used as indicators of *C. sublineolum* tolerance. This investigation also showed that *SbCHS8* was constitutively expressed in sorghum and its up-regulation is an indication of the severity of infection rather than a response *per se*. It was also demonstrated that genetic transformation of sorghum did not result in significant changes in the expression of the PR-genes in the transgenic lines. The PR genes could therefore be used as RNA-based marker system for tolerance of sorghum to fungal diseases.

REFERENCES

Able, J.A., Rathus, C., Godwin, I.D., (2001). The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. *In vitro* Cellular Development Biology—Plant 37, 341–348.

Albright, L. M., E. Huala, and F. M. Ausubel. (1989). Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu. Rev. Genet. 23:311–336.

Ali, M. E. K., and Warren, H. L., (1987). Physiological Races of *Colletotrichum graminicola* on Sorghum. *Plant Disease* 71:402-404.

Ali, M. E. K., and Warren, H. L., (1992). Anthracnose of Sorghum. In: de Millano W. A. J., Frederiksen, R. A., and Bengston, G. D., (eds), *Sorghum and Millet Diseases: A Second World Review*. ICRISAT, Patancheru, AP 502 324, India, pp. 203-208.

Awika JM, Rooney LW, Waniska RD (2004). Properties of 3-deoxyanthocyanins from sorghum. J Agric Food Chem. 52(14):4388-94.

Battraw M and Hall TC, (1991). Stable transformation of *Sorghum bicolor* protoplasts with chimeric neomycin phosphotransferase II and β-glucuronidase genes. *Theor. Appl. Genet.* 82:161–168.

Barker, R. F., K. B. Idler, D. V. Thompson, and J. D. Kemp. (1983). Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti-plasmid pTi15955. Plant Mol. Biol. 2:335–350.

Bartnicki-Garcia S (1968). Cell wall chemistry, morphogenesis and taxonomy of fungi. Annu Rev Microbiol 22:87-109.

Bailey, J. A., O'Connell, R. J., Pring, R. J., and Nash, C., (1992). Infection strategies of *Colletotrichum* species. In: Bailey, J. A., and Jeger, M. J., eds., *Colletotrichum: Biology, Pathology and Control*. C. A. B. International, Wallingford, pp. 88 - 120.

Bedell JA, et al. (2005) Sorghum genome sequencing by methylation filtration. *PLoS Biol* 3, 313.

Bechinger, C., Giebel, K.-F., Shnell, M. and Deising, H.B. and Bastmeyer, M. (1999) Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. Science, 285:1896–1899.

Bent, A. F. (1996). Plant disease resistance genes: function meets structure. Plant Cell 8:1757-1771.

Blaiseau PL, Lafay JF (1992) Primary structure of a chitinase-encoding gene (*chi1*) from the filamentous fungus *Aphanocladium album*: similarity to bacterial chitinases. Gene 120:243–248.

Bonnen, A. M., and Hammerschmidt, R., (1989). Role of cutinolytic enzymes in infection of cucumber by *Colletotrichum lagenarium*. *Physiological and Molecular Plant Pathology* 35:475 – 481.

Boyes, C.J., Vasil, I.K. (1984). Plant regeneration by somatic embryogenesis from cultured young inflorescences of *Sorghum arundinaceum* (Desv.) Stapf. var. sudanense (susangrass). Plant Science Letters 35, 153–157.

Brar, D.S., Rambold, S., Gamborg, O., Constabel, F. (1979). Tissue culture of corn and sorghum. Zeitschrift fur Pflanzenphysiologie 95:377–388.

Brettschneider R., Becker D., Lorz H. (1997). Efficient transformation of scutellar tissue of immature maize embryos. Theor. Appl. Genet. 1997;94:737-748.

Burkhard P, Stetefeld J, Strelkov SV. (2001). Coiled coils: a highly versatile protein folding motif. Trends Cell Biol. 11(2):82-88.

Bueso, F. J.; Waniska, R. D.; Rooney, W. L.; Bejosano, F. (2000). Activity of antifungal proteins against mold in sorghum caryopses in the field. *J. Agric. Food Chem. 4:* 810-816.

Byrne, M. C., J. Koplow, C. David, J. Tempe, and M.-D. Chilton. (1983). Structure of T-DNA in roots transformed by *Agrobacterium rhizogenes.* J. Mol. Appl. Genet. 2:201–209.

Cabib E (1987). The synthesis and degradation of chitin. In: Meisler A (ed) Advances in enzymology and related areas of molecular biology. Wiley, New York, pp 59-101.

Cai, T., Butler, L. (1990). Plant regeneration from embryogenic callus initiated from immature inflorescences of several high-tannin sorghums. Plant Cell Tissue Organ Culture 20, 101–110.

Cai, T., Daly, B., Butler, L. (1987). Callus induction and plant regeneration from shoot portions of mature embryos of high-tannin sorghum. Plant Cell Tissue Culture 9, 245.

Casas, A.M., Kononowicz, A.K., Zehr, U.B., Tomes, D.T., Axtell, J.D., Butler, L.G., Bressan, R.A., Hasegawa, P.M. (1993). Transgenic sorghum plants via microprojectile bombardment. Proceedings of the National Academy of Sciences USA 90, 11212–11216.

Casas, A.M., Kononowicz, A.K., Haan, T.G., Zhang, L., Tomes, D.T., Bressan, R.A., Hasegawa, P.M. (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. *In vitro* Cell Development Biology—Plant 33:92–100.

Celenza JL (2001). Metabolism of tyrosine and tryptophan - new genes for old pathways. Curr. Opin. Plant Biol. 4:234-240.

Chen AC, Mayer RT, De Loach JR (1982). Purification and characterization of chitinase from the stable fly, *Stomoxys calcitrans*. Arch Biochem Biophys 216:314-321.

Cheng, M., Fry, J.E., Pang, S., Zhou, H., Hironaka, C.M., Duncan, D.R., Conner, T.W., Wan, Y. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiology 115:971–980.

Cherif M and Benhamou N (1990). Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporium* f. sp. r*adicislycopersici*. Phytopathology 80:1406-1414.

Chet I, Schichler H, Haran S, Appenheim AB (1993). Cloned chitinase and their role in biological control of plant pathogenic fungi. Int Sym Chitin Enzymol, Senigallia (italy), pp 47-48.

Chiu WI, Niwa Y, Zeng W, Hirano T, Kobayashi H and Sheen J (1996). Engineered GFP as a vital reporter for plants. *Curr. Biol.* 6:325–330.

