EXPLORING HOST INDUCED GENE SILENCING OF KEY BIOSYNTHETIC PATHWAY GENES IN MANAGEMENT OF AFLATOXIN ACCUMULATION IN MAIZE (*Zea mays*) AND GROUNDNUTS (*Arachis hypogaea* L)

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Exploring host induced gene silencing of key biosynthetic pathway genes in management of aflatoxin accumulation in maize (z*ea mays*) and groundnuts (*arachis hypogaea* l)

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DECLARATION

This research thesis is my original work and has not been presented for a degree or any other award in any institution.

Signature…….. Date……

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This research thesis has been submitted for examination with our approval as supervisors.

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DEDICATION

To the late Prof. Jesse Simiyu Machuka. His love and passion for science will last in the hearts of those he mentored forever. Rest in Peace.

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ACRONYMS AND ABBREVIATIONS

µg/kg	Microgram per kilogram
µl	Microliter
2,4-D	2,4-Dichlrophenoxy acetic acid
AFB1	Aflatoxin B1
ANOVA	Analysis of variance
aflR	Aflatoxin reduction
BAP	6-Benzylaminopurine
bp	Base pair
$CaCl_2$	Calcium chloride
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CIMMYT	International Maize and Wheat Improvement Center
DMST	Dimethylsterigmatocystin
dsRNA	Double stranded RNA
E coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
Fas	Fatty acid synthase
HPLC	High performance liquid chromatography
hpRNA	Hairpin RNA
Hpt	Hygromycin phosphotransferase
KEBS	Kenya Bureau of Standards
LB	Luria and Bertani
MES	2-(*N*-morpholino) ethane sulfonic acid
miRNA	Micro RNA
MRBA	Modified Rose Bengal Agar
mRNA	Messenger RNA
NOR	Norsolorinic
nptII	Neomycin transferase II
NRDCA	Neutral red desiccated coconut agar
PCR	Polymerase chain reaction
PGR	Plant growth regulators
pksA	Polyketide synthase
PTGS	Post-transcriptional gene silencing
RdRp	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RNAi	RNA inteference
RT-PCR	Reverse transcriptase PCR
siRNA	Small interfering RNA
ST	Sterigmatocystin
stcA	Sterigmatocystin A

UV	Ultra violet
WHO	World Health Organization
YES	Yeast Extract Sucrose

ABSTRACT

Infestation of crops by mycotoxin-producing fungi is a major challenge in sub-Sahara Africa due to heavy economic losses and threats to human and animal health. Many crops are contaminated by fungi, particularly *Aspergillus spp,* that produce aflatoxins. Moreover, most studies on fungal contamination in plants have focused on maize due to its significance as a staple food in most parts of Africa. In Kenya, this has been driven by the outbreaks of aflatoxicosis in the eastern parts of the country, the worst in human history being in 2004 where 317 cases were reported with 125 deaths as a result of consumption of contaminated maize. Various pre and post-harvest control strategies have not effectively managed the aflatoxin menace in Africa therefore calling for a long term solution. This study aimed to develop transgenic maize and groundnut germplasm that accumulate low or no aflatoxins using host induced gene silencing approach. RNAi sequences targeting key enzymes and regulatory elements in the aflatoxin biosynthetic pathway were cloned into entry vector PCR8/TOPOTA and then introduced into monocot and dicot tailored binary vector pStargate and pHellsgate vectors respectively via the Gateway™ cloning technique. A regeneration protocol for ICGV-CG2 and CG2 groundnut varieties was first optimized using cotyledons for ease of regeneration of transgenics. Maize transformation was achieved by use of immature embryos co-cultivated with *Agrobacetrium tumefaciens* harboring the silencing constructs. Transgenic plants were confirmed via PCR using primers targeting respective selectable markers. Afterwards, transgenic maize were challenged using an aflatoxigenic *Aspergillus flavus* (MCKII) under controlled conditions in the glasshouse. Aflatoxin levels in transgenics and re-isolated fungi were first determined by fluorescence on neutral red desiccated coconut agar media and later quantified through enzyme-linked immunosorbent assay. Results showed reduced toxin levels in maize transformed with the constructs as compared to the wild type used as controls. *StcJ* tranagenic maize recorded the lowest aflatoxin levels of <1.75ppb followed by *aflR* and *stcA* at 10 and 12.4ppb respectively. Analysis of *in vitro* aflatoxigenecity of reisolated *A. flavus* also revealed higher levels of aflatoxins in fungal cultures reisolated from wild type and the

MCKII culture in acamparison to transgenics. Aflatoxin levels of 117 and 70.3ppb were recorded in *A. flavus* cultures from wild type and the MCKII respectively. Those from maize transformed with *stcJ, stcA* and *aflR* recorded <1.75, 29.4 and 43.6 ppb respectively. Interestingly, the *aflR* construct was observed to have detrimental effects on both maize and groundnuts.These results indicate that transgenic maize synthesized double stranded RNAs that were later converted into complementary small interfering RNAs against the respective target aflatoxin pathway mRNA sequences. On colonization by toxigenic *A. flavus*, the complementary siRNA molecules trafficked via the plant-fungal cellular interface into the fungi where they chopped the respective target esquences into siRNAs of 21-28bp hence resulting in downregulation of respective gene expression. In conclusion, this study successfully downregulated aflatoxin biosynthesis and accumulation in maize. The transgenic maize generated could help in alleviation of the aflatoxin problem in SSA without any further investment by farmers except on seed acquisition.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Food production in Africa is hampered by various constraints such as drought, crop pests and diseases, lack of inputs, suitable seeds and poor infrastructure (FAOSTAT 2010). These constraints pose a great challenge to attaining food self-sufficiency in the continent (Alakonya and Monda 2013). Among the greatest threat to food security is infestation of foodstuffs by mycotoxin-producing fungi. These pathogens produce secondary metabolites called mycotoxins that are a health concern (Lewis *et al*., 2005). Several mycotoxins including fumonisins, ochratoxins and aflatoxins exist and have been reported in food and feedstuffs worldwide. Of these, however, aflatoxins are the most extensively studied due to their harmful effects on human health, animal productivity and trade (WHO, 2006).