Cohen-Kupiec R, Broglie KE, Friesem D, Broglie RM, Chet I. (1999). Molecular characterization of a novel ß-1,3-exoglucanase related to mycoparasitism of *Trichoderma harzianum.* Gene 226:147–154.

Cordero MJ, Raventós D, San Segundo B (1994). Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound-response of a monocot gene. Plant J. 6(2):141-50.

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993). Plant Chitinases. Plant J 3:31-40.

Christie, P.J. (2004) Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. Biochim. Biophys. Acta 1694:219–234.

Christensen A.H., R.A. Sharrock and P.H. Quail, Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation, *Plant Mol Biol* **18** (1992), pp. 675–689.

Dana De las Mercedes, M, Limón MC, Mejías R, Mach RL, Benítez T, Pintor-Toro JA, Kubicek CP. (2001). Regulation of chitinase 33 (*chit33*) gene expression in *Trichoderma harzianum.* Curr Genet 38:335–342

Dangl JL, Jones JD. Nature (2001). Plant pathogens and integrated defence responses to infection. Nature 411(6839):826-33.

Darnetty, Leslie, J.F., Muthukrishnan, S., Swegle, M., Vigers, A., Selitrennikoff, C.P. (1993) Variability in antifungal proteins in the grains of maize, sorghum and wheat. Physiologia Plantarum 88: 339-349.

Davis SJ and Vierstra RD, (1998). Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol. Biol.* 36:521–528.

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21.

Deu M, Gonzalez-de-Leon D, Glaszmann J.C., Degremont I, Chantereau J., Lanaud C., Hamon R (1994). RFLP diversity in cultivated sorghum in relation to racial differentiation. Theoretical and Applied Genetics. 88:838-844.

De Vos, G., M. DeBeuckeleer, M. Van Montagu, and J. Schell (1981). Restriction endonuclease mapping of the octopine tumor-inducing plasmid pTiAch5 of *Agrobacterium tumefaciens* — addendum. Plasmid 6:249–253.

Donzelli BGG, Lorito M, Scala F, Harman GE. (2001). Cloning, sequence and structure of a gene encoding an antifungal glucan 1,3-ß-glucosidase from *Trichoderma atroviride* (*T. harzianum*). Gene 277:199–208.

Doty, S. L., M. C. Yu, J. I. Lundin, J. D. Heath, and E. W. Nester (1996). Mutational analysis of the input domain of the VirA protein of *Agrobacterium tumefaciens.* J. Bacteriol. 178:961–970.

Dunstan, D.I., Short, K.C., Thomas, E. (1978). The anatomy of secondary morphogenesis in cultured scutellum tissue of *Sorghum bicolor*. Protoplasma 97:251–260.

Dunstan, D.I., Short, K.C., Dhaliwal, H., Thomas, E. (1979). Further studies on plantlet production from cultured tissues of *Sorghum bicolor*. Protoplasma 101, 355–361.

Elliott AR, Campbell JA, Dugdale B, Brettell RIS and Grof CPL, (1999). Green-fluorescent protein facilitates rapid *in vivo* detection of genetically transformed plant cells. *Plant Cell Rep.* 18:707–714.

Ellis J, Jones D (1998). Structure and function of proteins controlling strain-specific pathogen resistance in plants. Curr Opin Plant Biol. 1(4):288-93.

Ellis J, Dodds P, Pryor T (2000). Structure, function and evolution of plant disease resistance genes. Curr Opin Plant Biol. 3(4):278-84.

Emani, C., Sunilkumar, G., Rathore, K.S., 2002. Transgene silencing and reactivation in sorghum. Plant Science 162:181–192.

FAO 2008. Sorghum FAO Statistics – 2006 Production. http://faostat.fao.org.

Ferreira, A. S., and Warren, H. L., 1982. Resistance of sorghum to *Colletotrichum graminicola*. *Plant Disease* 66:773-775.

Filichkin SA, Gelvin SB. (1993). Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1,D2 endonuclease. Mol Microbiol. 8(5):915–926.

Fry, W. E., and Evans, P. H. (1977). Association of formamide hydrolase with fungal pathogenicity to cyanogenic plants. *Phytopathology* 67:1001-1006.

Fry, W. E., and Munch, D. C., (1975). Hydrogen cyanide detoxification by *Gloeocercospora sorghi*. *Physiological Plant Pathology* 7:23-33.

Garfinkel, D. J., and E. W. Nester. (1980). *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. 144:732–743.

Gamborg, O.L., Shyluk, J.P., Brar, D.S., Constabel, F., (1977). Morphogenesis and plant regeneration from callus of immature embryos of sorghum. Plant Science Letters 10:67–74.

Gao, Z., Jayaraj, J., Muthukrishnan, S., Claflin, L., Liang, G.H. (2005). Efficient genetic transformation of sorghum using a visual screening marker. Genome 48:321–333.

Gelvin, S.B. (2000). Agrobacterium and plant genes involved in T-DNA transfer and integration. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:223–256.

Gelvin, S.B. (2003). *Agrobacterium*-Mediated Plant Transformation: the Biology behind the “Gene-Jockeying” Tool. Microbiology and Molecular Biology Reviews. 16–37.

Gerard, J.-C., J. Canaday, E. Szegedi, H. de la Salle, and L. Otten (1992). Physical map of the vitopine Ti plasmid pTiS4. Plasmid 28:146–156.

Girgi, M., O’Kennedy, M.M., Morgenstern, A., Smith, G., Lörz, H., Oldach, K.H., (2002). Transgenic and herbicide resistant pearl millet (*Pennisetum glaucum* L.) R.Br. via microprojectile bombardment of scutellar tissue. Molecular Breeding 10:243–252.

Girgi, M., Breese, W.A., Lörz, H., Oldach, K.H. (2006). Rust and downy mildew resistance in pearl millet (Pennisetum glaucum) mediated by heterologous expression of the afp gene from *Aspergillus giganteus*. Transgenic Research 15:313–324.

Girijashankar, V., Sharma, H.C., Sharma, K.K., Swathisree, V., Prasad, L.S., Bhat, B.V., Royer, M., Secundo, B.S., Narasu,M.L., Altosaar, I., Seetharama, N. (2005). Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*). Plant Cell Reports 24:513–522.

Godwin ID (2005) Sorghum genetic engineering: Current status and prospectus. Pages 1-8 *in* Sorghum tissue culture and transformation. N. Seetharama and Ian Godwin, eds. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi.

Godwin ID and Chikwamba R, (1994). Transgenic grain sorghum (*Sorghum bicolor*) plants via *Agrobacterium*. In: *Improvement of cereal quality by genetic engineering*, (eds RJ Henry and JA Ronalds), Plenum Press, New York, NY.

Hamer, J. E., Howard, R. J., Chumley, F. G., and Valent, B., (1988). A mechanism for surface attachment in spores of a plant pathogenic fungus. *Science* 239:288-290.

Heath, M. C. (1981). Non-host resistance. In: Staples, R. C., and Toenniessen, G. H., eds., *Plant Disease Control: Resistance and Susceptibility.* John Wiley and Sons, New York, pp. 201 - 217.

Hiei, Y., Ohta, S., Komari, T., Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. The Plant Journal 6:271–282.

Hammond-Kosack, K.E. and Jones, J.D.G. (1997) Plant disease resistance genes. Annual Review of Plant Physiology and Plant Molecular Biology 48:573-605.

Haran S, Schickler H, Oppenheim A and Chet I (1995). New components of the chitinolytic system of *Trichoderma harzianum*. Mycol Res 99:441-446.

Harris, H. B., and Cunfer, B. M. (1976). Observations on sorghum anthracnose in Georgia. *Sorghum Newsletter* 19:100-101.

Heath MC. (2000). Hypersensitive response-related death. Plant Mol Biol. 44(3):321-34.

Hendrix B. and Stewart J Mc D. (2002). Chitosanse may enhance anti-fungal defence response. AAES Research Series 507:2154-217.

Heim R and Tsien RY, (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescent resonance energy transfer. *Curr. Biol.* 6:178–182.

Hipskind, J. D., Hanau, R., Leite, B., and Nicholson, R. L. (1990). Phytoalexin accumulation in sorghum: Identification of an apigeninidin acyl ester. *Physiological and Molecular Plant Pathology* 36:381-396.

Howe A, Sato S, Dweikat I, Fromm M, Clemente T (2006). Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum. Plant Cell Rep 25:784–791.

Huang, S., Adams, W.R., Zhou, Q., Malloy, K.P., Voyles, D.A., Anthony, J., Kriz, A.L., Luethy, M.H. (2004). Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. Journal of Agricultural and Food Chemistry 52:1958–1964.

Hooykaas, P. J. J., M. Hofker, H. den Dulk-Ras, and R. A. Schilperoort (1984). A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid 11:195–205.

Horsch, R. B., H. J. Klee, S. Stachel, S. C. Winans, E. W. Nester, S. G. Rogers, and R. T. Fraley (1986). Analysis of *Agrobacterium tumefaciens* virulence mutants in leaf disks. Proc. Natl. Acad. Sci. USA 83:2571–2575.

Hu W and Cheng CL, (1995). Expression of Aequorea green fluorescent protein in plant cells. *FEBS Lett.* 369:331–334.

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (2008). An Endowment Fund for Sorghum and Pearl Millet Research Enhanced nutritional security, biodiversity and environmental sustainability. www.icrisat.org/Investors/Flyers/59_Endowment%20fund_D_scr.pdf.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T., Kumashiro, T., (1996). High efficiency transformation of maize (Zea mays L.) mediated *Agrobacterium tumefaciens*. Nature Biotechnology 6, 745–750.

Jefferson RA, Kavanagh TA and Bevan MW (1987). Gus fusion:β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901–3907.

Jeoung JM, Krishnaveni S, Muthukrishnan S, Trick HN, Liang GH. (2002). Optimization of sorghum transformation parameters using genes for green fluorescent protein and beta-glucuronidase as visual markers. Hereditas 137(1):20-8.

Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B(2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. 9(15):4004-4014.

Jones JD, Dangl JL (2006). The plant immune system. Nature. 444(7117):323-329.

Jones JDG, Grady K, Suslow T, Bedbrook J (1986) Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J 5:467–473.

KARI (Kenya Agricultural Research Institute) (1996). *Annual report.* KARI, Nairobi, Kenya.

Kempin, S. A., S. J. Liljegren, L. M. Block, S. D. Roundsley, M. F. Yanofsky, and E. Lam (1997). Targeted disruption in *Arabidopsis.* Nature 389:802–803.

Kobe B, Kajava AV. (2001). The leucine-rich repeat as a protein recognition motif. Curr Opin Struct. 11(6):725-32.

Kopp EB, Medzhitov R. (1999). The Toll-receptor family and control of innate immunity. Curr Opin Immunol. (1):13-8.

Krishnaveni, S., Jeoung, J.M., Muthukrishnan, S., Liang, G.H. (2001). Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. Journal of Genetics and Breeding 55:151–158.

Kubicek CP, Penttilä ME. (1998). Regulation of production of plant polysaccharide degrading enzymes by *Trichoderma.* In: Harman GE, Kubicek CP, eds. *Trichoderma* and *Gliocladium,* vol 2: enzymes, biological control and commercial application. London: Taylor & Francis. p 49–72.

Kuranda MJ and Robin PW (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J Biol Chem 266:19758-19767.

Lacroix Benoit , Tzvi Tzfira, Alexander Vainstein and Vitaly Citovsky (2006). A case of promiscuity: *Agrobacterium's* endless hunt for new partners 1 TRENDS in Genetics Vol.22 No.1.

Lee S, Choi H, Suh S, *et al*. (1999). Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. Plant Physiology 121:147-152.

Lemmers, M., M. DeBeuckeleer, M. Holsters, P. Zambryski, A. Depicker, J. P. Hernalsteens, M. Van Montagu, and J. Schell (1980). Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumours. J. Mol. Biol. 144:353–376.

Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C. (1996). Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr Biol. 6(4):427-37.

Linthorst, H. J. M. (1991). Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci. 10*:123-150.

Lo, S.-C., Weiergang, T., Bonham, C., Hipskind, J., Wood, K., and Nicholson, R. L., (1996). Phytoalexin accumulation in sorghum: Identification of a methyl ether of luteolinidin. *Physiological and Molecular Plant Pathology* 49:21-31.

Lo S-CC, de Verdier K, Nicholson RL (1999). Accumulation of 3-deoxyanthocyanidin phytoalexins and resistance to *Colletotrichum sublineolum* in sorghum. Physiol Mol Plant Pathol 55:263–273.

Lo C, Coolbaugh RC, Nicholson RL (2002). Molecular characterization and in silico expression analysis of a chalcone synthase gene family in sorghum. Physiol Mol Plant Pathol 61: 179–188.