Over 16 types of aflatoxins are reportedly produced by the *Aspergillus spp,* but the most commonly occurring are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2). This classification is based on their chromatographic and fluorescent characteristics (Lerda, 2010). The B type is produced by *Aspergillus flavus* which is considered the main source of aflatoxins in most communities. *Aspergillus parasiticus* produces both the B and G type aflatoxins (Vaamonde *et al*., 2003). AFB1 was implicated in Kenya where an outbreak of aflatoxicosis in the eastern parts of the country in 2004 that led to 317 cases with 125 deaths as a result of consumption of contaminated maize (CDC, 2005; Lewis *et al*., 2005). Aflatoxin biosynthesis follows a series of oxidation-reduction reactions and the process has been shown to involve 23 enzymatic reactions with at least 15 intermediates (Yu *et al*., 2004). Biosynthesis is believed to involve over 25 identified genes that are clustered within a

70-kb DNA region and transcriptional regulators termed *aflR* and *aflD* that control the entire pathway (Yu *et al*., 2004). Ingestion of aflatoxins may result in two health conditions; acute and chronic aflatoxicoses. Acute aflatoxicosis, which is a case of ingestion of large amounts of aflatoxins, is characterized by a decrease in liver function, edema, abdominal pains and eventual death of the patient. On the other hand, chronic aflatoxicosis occurs due to long term exposure to low concentrations of aflatoxins resulting in decreased growth rate in patients and immune suppression (Gong *et al*., 2004; Lewis *et al*., 2005).

Aflatoxin contamination has been reported in various crops including grains, nuts, cotton, spices and also in milk. Numerous reports, however, focus on aflatoxin contamination in maize (*Zea mays*) and groundnuts (*Arachis hypogaea* L.) since these are the most affected crops worldwide (Wagacha and Muthomi, 2008; Mutegi *et al.*, 2009). Predisposing factors that favor colonization of crops by fungi include high incidences of toxigenic strains in the soil, high kernel moisture during storage and delay in harvesting of the crop (Mutegi *et al.*, 2009). These factors are exacerbated by the existing weather conditions (wet and humid), poor harvesting, transporting and `handling techniques and storage conditions that favor growth of aflatoxin producing fungi (Robertson, 2005).

Farmers, consumers and government agencies in Africa have attempted various strategies for intervention in aflatoxin management aimed at reducing exposure of food crops to toxigenic fungi (Wagacha and Muthomi, 2008). This is in response to various regulations set by governments for the limit of aflatoxin levels allowed in foodstuffs that is deemed not harmful for human consumption. In Kenya, for instance, the Kenya Bureau of Standards, (KEBS), has set 10 and 5 parts per billion (ppb) for total and AFB1 respectively as the limits (KEBS 2007). Both pre-harvest prevention and post-harvest reduction measures tried out have not been effective in controlling the menace in Africa. More robust mitigation strategies are therefore needed in this field to ensure a reduction in aflatoxin levels and therefore safety of food and feed (Wagacha and Muthomi, 2008).

Given the current body of knowledge and various scientific breakthroughs in controlling biotic and abiotic stresses in plants, it is almost certain that genetic engineering is the way forward. Genetic transformation, which allows the introduction of agronomically important genes into the genomes of plants for improvement, could offer a long lasting solution (Sharma & Anjaiah, 2000). A pre-requisite for utilization of this approach, however, is the availability of an efficient protocol to transform and regenerate whole plants *in vitro*. Several protocols have been developed for transformation and regeneration of tropical maize making their improvement via genetic transformation easier (Oduor *et al.,* 2006; Ombori *et al.,* 2008). This is however not the case in groundnuts where the existing protocols for transformation and regeneration are genotype-dependent requiring optimization for each variety (Sharma and Anjaiah, 2000). Many varieties of groundnuts exist worldwide but protocols for regeneration have only been reported for a handful. This study sought to first optimize a regeneration protocol for selected groundnut varieties adapted to East Africa with a view of setting up a platform for their improvement through genetic transformation. ICGVCG2 and CG2 lines were developed by Leldet (Ltd.) Seed Company. They are high yielding, well adapted to fields in East Africa and preffered by farmers in Kenya (Maina *et al*., 2010).

The prospect of using RNA interference (RNAi) to deal with the aflatoxin problem in maize and groundnuts could spell new life to management of mycotoxins (Alakonya & Monda, 2013). The mechanism, which has the ability to knock down genes, naturally plays an important role in cellular defense; protecting host plant cells from unwanted expression of repetitive sequences, viral infections and damage by transposons (Lippman and Martiensen, 2004). Recently, host induced gene silencing (HIGS) has been applied in crop improvement to target degradation of specific fungal target sequences and the results support the possibility of using the technique in controlling fungal aflatoxin production through host plants (Nowara *et al.,* 2010; Tinoco *et al.,* 2010; Yin *et al.,* 2011; Koch *et al.,* 2013).

HIGS is achieved via expression of double-stranded (ds) RNAs into plants through transient or stable transformation protocols using RNAi vectors (Wesley *et al*., 2001). This type of post-transcriptional gene silencing (PTGS), termed co-suppression in plants and quelling in fungi, is mediated by 21–24 nt dsRNA molecules known as siRNAs produced by an endonuclease called Dicer (Hamilton and Baulcombe 1999). The siRNAs are incorporated in an RNA-induced Silencing Complex (RISC) that then guides them to degrade complementary target mRNAs in a sequence specific manner (Baulcombe 2004). In theory, HIGS through RNAi can be achieved by stably transforming a host plant with a transgene that encodes a hairpin RNA sequence targeted against problem genes such as those that control aflatoxin biosynthesis (Koch and Kogel 2014). Macromolecular trafficking may occur between the transformed host and the fungus via a plant-fungal cellular interface between the two and therefore ensure delivery of double stranded RNA (dsRNA) into the pathogen (Voegele and Mendgen, 2003; Micali *et al.,* 2011). Once inside the fungus, gene silencing would occur due to production of secondary small interfering RNAs (siRNA) that guide degradation of the homologous sequences in the fungus inhibiting aflatoxin biosynthesis.

To add strength to this, studies have reported success in engineering plants against viruses (Duan *et al*., 2012), insects (Huvenne and Smagghe 2009), nematodes (Niu *et al.,* 2006), parasitic plants (Alakonya *et al*., 2012), bacteria (Dunoyer *et al.*, 2006) and, recently, fungi (Nunes & Dean 2012). These works therefore set up a platform for controlling other plant challenges that have not been reported such as contamination of plants with aflatoxins. Studies have shown that a cluster of aflatoxin and sterigmatocystin (ST) genes control the biosynthesis of aflatoxins in fungi with the rate limiting steps being catalyzed by *stcJ, stcK* and *stcA* genes (Yu *et al*., 2012). There also are transcription factors, aflatoxin regulatory gene (*aflR*) and *aflD* among others, which are responsible for activating and controlling the pathway for gene transcription (Yu *et al.,* 2004). Down-regulating these genes would have a possible positive effect on the pathway since in their absence, it is postulated that no induction of aflatoxin can occur in the fungi (Brown *et al.,* 1996).