Masteller, V.J., Holden, D.J., (1970). The growth of and organ formation from callus tissue of sorghum. Plant Physiology 45:362–364.

Lorito M, Peterbauer C, Hayes CK, Harman GE (1994). Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology.140 (3):623-9.

Lorito M, Farkas V, Rebuffat S, Bodo B, Kubicek CP (1996). Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. J Bacteriol. 178(21):6382-6385.

Lorito M, Woo SL, Garcia I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci U S A. 95(14):7860-7865.

Lundquist, R. C., T. J. Close, and C. I. Kado (1984). Genetic complementation of *Agrobacterium tumefaciens* Ti plasmid mutants in the virulence region. Mol. Gen. Genet. 193:1–7.

Mercer, P. C., Wood, R. K. S., and Greenwood, A. D. (1975). Ultrasrtucture of parasitism of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. *Physiological Plant Pathology* 5:203-214.

Mercure, E. W., Kunoh, H., and Nicholson, R. L. (1995). Visualization of materials released from adhered, ungerminated conidia of *Colletotrichum graminicola*. *Physiological and Molecular Plant Pathology* 46:121-135.

Money, N.P. (1999a) Fungus punches its way in. Nature, 401:332–333.

Money, N.P. (1999b) To perforate a leaf of grass. Fungal Genet. Biol. 28:146–147.

Morel JB, Dangl JL (1999). Suppressors of the arabidopsis lsd5 cell death mutation identify genes involved in regulating disease resistance responses. Genetics 151(1):305-319.

Mysore, K. S., J. Nam, and S. B. Gelvin (2000). An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. Proc. Natl. Acad. Sci. USA 97:948–953.

Nicholson, R. L., Jamil, F., Snyder, B. A., Lue, W. L., and Hipskind, J. (1988). Phytoalexin synthesis in the juvenile sorghum leaf. *Physiological and Molecular Plant Pathology* 33:271-278.

Nicholson, R. L., and Epstein, L. (1991). Adhesion of fungi to the plant surface. Prerequisite for pathogenisis. In: Cole, G. T., and Hoch, H. C., eds., *The fungal spore and disease initiation in plants and animals.* Plenum Press, New York.

Oldach, K.H., Morgenstern, A., Rother, S., Girgi, M., O'Kennedy, M.M., Lo¨ rz, H. (2001). Efficient *in vitro* plant regeneration from immature zygotic embryos of pearl millet [*Pennisetum glaucum* (L.) R. Br.] and *Sorghum bicolor* (L.) Moench. Plant Cell Reports 20:416–421.

Pang AZ, DeBoer DL, Wan Y, Ye G, Layton JG, Neher MK, Armstrong CL, Fry JE, Hinchee MA and Fromm ME, (1996). An improved green fluorescent protein gene as a marker in plants. *Plant Physiol.* 112:893–900.

Pastor-Corrales, M. A., and Frederiksen, R. A. (1980). Sorghum anthracnose. Pages 289-294 in: Sorghum Diseases: A World Review. Proc. Int. Workshop Sorghum Dis. R. J. Williams, R. A. Frederiksen, and L. K. Mughogho, eds. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India.

Papavizas CG (1985). Trichoderma and Gliocladium: biology, ecology and potential for biocontrol. Annu Rev Phytopathol 23:23-54.

Pedersen, J.F., Marx, D.B., Funnell, D.L. (2003). Use of A3 cytoplasm to reduce risk of gene flow through sorghum pollen. Crop Science 43:1506–1509.

Rasmussen, J. B., and Hanau, R. M. (1989). Exogenous scytalone restores appressorial melanization and pathogenicity in albino mutants of *Colletotrichum graminicola*. *Canadian Journal of Plant Pathology* 11:349-352.

Reddy, B.V.S., Ramesh, S., Longvah, T. (2005). Prospects of breeding for micronutrients and b-carotene-dense sorghum. International Sorghum and Millets Newsletter 46:11–14.

Repellin A, Baga M, Jauhar P and Chibbar RN, (2001). Genetic enrichment of cereal crops via alien gene transfer: new challenges. *Plant Cell Org. Cul.* 64: 159–183.

Ridout CJ, Coley Smith JR, Lynch JM (1986). Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizoctonia solani*. J Gen Microbiol 132:2345-2352.

Rodriguez-Herrera, R. (1999). Grain mold resistance in sorghum (*Sorghum bicolor* (L) Moench): Genetic, physical and biochemical analysis. Ph.D. Dissertation, Texas A&M, University, College Station, TX, 1999; 143 pp.

Rodriguez-Herrera, R.; Waniska, R. D.; Rooney, L. W. Antifungal proteins and grain mold resistance in Seetharaman, K.; Waniska, R. D.; Rooney, L. W. (1996). Physiological changes in sorghum antifungal proteins. *J. Agric. Food Chem. 44*,2435-2441.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2nd edn, Cold Spring Habour Laboratory, Cold Spring Harbor, New York.

Salzman RA, Brady JA, Finlayson SA, Buchanan CD, Summer EJ, Sun F, Klein PE, Klein RR, Pratt LH, Cordonnier-Pratt MM, Mullet JE (2005). Transcriptional profiling of sorghum induced by methyl jasmonate, salicylic acid, and aminocyclopropane carboxylic acid reveals cooperative regulation and novel gene responses. Plant Physiol.138(1):352-68.

Saskia C. M. van Wees, Erik A. M. de Swart, Johan A. van Pelt, Leendert C. van Loon, and Corné M. J. Pieterse (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana.* PNAS July 18, 2000 vol. 97 no. 15 8711-8716

Sela-Buurlage, M., Epstein, L., and Rodriguez, R. J. (1991). Adhesion of ungerminated *Colletotrichum musae* conidia. *Physiological and Molecular Plant Pathology* 39:345-352.

Schirmböck M, M Lorito, Y L Wang, C K Hayes, I Arisan-Atac, F Scala, G E Harman and C P Kubicek (1994). Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of Trichoderma harzianum against phytopathogenic fungi. Appl Environ Microbiol. 60(12):4364-4370.

Smith, R.H., Bhaskaran, S., Schertz, K., (1983). Sorghum plant regeneration from aluminium selection medium. Plant Cell Reports 2:129–132.

Snyder, B. A., and Nicholson, R. L. (1990). Synthesis of phytoalexins in sorghum as a site specific response to fungal ingress. *Science* 248:1637-1639.

Snyder, B. A., Leite, B., Hipskind, J., Butler, L. G., and Nicholson, R. L. (1991). Accumulation of sorghum phytoalexins induced by *Colletotrichum graminicola* at the infection site. *Physiological and Molecular Plant Pathology* 39:463-470.

Srinivasula SM, Ahmad M, MacFarlane M, Luo Z, Huang Z, Fernandes-Alnemri T, Alnemri ES (1998). Generation of constitutively active recombinant caspases-3 and -6 by rearrangement of their subunits. J Biol Chem. 273(17):10107-11.

Steyaert, J. M., Stewart, A., Jaspers, M. V., Carpenter, M., Ridgway, H. J. (2004). Co-expression of two genes, a chitinase (chit42) and proteinase (prb1), implicated in mycoparasitism by *Trichoderma hamatum*. *Mycologia* 96:1245-1252.

Stachel, S. E., E. W. Nester, and P. C. Zambryski (1986). A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc. Natl. Acad. Sci. USA 83:379–383.

Stam, M., R. de Bruijn, S. Kenter, R. A. L. van der Hoorn, R. van Blokland, J. N. M. Mol, and J. M. Kooter (1997). Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. Plant J. 12:63–82.

Strauch E, Wohlleben W, Pühler A (1988) Cloning of a phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene 63: 65-74

Sundheim L (1992). Effect of chitinase-encoding genes in biocontrol of *Pseudomonas* spp. In James EC, Papavizas GC, Cook RJ (eds) Biological control of plant diseases. NATO ASI series, 230. Plenum Press, New York London, pp 331-332.

Sutton, B. C. (1980). *The Coelomycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata.* Commonwealth Mycological Institute, Kew, London, 696 pp.

Suzuki, K., Y. Hattori, M. Uraji, N. Ohta, K. Iwata, K. Murata, A. Kato, and K. Yoshida (2000). Complete nucleotide sequence of a plant tumorinducing Ti plasmid. Gene 242:331–336.

Stephanie Lüticke (2006). Personal Communication.

Tadesse, Y.S., (2000). Genetic transformation of sorghum (*Sorghum bicolo*r (L.) Moench) towards improving nutritional quality. Ph.D. thesis. Vrije Universiteit Brussel Instituut Voor Moleculaire Biologie, Faculteit Wetenschappen, Belgium.

Tadesse, Y., Sgi, L., Swennen, R., Jacobs, M. (2003). Optimisation of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) via microparticle bombardment. Plant Cell, Tissue and Organ Culture 75, 1–18.

Tarr S. A. J. (1962) *Diseases of Sorghum, Sudan Grass and Broom Corn.* The Commonwealth Mycological Institute, Kew, Surrey, 298 pp.

Thomas CM, Jones DA, Parniske M, Harrison K, Balint-Kurti PJ, Hatzixanthis K, Jones JD.(1997). Characterization of the tomato Cf-4 gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. Plant Cell (12):2209-24.

Thomas, M.D., I. Sissoko and M. Sacko (*1996)*. Development of leaf anthracnose and its. effect on yield and grain weight of sorghum in West Africa. Plant Dis 89:151-153.

Thomson JA (2008). The role of biotechnology for agricultural sustainability in Africa. Philos Trans R Soc Lond B Biol Sci. 363(1492):905-13.

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S., Brettell, R. (1997). *Agrobacterium tumefaciens*-mediated barley transformation. The Plant Journal 11:1369–1376.

Turk, S. C. H. J., R. P. van Lange, T. J. G. Regensburg-Tuink, and P. J. J. Hooykaas (1994). Localization of the VirA domain involved in acetosyringone- mediated vir gene induction in *Agrobacterium tumefaciens.* Plant Mol. Biol. 25:899–907.

Tzfira, T. and Citovsky, V. (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol. 12:121–129.

Tzfira, T., M. Vaidya, and V. Citovsky (2002). Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. Proc. Natl. Acad. Sci. USA 99:10435–10440.

UN (2007). World population will likely increase by 2.5 billion by 2050. Press Release POP/952 13 March 2007, *World Population Prospects: The 2006 Revision, Highlights* (United Nations, February 2005), www.unpopulation.org.

UN (2008). 25th Regional Conference for Africa. 19 June 2008 Nairobi/Rome.

Unger, L., S. F. Ziegler, G. A. Huffman, V. C. Knauf, R. Peet, L. W. Moore, M. P. Gordon, and E. W. Nester (1985). New class of limited-host-range- *Agrobacterium* mega-tumor-inducing plasmid lacking homology to the transferred DNA of a wide-host-range, tumor-inducing plasmid. J. Bacteriol. 164:723–730.

Vander P, V rum KM, Domard A, Eddine El Gueddari N, Moerschbacher BM. (1998). Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. Plant Physiol. 118(4):1353-9.

Vergunst, A. C., B. Schrammeijer, A. den Dulk-Ras, C. M. T. de Vlaam, T. J. G. Regensburg-Tuink, and P. J. J. Hooykaas (2000). VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. Science 290:979–982.

Waniska RD, Venkatesha RT, Chandrashekar A, Krishnaveni S, Bejosano FP, Jeoung J, Jayaraj J, Muthukrishnan S, Liang GH. (2001). Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold. J Agric Food Chem. 49(10):4732-42.

Ward, E. R., and W. M. Barnes (1988). VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5´ end of T-strand DNA. Science 242:927–930.

Watanabe T, Oyanagi W, Suzuki H, Tanaka H (1990). Gene cloning of chitinse AI from *Bacilllus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type-III homology units of fibronectin J Biol Chem 265:15659-15665.

Watanabe T, Oyanagi W, Suzuki H, Tanaka H (1992). Structure of the gene encoding chitinase-D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class-III plant chitinases. J Bacteriol 174:408-414.

Wernicke, W., Brettell, R. (1980). Somatic embryogenesis from *Sorghum bicolor* leaves. Nature 287, 138–139.
Winans, S. C. 1991. An *Agrobacterium* two-component regulatory system for the detection of chemicals released from plant wounds. Mol. Microbiol. 5:2345–2350.