1.2 Statement of the Problem

Maize is Africa's most important food crop with an estimated production rate of 2.7 million tons per year (FAOSTAT, 2010). Groundnuts on the other hand generate up to 60% cash earnings and over 70% of rural employment in developing countries (Nigam *et al*., 2012). In particular, Asia accounts for 71.7% while Africa produces 18.6% of global groundnut production. Productivity of these crops, however, has remained low due to various production constraints, key among them being infestation by fungal pathogens that produce aflatoxins. In Africa, aflatoxins have attracted wide attention because of their negative impacts on the yield, human health, animal productivity and trade (Lewis *et al*., 2005). Heavy losses resulting from discarding contaminated products, cancer cases among people and bans on imports and exports are a threat to human wellbeing and the economy. Prevailing tropical environmental conditions of high temperatures and high relative humidity predispose crops to fungal infections and worsen the situation. Aflatoxin contamination is therefore a major problem in the world needing an alternative approach that can offer a long term solution that is feasibly acceptable in the poverty ravaged SSA.

1.3 Justification

Reducing aflatoxin levels in foodstuffs in Africa is imperative. This will improve the quality of food, reduce risks to human and livestock health and improve trade. Conventional control strategies such as early harvesting, proper drying of the harvest and development and use of non-toxigenic fungi to outcompete the toxigenic *Aspergillus Spp* have not worked effectively. They are also laborious, time consuming and expensive for small scale farmers in developing countries. There is need, therefore, for a solution to the aflatoxin menace that is practical, efficient and cost effective to farmers in such countries. Since the aflatoxin biosynthetic pathway and its genes have been identified and understood, there is a platform for application of genetic engineering techniques in aflatoxin control through RNAi-mediated gene silencing. This study used HIGS technology in attempts to reduce the levels of aflatoxin accumulating in maize and

groundnuts infected with *Aspergillus flavus* by silencing the key pathway genes. This technology has the potential to save time and is also affordable to scientists worldwide.

1.4 Hypotheses

1. Groundnut genotypes are regenerable through variation of 2,4-D and BAP concentrations.
2. Downregulation of transcription factor *aflR* suppresses aflatoxin biosynthesis in *A. flavus* and its accumulation in maize and groundnuts.
3. There is an effect of HIGS cassettes targeting *stcA* and *stcJ* on aflatoxin biosynthesis and its accumulation in the transgenic maize.

1.5 Objectives

1.5.1 General objective

To develop maize and groundnut germplasm exhibiting low or no aflatoxin levels through host induced gene-silencing technology.

1.5.2 Specific objectives

1. To optimize a regeneration protocol for ICGV-CG2 and CG2 groundnut lines using varying concentrations of BAP and 2, 4-D.
2. To investigate the effect of downregulating transcription factor *aflR* on aflatoxin biosynthesis and accumulation in maize inbred line CML 144 and groundnut lines ICGV-CG2 and CG2.
3. To assess the effect of HIGS cassettes against genes coding for aflatoxin biosynthesis rate limiting enzymes *stcA* and *stcJ* and aflatoxin accumulation in maize inbred line CML 144.

CHAPTER TWO

LITERATURE REVIEW

2.1 Maize production dynamics in Kenya and Africa

Maize is Africa's most important food crop, grown by both large and small scale farmers, due to its attributes including high yield per seed planted and its adaptation to prevailing climatic and soil conditions (Machuka, 2005). In Kenya, the average annual maize production is 2.7 million tons but this does not meet the consumption needs of the country, estimated to be around 4.5 million tons (FAOSTAT, 2010). A large percentage of the total maize production in Kenya (85%) is mainly by small scale farmers with the rest being produced on a large scale (Muhammad & Underwood, 2004).

2.2 Maize production constraints in Kenya

Various production constraints that adversely affect maize production in Kenya under the prevailing conditions have been identified and reported. These constraints result from both biotic and abiotic stresses coupled with poor agronomic practices by the farmers. Abiotic stresses such as drought, unreliable rainfall and salinity cause a reduction in maize yields therefore impacting negatively on its economic importance (Thomson 2008). Of these, however, drought and unreliable rainfall have had a greater effect on production systems in Kenya since maize is grown almost exclusively under rain-fed conditions (Anami *et al.*, 2008). Irrigation has been used in various areas of the country to increase production.

Other abiotic stresses are occurrence of floods in some maize growing areas and low soil nutrient levels. Biotic constraints to maize production in Kenya include pests (stem borer and the larger borer), parasitic weeds (*Striga spp*) and diseases (maize steak virus disease, rust and head smut) (Mugo *et al.*, 2005; Shepherd *et al*., 2010). Infestation of maize by fungal pathogens, especially *Fusarium spp* and *Aspergillus spp*, also has a huge impact on maize production due to occurrence of mycotoxins that are harmful to

human and livestock health (Alakonya & Monda, 2013). These pests and microbes cause significant post-harvest grain losses that impact negatively on the economic importance of the crop including bans on exportation.

2.3 Overview of groundnut production in Kenya and the world

Cultivated groundnut (*Arachis hypogaea* L.) is a native of South America belonging to family *Fabaceae* (*Leguminosae*). It is further classified into two subspecies; *fastigiata* and *hypogaea* (Tillman & Stalker 2009). Groundnuts are mainly distributed in the tropical, sub-tropical and warm temperate zones of the world. The crop grows well in areas with well distributed annual rainfall of at least 500 mm and requires an optimum air temperature of between 25-30ºC (Janila *et al.,* 2013). Groundnuts are cultivated on nearly 23.9 million hectares worldwide with a total annual production of 36.4 million tons at an average of 1520 kg/ha (FAOSTAT, 2011).

The major groundnut growing countries in the world are China, India, Nigeria, USA and Myanmar. Developing countries account for over 97% of global groundnut area and 95% of the total production where it is cultivated under rain-fed conditions with low inputs by resource poor farmers (Janila *et al.,* 2013). Nigeria and Senegal are the largest groundnut producers in Africa although Mali, Niger, Kenya and Burkina Faso are also significant producers. In Kenya, the crop is cultivated mainly in western parts but its trade and consumption is all over the country (Mutegi *et al.,* 2012). Production is mainly small scale with western and Nyanza provinces being the leading producers. Homabay local and Valencia Red are the commonly grown varieties in Kenya although other improved genotypes such as ICGV 12991, JL-24 and CG7 have been introduced by ICRISAT (Mutegi *et al*., 2009).

2.4 Economic importance of groundnuts

Groundnut plays an important role in various economies of the world where it generates income to livelihoods through production of food and oil (Janila *et al.,* 2013). The groundnut seed, consumed in the raw state, boiled or roasted, is rich in energy and proteins. It has been reported to contain 38.6% protein and 47% oil emphasizing its role in the nutrition of its consumers and trade (Atasie *et al.,* 2009). Over 60% of global groundnuts are used for extraction of oil while the rest is consumed as food among other uses (Birthal *et al.,* 2010). Peanut butter is the most common processed product of groundnuts and is used on bread among other uses in the confectionery industry. Groundnut haulms are rich in lipids, minerals and carbohydrates and these are important sources of nutritious fodder for livestock. Furthermore, groundnut, being a legume, fixes nitrogen to the soil helping improve its fertility. Shells from harvested groundnuts are used as fuel in fertilizer industries (Janila *et al*., 2013).