Wood, D. W., J. C. Setubal, R. Kaul, D. E. Monks, J. P. Kitajima, V. K. Okura, Y. Zhou, L. Chen, G. E. Wood, N. F. Almeida, L. Woo, Y. Chen, I. T. Paulsen, J. A. Eisen, P. D. Karp, D. Bovee, P. Chapman, J. Clendenning, G. Deatherage, W. Gillet, C. Grant, T. Kutyavin, R. Levy, M.-J. Li, E. McClelland, A. Palmieri, C. Raymond, G. Rouse, C. Saenphimmachak, Z. Wu, P. Romero, D. Gordon, S. Zhang, H. Yoo, Y. Tao, P. Biddle, M. Jung, W. Krespan, M. Perry, B. Gordon-Kamm, L. Liao, S. Kim, C. Hendrick, Z.-Y. Zhao, M. Dolan, F. Chumley, S. V. Tingey, J.-F. Tomb, M. P. Gordon, M. V. Olson, and E. W. Nester (2001). The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science 294:2317–2323.

Yanai K, Takaya N, Kojima N, Horiuchi H, Ohta A, Takagi M (1992). Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. J Bacteriol 174: 7398-7406.

Young , D. H., and Kauss, H. (1984). Adhesion of *Colletotrichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and to polystyrene. *Applied Environmental Microbiology* 47:616-619.

Yu TT, Skinner DZ, Trick HN, Huang B, Muthukrishnan S and Liang GH (2001). *Agrobacterium*-mediated transformation of creeping bentgrass using GFP as a reporter gene. *Hereditas* 133:229–233.

Yu, Christine K.Y., Karin Springob, Jürgen Schmidt, Ralph L. Nicholson, Ivan K. Chu, Wing Kin Yip and Clive Lo (2005). Stilbene Synthase Gene (*SbSTS1*) Is Involved in Host and Nonhost Defense Responses in Sorghum. *Plant Physiology* 138:393-401.

Zambryski, P., M. Holsters, K. Kruger, A. Depicker, J. Schell, M. Van Montagu, and H. M. Goodman (1980). Tumor DNA structure in plant cells transformed by *A. tumefaciens.* Science 209:1385–1391.

Zambryski, P., P. H. Joos, C. Genetello, J. Leemans, M. Van Montagu, and J. Schell (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. 2:2143–2150.

Zhao, Z.-U., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., Pierce, D. (2000). *Agrobacterium*-mediated sorghum transformation. Plant Molecular Biology 44:789–798.

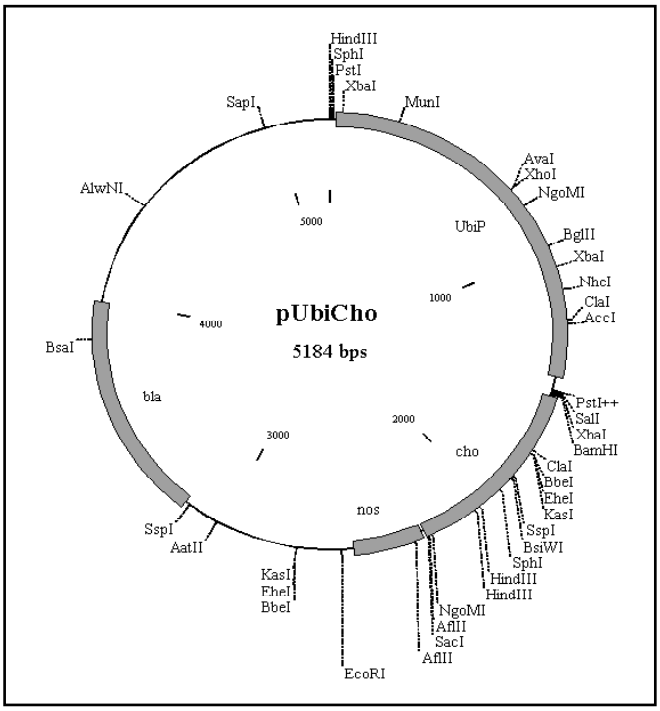
Zhong, H., Wang, W., Sticklen, M. (1998). *In vitro* morphogenesis of *Sorghum bicolor* (L.) Moench: efficient plant regeneration from shoot apices. Journal of Plant Physiology 153:719–726.

Zhu, H., Muthukrishnan, S., Krishnaveni, S., Wilde, G., Jeoung, J.M., Liang, G.H. (1998). Bioilistic transformation of sorghum using a rice chitinase gene. Journal of Genetics and Breeding 52:243–252.

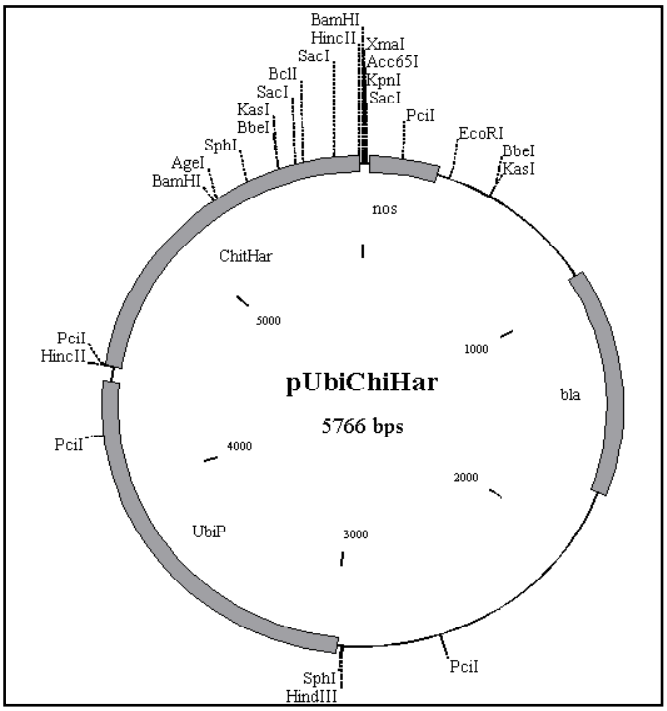
Zhu, X., Galili, G. (2003). Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also transregulates the metabolism of other amino acids in Arabidopsis seeds. Plant Cell 15:845–853.