2.5 Challenges in groundnut production and breeding for improvement

A combination of biotic and abiotic factors has greatly affected groundnut production in many countries leading to reduced yields. Harsh environmental conditions such as drought, low soil pH and low temperatures play a significant role in limiting production from the crop. Apart from this, poor management practices and soil fertility issues also constrain production (Janila *et al*., 2013). Contamination of groundnuts by mycotoxins, particularly aflatoxins is one of the most common challenges facing the crop in Africa (Mutegi *et al*., 2009). *Aspergillus flavus* attack crops during growth and development or during harvesting and post harvesting time and produce aflatoxins which are toxic to human and livestock feeding on the harvested crop (Wagacha & Muthomi, 2008). The aim of breeding programs in groundnut improvement worldwide has been to develop new varieties that meet the requirements of farmers, processors and consumers.

In developing countries, the focus has been more on removing trade barriers besides improving the yield itself (Janila *et al*., 2013). The targeted traits for improvement have included yield parameters such as pod yield per plant, number of pods per plant and seed weight. Adaptation parameters such as stress tolerance or resistance that offer protection against losses caused by biotic and abiotic stresses have been targeted in improvement programs. These affect pod yield and therefore the quality of produce (Craufurd *et al*., 2002; 2003). Diseases have also been targeted in groundnut breeding (Upadhyaya *et al.*, 2005). These include early and late leaf spot, groundnut rust, stem rot, groundnut rosette virus, peanut bud necrosis and peanut clump virus disease (Nigam *et al*., 2012). Insect pests such as aphids, root-knot nematodes and thrips attack groundnuts causing serious damage.

Breeding for resistance to diseases has received more attention worldwide than breeding for resistance to insect pests (Janila *et al*., 2013). Desirable traits for confectionery uses such low oil and high protein content for food use and high oil content for production of industrial oil have also been targeted in advanced breeding programs (Misra *et al.*, 2000). In the past, breeding programs for self-pollinated crop improvement have involved methods such as mass pedigree selection, single seed descent and back-crossing. These conventional breeding methods have been successful in improving groundnuts but their progress has been limited for some of the traits. They also take long to produce an improved cultivar. ICRISAT has released cultivars with resistance to rust and groundnut rosette virus in Africa (Singh *et al*., 2003). Targeting specific traits for improvement is now possible through the use of molecular breeding programs (Janila *et al*., 2013).

2.6 Aflatoxins occurrence and diversity

Aflatoxins are toxic and carcinogenic secondary metabolites produced by fungi. They are classified as the most significant mycotoxins in Africa and other tropical developing countries (WHO, 2006). These mycotoxins have a negative impact on crop yield, human

health, animal productivity and trade. Many species of fungi produce aflatoxins; *A. flavus, A. parasiticus* and *A. nomius* (Dorner, 2002; Vaamonde *et al.*, 2003). Of these however, *A. flavus* and *A. parasiticus* are the most common (Yu, 2012). Pre and post-harvest contamination of crops including maize, groundnuts, cotton and tree nuts by *Aspergillus spp* is common. Over 16 types of aflatoxins have been reported worldwide but the most commonly occurring are AFB1, AFB2, AFG1 and AFG2, classified according to their chromatographic and fluorescent characteristics (Lerda, 2010). The B type aflatoxins are produced by *A. flavus,* which is considered the main source of aflatoxins in most communities. *A parasiticus* produces both the B and G type aflatoxins (Vaamonde *et al*., 2003).

2.7 Aflatoxin biosynthesis

Biosynthesis of aflatoxins involves a series of oxidation-reduction reactions. The process has been shown to involve 23 enzymatic reactions and at least 15 intermediates (Yu *et al*., 2004; Yu, 2012) (Figure 1). Studies have demonstrated that over 25 identified genes are involved in the biosynthesis and are clustered within a 70-kb DNA region in the chromosome (Yu *et al*., 1995; Bhatnagar *et al*., 2000). Biosynthesis begins with conversion of malonyl-CoA to a polyketide called noranthrone, by the products of fatty acid synthase genes (*fas-1* and *fas-2*) controlled by *stcJ* and *stcK* and polyketide synthase gene (*pksA*) encoding *stcA* (Cary *et al*., 2000a).

Noranthrone is converted to norsolorinic acid (NOR), the first stable intermediate in the pathway. NOR is then converted to averantin (AVN) through the action of *nor-1, norA* and *norB* genes. This is then converted to averufin (AVR) and later to versicolorin B (VERB) through the action of 3 genes; *cypX, moxY* and *avfA*, that catalyze individual steps. Two (2) other genes; *ver1* and *verA* are required for the conversion of versicolorin A (VERA) to dimethyl-sterigmatocystin (DMST). The final step involves conversion of O-methylsterigmatocystin (OMST) or dihydro-O-methylsterigmatocystin (DHOMST) to aflatoxins in the presence of a NADPH-dependent mono-oxygenase; *ordA* (Yu *et al*.,

2004). Sterigmatocystin is the penultimate precursor in the biosynthesis of aflatoxin (Figure 1).

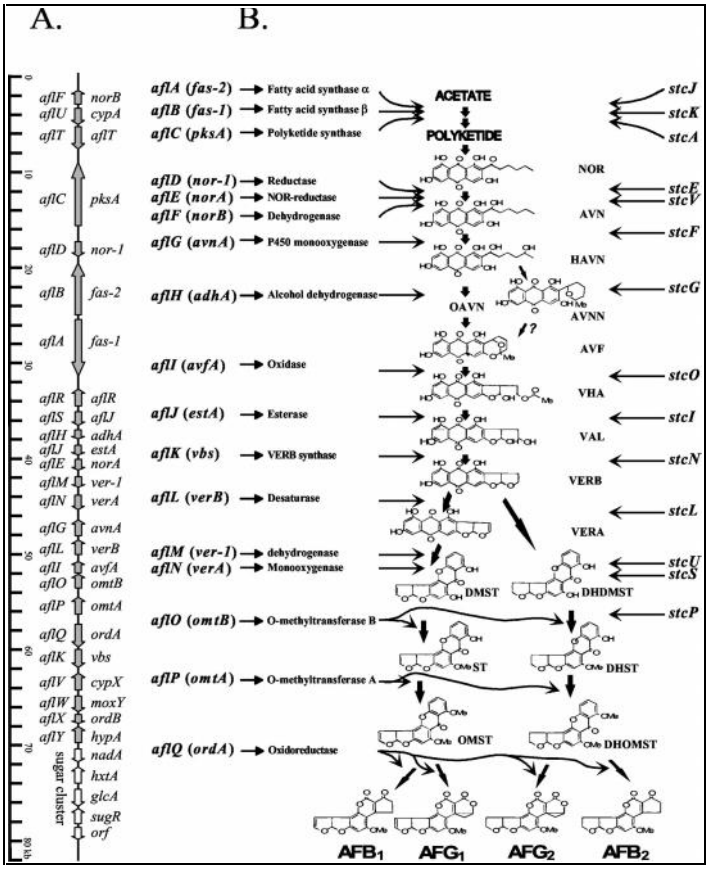

Figure 1. Clustered genes **(A)** and the aflatoxin biosynthetic pathway **(B).** (Source: Yu *et al*., 2004).

2.8 Toxicity and health implications of aflatoxins

Humans and livestock are exposed to aflatoxins through feeding on contaminated food and feed respectively (Chulze, 2010). Public health problems have been shown to result from ingestion of products contaminated with aflatoxins (Bandyopadhyay and Cotty, 2011). The effects of aflatoxins on health are classified into two general forms; acute and chronic aflatoxicoses. Acute aflatoxicosis results from ingestion of large amounts of aflatoxins in food. Its effects include a decrease in liver function leading to blood clotting and jaundice manifested through symptoms like oedema, abdominal pains,

vomiting and later, death of the patient follows. This type of aflatoxicosis was observed in Eastern Kenya where 125 deaths and 317 cases were reported due to consumption of contaminated maize (CDC, 2005; Lewis *et al*., 2005).

The second form of toxicity, chronic aflatoxicosis, occurs when individuals have long term exposure to low concentrations of aflatoxins. Symptoms reported have included a decrease in growth rate in patients and immune suppression (Gong *et al*., 2004). Affected domestic animals exhibit a reduction in milk and egg production and immune suppression. Some studies have confirmed that children affected by aflatoxin contamination show a higher risk for infections such as diarrhea (Gong *et al.*, 2004). Aflatoxins have further been shown to work synergistically with hepatitis viruses in raising the risk of liver cancer (Turner *et al*., 2003). Regulation of aflatoxin levels in foodstuffs is imperative with various countries having set limits for the maximum level of aflatoxins acceptable in food and feed. For instance, the European Union and Canada have set 15ppb as the limit of aflatoxins in groundnuts while Brazil has a 20ppb limit (Alakonya &Monda, 2013). In Kenya, the limit has been set at 10 and 5ppb for total aflatoxins and AFB1 respectively in all foodstuffs for human consumption (KEBS, 2007).

2.9 Factors affecting aflatoxin accumulation in crops

The factors that predispose crops to aflatoxin producing fungi and hence contamination are classified into environmental, agronomic and socio-cultural categories (Mutegi *et al*., 2012). Reports have shown that high humidity and temperatures provide optimal conditions for fungal contamination and toxin formation. For instance, Mutegi *et al* (2009) showed that groundnuts from wet, more humid environments were more likely to be contaminated than those from drier areas. Contamination has also been found to be widespread to crops under rain fed conditions as compared to those under irrigation. End of season drought stress and elevated soil temperatures have also been shown to promote aflatoxin contamination (Bankole *et al*., 2006). This is a common phenomenon in sub Saharan Africa. Infestation of groundnut pods by pests coupled with mechanical damage

during harvesting make way for fungi to colonize crops (Waliyar *et al*., 2008). Kernel moisture has also been shown to play a role in fungal contamination. For instance, aflatoxin contamination was reported to increase, 10 fold in a 3-day period, when grains were stored with high moisture content (Hell *et al.,* 2008). Other factors include high moisture during storage (Jeurissen *et al.,* 2011), susceptibility of cultivars to fungal contamination (Mutegi *et al.,* 2009) and poor aeration in storage houses coupled with dirty floors that promote fungal growth (Robertson, 2005).

2.10 Current status of aflatoxins contamination in food and feed in Africa

Reports indicate that aflatoxin poisoning occurs in sub Saharan Africa especially in staple foods such as maize (Wagacha and Muthomi, 2008). Crops such as groundnuts have received little attention regarding fungal contamination and the effects of aflatoxins since greater focus is on maize. Due to this high exposure to aflatoxins, Kenya does not export groundnuts to other markets hence impacting on the economy (Mutegi *et al.,* 2012). To add strength to this, most of the hepatocellular carcinoma deaths associated with aflatoxins have occurred in this region. For instance, 55% of cancer deaths are reported to occur in developing countries (MERCK 2006).

Apart from the health risks, a larger percentage of economic losses as a result of mycotoxins lie in Africa (Fellinger 2006; MERCK 2006). The largest documented case of human aflatoxin poisoning occurred in Kenya in 2004 where 317 cases and 215 deaths were reported (CDC 2004; Lewis *et al.,* 2005). One hundred people were also reported dead in Nigeria due to ingestion of AFB1 (Afla-guard, 2005). The legal aflatoxin limits permitted in foodstuffs in Kenya by KEBS are 10 and 5ppb for total and AFB1 respectively (KEBS 2007). These are however neglected by farmers, processors and consumers due to the high cost of testing of the products. In line with this, very high concentrations of AFB1 (as high as 4400ppb) have been reported in maize especially in Eastern province (Lewis *et al.,* 2005). Moreover, groundnuts and groundnut products such as peanut butter have also been reported to have very high concentrations of AFBI

which is of great concern (MERCK 2006; Mutegi *et al*., 2012). West African countries such as Benin and Togo have also been implicated with household commodities having AFB1 levels 5 times above the permitted levels (Hell 2005).

2.11 Management strategies against aflatoxins in food and feed

Aflatoxins, among other mycotoxins contaminate crops in the field and accumulate during harvest, storage, transportation and marketing. Given the negative impacts of aflatoxins to food production and health in developing countries, farmers, governments and consumers have attempted to reduce the levels of this mycotoxin in foodstuffs. In Africa, possible strategies for intervention in aflatoxin management have aimed at reducing exposure of food crops to toxigenic fungi at various stages of plant development (Wagacha & Muthomi, 2008). Physical pre-harvest prevention strategies have included good agricultural practices such as early harvesting (Rachaputi *et al*., 2002) to reduce fungal infection in the field and proper drying of the harvested produce to reduce moisture content as moist conditions make it favorable for fungal growth (Lanyasunya *et al*., 2005).

Physical treatments have been employed in some countries to reduce crop residues that harbor saprophytic pathogens (Awad *et al*., 2010). For instance, sorting, winnowing and washing have been employed in developing countries to remove significant amounts of mycotoxins (Fandohan *et al*., 2005). Various biocontrol strategies have also been used in the past to deal with the aflatoxin menace in Africa. For instance, development of atoxigenic biocontrol fungi that out-compete the toxigenic strains in the field has seen much success (Cleveland *et al*., 2003). Atoxigenic strains of *A. flavus* have had a significant impact to reduction of aflatoxin levels in West Africa (IITA 2003). The atoxigenic strains that have been applied in fields in a number of African countries including Nigeria, Kenya, Zambia and Senegal include NRRL 21882, which is an active ingredient in Afla-Guard and Aflasafe^{TM} (consist of a combination of local atoxigenic strains) (Dorner *et al*., 1999). A potential drawback of biocontrol is the possibility of

cytoplasm fusion and nuclear fusion between toxigenic and atoxigenic *A. flavus* strains leading to production of ascospores, where the atoxigenic biocontrol strains could acquire aflatoxin pathway genes through vegetative fusion and this could exacerbate the aflatoxin contamination problem (Horn *et al*., 2013). Apart from the above strategies, chemical control has also been tried out. The use of pesticides during crop production has the potential to reduce damage by insect pests that lead fungal contamination (Haidukowski *et al.,* 2004). Similarly, fungicides have also been used to control aflatoxigenic fungi. The use of chemicals, however, has not been extensively used due to environmental and food safety concerns.