APPENDICES

Appendix 1.1 pUbiHarChit



Appendix 1.2 pUbiCho



Appendix 1.3 Biolistic Transformation Tissue Culture Media

	Callus induction medium (CIM)	**Regeneration medium (REM)**	**Rooting medium (ROM)**
Macroelements (mg/l)			
NH_4NO_3	200	200	100
KNO_3	1750	1750	875
KH_2PO_4	200	200	100
$CaCl_2.2H_2O$	350	350	175
$MgSO_4$	450	450	225
Na_2SO_4	37	37	18.5
$FeSO_4.7H_2O$	28	28	14
Microelements (mg/g)			
H_3BO_3	5	5	2.5
$MnSO_4.\ 4H_2O$	15	15	7.5
$ZnSO_4\ .4H_2O$	7.5	7.5	3.75
KI	0.75	0.75	0.375
$NaMoO_4\ .2H_2O$	0.25	0.25	0.125
$CuSO_4\ .5H_2O$	0.025	0.025	0.0125
$CoCl_2\ .6H_2O$	0.025	0.025	0.025
Vitamins (mg/l)			
Thiamin HCl	10	10	5
Nicotinic acid	1	1	0.5
Pyridoxin acid	1	1	0.5
D-Calcium Pentothenate	0.05	0.5	0.25
Folic acid	0.2	0.2	0.1
p-Aminobenzoic acid	1	1	0.5
Biotin	0.005	0.005	0.0025
Cholinchloride	0.5	0.5	0.25
Riboflavin	0.1	0.1	0.05
Ascobic acid	1	1	0.5
Myo-inositol	100	100	50
Amino acids mg/l)			
L-Glutamin	750	750	375
L-Prolin	150	150	75
L-Asparagin	100	100	50
Sucrose (g/l)	**30**	**30**	**1515**
BAP (mg/l)	**0.1**	**1**	**0**
2,4-D (mg/l)	**2.5**	**0**	**0**

Appendix 1.4 Agrobacterium Transformation Media

		A	B	C	D
Inoculation Medium	MS Salts	MS Salts - 4.3g/l	1/10 MS	MS Salts	MS Salts
	N6 Vitamins	0	N6 Vitamins	0	N6 Vitamins
	Nicotinic acid	0.5mg	0	0	0
	Pyridoxine-HCl	0.5mg	0	0	0
	Thiamine-HCl	1.0mg	0	1mg	0
	Myo Inositol	0.1g	0	250mg	0
	Vitamin Assay Casamino Acids	1.0g	0	1g	0
	Sucrose	68.5g	30g	0	68.4g
	Maltose	0	0	30g	0
	Glucose	36.0g	10g	0	36.0g
	L-Prolin	0	0	680mg	0.7g
	Glycin	0	0	0	2mg
	3 AA	0	0	25ml (40x 3 AA)	0
	Ascobic Acid	0	0	50mg	0
	L-Cystein	0	0	200mg	0
	MES	0	0.25g	3.9g (10mM)	0
	Pluronic F-68	0	0.03%	0	0
	2,4-D	0	0.002g	200µM	0
	Silwet L-77	0	0	0.01%	0
	Acetosyringone	100µM	0	200µM	100µl
	pH	pH 5.2	pH 5.6	pH 5.4	pH 5.2
		Zhao *et al* 2000	Cavalho *et al* 2002	S. Lütticke 2004	Bronwyn 2006
Co-Cultivation Medium	MS Salts	MS Salts	MS Salts	MS Salts	N6 Salts
	N6 Vitamins	0	N6 Vitamins	0	N6 Vitamins
	Nicotinic acid	0.5mg	0	0	0
	Pyridoxine-HCl	0.5mg	0	0	0
	Thiamine-HCl	1.0mg	0	1mg	0
	Myo Inositol	0.1mg	0	250mg	0
	Vitamin Assay Casamino Acids	1.5mg	0	1g	0
	Sucrose	20.0g	30g	0	30g
	Maltose	0	0	30g	0
	Glucose	10.0g	10g	0	0
	L-Prolin	700mg	0	690mg	700mg
	Glycin	0	0	0	0
	3 AA	0	0	25ml	0
	Ascobic Acid	10.0mg	0	50mg	0
	L-Cystein	0	0	200mg	300mg
	Silver Nitrate	0	0	0	0.85mg
	MES	0.5g	0.25g	3.9g	0
	Pluronic F-68	0	0	0	0
	Coconut Water	0	10%	0	0
	PVPP	0	0.5 or 1%	0	0
	DTT	0	0.2%	0	0
	Kinetin	0	0	0	0
	2,4-D	2.0mg	2.0mg	1.25mg	1.5mg
	Acetosyringone	100µM	200µM	200µM	100µM
	pH	pH 5.8	pH 5.6	pH 5.4	pH 5.8

Appendix 1.5 Preparations of Gold Suspension and DNA Coating

(a) Gold Suspension

(i) Wash 40mg gold in 1ml absolute ethanol 3 times by vortexing

(ii) Wash gold in sterile ddH20 3 time by vortexing

(iii) Re-suspend gold in 1ml sterile ddH20

(iv) Ultrasonicate for 20 sec.

(v) Freeze in 50µl aliquots (approx. 2mg/50µl) in 2ml Eppendorf tubes.

(vi) Sonicate before use

(b) DNA Coating

(i) Add 5-6µl of µg/µl plasmid DNA into the 50µl gold suspension and vortex.

(ii) In Eppendorf lid, add 50µl 2.5M CaCl2 and 20µl 0.1M spermidin.

(iii) Close lid and vortex immediately and set on ice for 5sec and centrifuge for 30sec.

(iv) Pipette-off the supernatant and re-suspend in 250µl absolute ethanol.

(v) Centrifuge for 5sec and pipette-off the supernatant.

(vi) Re-suspend DNA-coated gold in 240 µl absolute ethanol.

(vii) Load 3.5-10µl on macrocarrier for particle bombardment.

Appendix 1.6 LB Luria-Bertabi (LB) medium

Bacto-Tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
ddH2O to	1l
Agar (for solid Medium)	20g

pH adjusted to 7.0 and autoclaved to sterilize

Appendix 1.7 YEB Medium

Beef extract	*0.5 g*
Yeast extract	*0.1 g*
Peptone	*0.5 g*
Sucrose	*0.5 g*
MgSO4.7H2O	*30.0 mg*
Distilled water	*100.0 ml*
Agar	*2.0 g*

Appendix 1.8 AB Medium

AB Buffer (20x) pH adjusted to 7.0 with KOH and sterile filtered	K2HPO4	*60g/l*
	NaH2PO4	*20g/l*
AB Salt (20X) Sterilised through autoclaving	NH4Cl	*20g/l*
	MgSO4.7H2O	*6g/l*
	CaCl2	*0.2g/l*
	FeSO4.7H2O	*0.05g/l*
Glucose (100mM) - Sterile filtered	Glucose	*18.01g/l*
AB Medium	AB Buffer (20X)	*10ml*
	AB Salt (20X)	*10ml*
	Glucose (100mM	*100ml*
	H2O (dd sterile)	*80ml*

Appendix 1.9 GUS Staining Buffer and Histochemical staining

A. Preparation of GUS staining solution

(i) 80 mM sodium phosphate buffer [pH 7.0]

(ii) 0.4 mM potassium ferricyanide.