2.12 Groundnuts regeneration

Genetic transformation has been shown to introduce genes into groundnuts that have provided a solution to many of the problems affecting the groundnut crop (Anuradha *et al.,* 2006). However, as in other crops the lack of an efficient and reproducible regeneration protocol makes improvement via genetic engineering a challenge. This is because groundnut regeneration is genotype dependent and therefore optimization of an efficient *in vitro* regeneration protocol for specific genotypes is imperative to their micro propagation and transformation (Iqbal *et al.,* 2011). Regeneration of groundnuts from various tissues such as leaf discs, cotyledons, cotyledonary nodes, hypocotyls, epicotyls and embryos has been reported (Kim *et al*., 2003; Anuradha *et al*., 2006; Iqbal *et al.,* 2011). However, the frequencies of plants regenerated have been low and this limits rapid concept testing of candidate genes targeted for introduction via genetic transformation. Direct organogenesis using cotyledons as explants is preferred since shoot regeneration occurs at the cut edges and this may allow easy transfer of genes of interest through *Agrobacterium*-mediated transformation into the tissues. Regeneration protocols for groundnuts adapted to Eastern and Southern Africa have only been reported for a handful of genotypes hence the importance of optimizing a protocol for the other genotypes (Maina *et al.,* 2010).

2.13 Approaches used in genetic improvement of plants through transformation

Plant genetic engineering entails transfer of genes with known functions into an existing plant genome for improvement purposes. This technique of crop improvement has been successful since new traits such as resistance to insect pests, viruses and herbicides have been genetically introduced into crops from other organisms (Sharma *et al.*, 2004). These traits have been acquired via genes from sources such as plants, animals, viruses, bacteria and fungi. Transfer of these genes into crops requires an efficient and reproducible transformation protocol to introduce foreign DNA into the plant genomes. Such protocols entail the development of a feasible tissue culture system for regeneration of the plants *in vitro*, making of gene constructs in suitable transformation vectors, transforming the plant material with the constructs and the subsequent recovery of putatively transformed plants on suitable selection media (Sharma and Anjaiah, 2000).

Molecular and genetic characterization of the transgenic plants for gene expression and evaluation of their effectiveness in dealing with the environmental stresses is the last step (Sharma *et al.*, 2004). In plants, the methods for gene transfer are classified into direct and indirect with the focus being on maximizing the efficiencies of recovered transformed plants. Direct methods include polyethylene glycol (PEG)-induced DNA uptake, microinjection of DNA into cultured cells, electroporation and particle bombardment (biolistics) (Rao 2009). *Agrobacterium*-mediated transformation is an indirect method of plant transformation and together with particle bombardment have been the extensively used worldwide (Chandra & Pental, 2003; Rao 2009).

2.14 *Agrobacterium*-mediated transformation of plants

This is the most dominant method for production of transgenic plants that involves the use of *Agrobacterium tumefaciens*, a naturally occurring bacterium that is involved in crown gall formation at wounded sites of many dicotyledonous plants (Gelvin, 2003; Tzfira & Citovsky 2006). It forms tumors due to a tumor-inducing (Ti) plasmid present

in the virulent strains of *Agrobacterium.* This method is cheaper as compared to other transformation techniques such as particle bombardment (Tzfira and Citovsky 2006). It also results in minimum re-arrangement of transgenes due to integration of fewer copy numbers of these transgenes into the genome of the host plant. During transformation, the T-DNA region of the *Agrobacterium*, usually with the genes of interest, is transferred into host cell's genome. The process of transformation starts when the *Agrobacterium* attaches to the host plant and the virulent (*vir)* proteins are induced by expression of specific host plant signals. The T-DNA is then delivered together with several other *vir* proteins into the host cell cytoplasm and later into the nucleus where it is integrated into the plant genome (Tzfira & Citovsky 2006).

2.15 Genetic transformation of tropical maize

An efficient and reproducible transformation protocol is a prerequisite for any plant improvement program via genetic engineering. However, such protocols require efficient regeneration protocols since recovering a whole plant from transformed tissues is a critical aspect of any transformation technique. Tropical maize lines have been successfully regenerated using immature zygotic embryos as explants (Oduor *et al.,* 2006; Ombori *et al.,* 2008). Such protocols have set up a platform for the improvement of these maize lines through transformation. Immature embryos are preferred as explants for many transformation techniques although other tissues including mature embryos, young leaf discs from seedlings and shoot apical meristems have been tried out (Ahmadabadi *et al.,* 2007; Abebe *et al.,* 2008; Muoma *et al.,* 2008).

Regeneration of transformed maize begins with callus induction following infection with target genes using *A. tumefaciens*. Callus induction is achieved on basal medium with appropriate levels of plant growth regulators (PGRs) such as 2,4-D. The resulting calli are then maintained on media with the PGRs with sub culturing until they mature. Later on, the somatic embryos formed are matured and regenerated into whole maize plantlets (Oduor *et al.,* 2006). Tropical maize inbred lines CML 216 and CML 144 are reported to be transformable through *A. tumefaciens* although their efficiencies have

been low (Anami *et al.*,2008). This is attributed to reduced frequencies due to resistance of maize to infection by *Agrobacterium* and also due to the use of chemicals for selection of transformed tissues such as antibiotics in the transformation media. This work set out to transform CML 144 maize line with various RNAi cassettes against aflatoxin biosynthesis and accumulation.

2.16 Genetic transformation of groundnuts

The transfer of genes from alien sources into groundnuts and subsequent regeneration of transgenic plants has been demonstrated in attempts to confer resistance to biotic and abiotic stresses. *Agrobacterium*-mediated transformation is the most commonly used technique for genetic improvement in groundnuts although other methods such as particle bombardment have been tried out (Sharma & Anjaiah, 2000). Various groundnut transformation protocols employing the use of diverse tissues as explants have been developed. Leaf discs, leaf sections, embryos and cotyledons have all been used in transformation procedures (Rohini & Rao, 2000; Sharma & Anjaiah, 2000; Anuradha *et al.,* 2006). However, the frequencies of transformation are low therefore not much success has been achieved.

Cotyledonary nodes have been shown to have a higher response to transformation and regeneration than any other explant used hence they are the explants of choice in transformation systems (Anuradha *et al.,* 2006). These explants contain axillary meristematic cells at the junction between the cotyledon and the embryo axis which facilitates rapid regeneration and therefore are targeted for gene delivery. This system of transformation, for instance, was utilized by Sharma and Anjaiah (2000) where they successfully transformed JL-24 with marker genes (*nptII*;*vid*A) and a coat protein gene of the Indian clump virus (IPVCcp) for resistance against the virus.

2.17 Selectable markers in *Agrobacterium*-mediated transformation

Systems to select transformed cells and tissues from untransformed ones are a pre-requisite for regeneration of genetically transformed plants when using *A. tumefaciens*. Selectable marker genes are vital to the transformation process. Antibiotic and herbicide resistance genes allow transformed cells that express them to survive on medium containing the selective agent while those not transformed are killed (Sakthivel & Sundar, 2008). These genes may be the genes of interest or can be cloned together with other genes and transformed into the plant tissues. A selective agent should fully inhibit growth of untransformed cells but should not cause harmful effects to the transformed ones. The most widely used selection agents in plant transformation are antibiotics (kanamycin and hygromycin) and herbicide (phosphinothricin, PPT) (Rafiq *et al.,* 2004).

Nearly all antibiotic selectable marker genes used in transformation systems are from bacterial sources. Hygromycin phosphotransferase (*Hpt)* is an amino glycoside antibiotic which inhibits protein synthesis by interfering with translocation and causing mistranslation at the 80S ribosome (Rafiq *et al.,* 2004). The *hpt* gene from *E. coli* confers resistance to antibiotic hygromycin B. Hygromycin B has been used to select various plants including maize, soybean, *Phytopthora parasitica* and *Triphysaria versicolor* (Hazel *et al*., 1998; Zhang *et al*., 2011). The neomycin phosphotransferase (*nptII)* gene from *E. coli* is a protein synthesis inhibitor and confers resistance to kanamycin (Rafiq *et al.,* 2004). Kanamycin has been successfully used to recover transgenic groundnuts transformed with various genes (Sharma and Anjaiah, 2000; Anuradha *et al.,* 2006). Herbicide phosphinothricin *N*-acetyltransferase is also an effective selectable marker. The bialaphos resistance genes *bar* from *Streptomyces hygroscopicus* and *pat* from *Streptomyces viridochromogenes* encode the enzyme PAT. The enzyme disrupts the glutamate synthetase pathway and hence inhibits photosynthesis resulting in the death of plant cells (Sakthivel and Sundar, 2008). Omer *et al.* (2013) reported the use of phosphinothricin to effectively recover transgenic maize.

2.18 RNA interference mechanism in plants

RNAi-mediated gene silencing occurs via two pathways: post transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) (Vaucheret *et al.,* 2001; Vaucheret and Fagard, 2001; Castel and Martienssen, 2013). The mechanism starts with initial processing of precursor dsRNA into short 21–25 nucleotides called small interfering RNAs (siRNA) by an RNaseIII-like enzyme called Dicer (Hamilton and Baulcombe, 1999; Baulcombe, 2004; Ketting, 2011). PTGS takes place in the cytoplasm at the mRNA level. Small iinterfering RNAs are then incorporated into an RNA-induced silencing complex (RISC) containing an Argonaute protein that has a small RNA-binding domain (Vaucheret *et al*., 2004). RISC subsequently unwinds and generates sense and antisense strands. The sense strand becomes degraded while RISC guides the antisense strand to target complementary mRNA transcripts via base pairing leading to degradation of the mRNA and thereby inhibiting protein biosynthesis (Brodersen and Voinnet, 2006; Ghildiyal and Zamore, 2009).

2.19 Host induced gene silencing (HIGS)

RNAi has been used as a key strategy in functional genomics. For instance, the technique has been employed in engineering crops with enhanced or reduced ability to accumulate specific metabolites hence altering their nutritional value (Davuluri *et al*., 2005; Kim *et al*., 2009). Apart from this, RNAi has been used to develop plants with improved resistance to biotic and abiotic stresses (Koch & Kogel, 2014). Scientists have exploited this innate RNA silencing machinery, termed host induced gene silencing, that plants and other eukaryotes use to regulate developmental processes and also protect themselves from invasion by nucleic acids, control diseases, insects (Price and Gatehouse, 2008), viruses (Duan *et al*., 2012) and fungi (Nunes and Dean, 2012). HIGS occurs when *in vitro* uptake of dsRNA signals PTGS of target genes in pathogenic microorganisms, pests and parasitic plants leading to protection of host plants. The process involves identification of suitable target genes in stress agents, production of

corresponding dsRNAs through construction of hairpin RNA constructs and their subsequent expression into host plants. Assessment of the activity of the construct on the phenotypes of target organisms is the final step (Koch & Kogel, 2014).

2.20 HIGS against plant biotic and abiotic stresses

RNAi holds promise in agriculture by providing solutions for controlling a vast array of crop pests and disease-causing pathogens (Runo, 2011). This has been demonstrated through silencing of genes against viruses (Duan *et al.,* 2012), insects (Huvenne and Smagghe 2009), nematodes (Niu *et al.,* 2006), parasitic plants (Alakonya *et al.,* 2012) and fungi (Nunes and Dean 2012) with studies elucidating the mechanisms of dsRNA uptake by these plant parasites hence setting up a platform for use of HIGS as a control strategy. For instance, more than 30 species of insects have been controlled using this technique with Mao *et al.* (2007) successfully controlling insect predation by engineering plants that synthesize secondary metabolites aimed at reducing predation upon insect attack. Similarly, nematodes have been effectively controlled in various plants through targeting of genes involved in parasitism (Lilley *et al.,* 2012).

2.21 Application of HIGS in downregulating aflatoxin biosynthesis in maize and groundnuts

Recent studies have reported trafficking of molecular material between plants and fungi (Panstruga, 2003). To add strength to this, a plant-fungal cellular interface has also been proposed, which has the potential to enable host-fungal translocation of molecules (Micali *et al.,* 2011; Voegele & Mendgen, 2003). Among the trafficked molecules are siRNAs, which can be used for targeted down-regulation of problem genes. It is understood that fungal pathogens and their plant hosts interact via haustoria; highly specialized cells through which signal exchanges and nutrient uptake occur. These connections, just like in the case with parasitic plants, facilitate movement of mRNA signals between the host plant cells and fungal pathogens thereby initiating silencing (Duan *et al.,* 2012). Nowara *et al.* (2010) reported a reduced infection by powdery

mildew through production of transgenic barley expressing hairpin RNA (hpRNA) cassette.