(iii) 0.4 mM potassium ferrocyanide

(iv) 8 mM EDTA

(v) 0.05% Triton X-100

(vi) 0.8 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

(vii) Filter-sterilize using 0.2 micro filter.

(viiii) For 100 ml of GUS staining solution, add 25 ml MeOH (to give 20% MeOH concentration)

(ix) Before first use, add 60 ul of Triton-X for each 100 ml.

B. Histochemical assay

1 Dip the sample in GUS staining solution.

2 Incubate for one hour to overnight at 37C.

3 Remove GUS staining solution.

4 Add 70 – 100% ethanol.

5 Incubate at room temperature to remove chlorophyll.

6 Repeat steps 4 and 5 several times

Appendix 1.10 DNA Extraction Buffer

End Concentration	**Stock solution**	***Quantity for 200ml***
1% Lauryl Sarkosyl	30	*6.6ml*
100mM Tris-HCl	1M	*20ml*
100mM NaCl	1M	*20ml*
10mM EDTA	*0.5M*	*4ml*

Appendix 1.11 peqGold Trifast RNA Isolation

1	Homogenisation	1.0 ml peqGOLD TriFast™ + 50-100 mg leaf sample
2	Phase separation	Homogenate + 0.2 ml Chloroform
3	RNA-Precipitation	Watery Phase + 0.5 ml Isopropanol
4	RNA washing	1 ml 75 % Ethanol
5	Dissolving RNA	Formamide, 0.5 % SDS or Water

Appendix 1.12 1st Strand cDNA Synthesis

1.	In sterile tube:	Total RNA	-	0.1 – 5µg
		Primer oligo(dT)18	-	1.0µl
		H20, nuclease-free	-	9.0µl

2. Incubate the mix at 70℃ for 5min and chill on ice.

3. Add in the order:	5X M-MuLV Buffer	-	4µl
	10mM 4dNTPs Mix	-	2µl
	Ribonuclease inhibitor	-	0.5µl (20U)
	DEPC-treated H20	-	12.5µl

4. Incubate at 37℃ for 5min

5. Add 200U of RevertAid™ H Minus M-MuLV Reverse Transcriptase = 1µl

6. Incubate reaction mix at 42℃ for 60min

7. Stop reaction by heating at 70℃ for 10min

8. Chill on ice.

Appendix 1.13 PCR Reaction Mix

Reagent	**Final Concentration**
Sterile deionized water	to reaction volume
10X *Taq* buffer	1X
2mM dNTP mix	0.2mM of each
Primer I	0.1-1µM
Primer II	0.1-1µM
Taq DNA Polymerase	1.25U/50µl
25mM $MgCl_2$	1-4mM
Template DNA	10pg-1µg

ACKNOWLEDGEMENTS

This work was undertaken at Hamburg University, Department of Applied Molecular Plant Biology (AMPII). The work was made possible through a Deutscher Akademischer Austausch Dienst (DAAD) scholarship and a study leave from my employer Kenya Industrial Research and Development Institute (KIRDI).

I am grateful to Prof. Dr. Horst Lörz who gave me the opportunity to join his research group and pursue my long held ambition to work in the field of biotechnology. This ambition was realised under the patient tutelage of Dr. Maram Bader and Dr. Dirk Becker to whom I am immensely grateful. Much thanks to Prof. Dr. Erhard Kranz for making sure that my work was successfully finalised.

The good working relationship in the AMPII research group, especially the hospitality of Dr. Stephan Scholten, Dr. Stephanie Meyer, Iqrar Ahmad Rana, Tobias Schenk, Marlis Nissen, Margarete Hunt, Ursula Reinitz, Kirsten Kollek, Sabina Miaskowska, Julia Sandberg-Meinhardt, Petra von Wiegen Bärbel Hagemann and Simone Amati made this travail much easier than it could have been.

A word of gratitude to Louis Prom, PhD of USDA-ARS, College Station, TX, who provided the *Colletotrichum sublineolum* strains used in this study. I would also like to thank Calleb Olweny of Kenya Sugar Research Foundation (KESREF) at Kibos; Kenya; Evans Mutegi of Kenya Agricultural Research Institute (KARI)-Genebank at Mugugaa, Kenya; P.K. Kamau, Silas Nzioki and Rachel Maswili of KARI-Katumani who made sure that I got all the sorghum seeds I needed and proffered background information on disease reaction of the lines used in this study.

CURRICULUM VITAE

Name	Linus Moses Kosambo Ayoo
Place of Birth	Kisumu, Kenya
Contact	KIRDI P.O. Box 30650-00100 Nairobi, Kenya

PhD Studies

2005-2008	PhD in Bioengineering – Genetic Transformation of Sorghum for Fungal Diseases Resistance. University of University, Department of Applied Molecular Biology (Biozentrum – Klein Flottbek).

Graduate Studies

1995 - 1998	MSc. Botany - Plant Biochemistry and Physiology. University of Nairobi

Undergraduate Studies

1990 - 1994	B.Sc. in Botany and Zoology. University of Nairobi, Kenya.

Employment

1999 – Present:	Research Officer - Kenya Industrial Research and Development Institute (KIRDI). Nairobi, Kenya.

Other Training

2004	Evaluation of National Research and Development Projects JICA – Mitsubishi Research Institute, Inc. (MRI) and Industrial Science and Technology Policy and Environment Bureau of the Japanese Ministry of Economy, Trade and Industry (METI). Tokyo, Japan.
2003	Management Functions, Principles and Practices - Kenya Institute of Administration (KIA), Kabete, Nairobi
2002	Hazard Analysis and Critical Control Point (HACCP) and Quality Systems Development. World Association of Industrial and Technological Research Institute (WAITRO) sponsored training held in Impala Hotel, Arusha - Tanzania

Publication

L. M. K'osambo, E. E. Carey, A. K. Misra, J. Wilkes and V. Hagenimana. Influence of Age, Farming Site, and Boiling on Pro-Vitamin A Content in Sweet Potato (*Ipomoea batatas*(L.) Lam.) Storage Roots. Volume 11, Issue 4, December 1998, Pages 305-321.