An RNAi construct with the target sequences in the sense and antisense orientations separated by an intron allows formation of siRNAs that bring about silencing (Alakonya & Monda, 2013). In this study, the aim was to make hpRNA constructs using *stcA, stc J* and *aflR* sequences in the sense and antisense directions and transform them into maize and groundnuts. It is postulated that colonization of transgenic plants by aflatoxigenic *A. flavus* would trigger translocation of the siRNAs from the transgenics into the fungi via the plant-fungal cellular interface. These siRNAs would then target and cleave the complementary sequences in the fungi leading to downregulation or inhibition of aflatoxin biosynthesis (Alakonya & Monda 2013).

2.22 Techniques for construction of hairpin RNAi cassettes

Construction of RNAi vectors for post transcriptional gene silencing has been successfully demonstrated using both conventional and Gateway™ cloning systems. RNA-mediated gene silencing is a powerful tool for dealing with many of the problems affecting plants and this has been achieved through transformation. The strategy involves making of constructs expressing self-complimentary or hairpin (hp) RNA with sequences homologous to the target gene (s) and then transforming them into plants (Zhong *et al.,* 2012). The making of an RNAi construct entails amplification of the target gene via PCR and then cloning it into a suitable vector as an inverted repeat. The inverted repeats are usually separated by a spacer region encoding an intron that makes the construct stable and also increases the silencing efficiency (Helliwell & Waterhouse, 2003). Several silencing systems have been developed but the means for insertion of the PCR product of target genes into the vectors differ. For instance, some vectors such as pSilent1 utilize the conventional cloning technique where the PCR products of target genes are inserted into the vector by restriction enzyme digestion and ligation. Other techniques utilize the Gateway™ recombination system (Zhong *et al.,* 2012).

2.23 The Gateway cloning technology

This is a two-step process based on the site specific recombination system by a phage λ to integrate its DNA into the *E. coli* chromosome (Karimi *et al.,* 2002). The first step involves cloning the gene of interest into an entry vector using the BP reaction. This is then followed by sub cloning of the gene from the entry clone in step one into a destination vector through an LR reaction to produce the expression clone (Karimi *et al.,* 2002). An entry clone is a donor plasmid made by recombining the PCR product of the gene of interest with flanking *att*B sites into the *att*P site mediated by the BP Clonase™ enzyme mix. The fragment in the entry clone is then transferred to a destination vector containing the *att*R sites by mixing both plasmids and using the LR Clonase enzyme mix (Wesley *et al.,* 2001).

Destination vectors often contain one of various selectable marker genes: *nptII, hpt* and *bar,* which confer resistance against kanamycin, hygromycin and glufosinate ammonium respectively. The markers are regulated by a promoter such as nopaline synthase (NOS) or cauliflower mosaic virus (CaMV) 35S and a terminator (Karimi *et al.,* 2002). The Gateway cloning technology has an advantage over conventional cloning in that it is less tedious and it takes less time to produce the expression clone. Furthermore, once an entry clone for the gene of interest has been made, it can easily be sub cloned into a wide variety of available destination vectors via the LR™ reaction. Generic gene silencing vectors such as the pHANNIBAL, pHELLSGATE and pSTARGATE series have been be used in construction of expression clones (Helliwell & Waterhouse, 2003).

2.24 Detection of aflatoxigenic fungi, determination and quantification of aflatoxins

Afltoxigenic fungi can first be identified by fluorescence on agar before they are tested chemically as this is easier and economically viable (Atanda *et al*., 2011). Desiccated coconut agar is the conventional medium used for detection of aflatoxigenic fungi and direct visualization of aflatoxins. Palm kernel agar has also been used for this work (Atanda *et al.,* 2006). However, several limitations of these media have been identified

such as shorter shelf life and weak fluorescence intensities and these affect visibility. Neutral red desiccated coconut agar (NRDCA) introduces a dye that has been widely used in many other staining methods that enhances aflatoxin fluorescence and detection ability. It produces a light pink background with bright fluorescence intensity making visibility clearer and aflatoxin detection faster. This method has been used to successfully identify *A. nomius* and detect aflatoxins as reported by Atanda *et al.* (2011). Once the aflatoxins have been identified, other quantification techniques need to follow to establish the levels of toxins accumulation such as enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC).

CHAPTER THREE

OPTIMIZATION OF A REGENERATION PROTOCOL FOR ICGV-CG2 AND CG2 GROUNDNUT LINES USING BAP AND 2, 4-D

3.1 Introduction

Groundnut (*Arachis hypogaea* L) is an important leguminous plant that is used as food and in generation of cash for the rural poor (Sharma & Anjaiah, 2000; FAOSTAT, 2010). The yields from *Arachis hypogaea* are estimated to be 38 million tons annually (FAOSTAT, 2010). These yields however face quality and quantity limitations as a result of biotic and abiotic stresses such as soil fertility, weeds, pests and diseases (Pandey *et al.,* 2012). More importantly is the contamination of groundnuts by aflatoxigenic fungi that produce toxic aflatoxins resulting in major food security and safaety concerns especially in rural populations in Africa where aflatoxin regulations are difficult to enforce (Bankole *et al.,* 2006). Genetic transformation of this crop could introduce genes that could provide a solution to these problems. However, there is a lack of efficient and reproducible regeneration protocols for most of the germplasm grown in sub Sahara Africa and this hinders their improvement through genetic engineering (Anuradha *et al*., 2006; Maina *et al.,* 2010).

Groundnut regeneration is genotype dependent and therefore optimization of an efficient *in vitro* regeneration protocol for genotypes adapted for sub Sahara Africa is imperative to their improvement (Iqbal *et al.,* 2011). Regeneration of groundnuts from various tissues such as leaf discs, cotyledons, cotyledonary nodes, hypocotyls, epicotyls and embryos has been reported (Kim *et al.,* 2003; Anuradha *et al.,* 2006; Iqbal *et al.,* 2011). However, the frequencies of plants regenerated have been low and this limits rapid concept testing of candidate genes targeted for introduction through genetic transformation. Direct organogenesis using cotyledons can circumvent the limitation possed by using other explants since transformants are obtained within a short period.

The aim of this study was therefore to optimize a regeneration protocol for sub Sahara region-popular groundnuts genotype ICGV-CG2 and CG2 through direct organogenesis using cotyledons.

3.2 Materials and Methods

3.2.1 Groundnut regeneration

3.2.1.1 Plant material and explant preparation

This work was done at the Plant transformation Laboratory at Kenyatta University. Summarily, mature groundnut seeds genotypes ICGV-CG2 and CG2 **(**Plate 1) were kindly provided by Dr. Santie de Villiers from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and used in regeneration. Murashige and Skoog (MS) salts (Murashige & Skoog, 1962) with vitamins were used in all experiments. For sterilization of plant materials, dry groundnut seeds of ICGV-CG2 and CG2 were first washed with running tap water for 20 minutes to remove adherent particles. Excess water was then blotted off before vapor sterilization using 100ml of 3.85% (v/v) sodium hypochlorite (NaOCl) from local bleach (Jik) with 3ml concentrated hydrochloric acid in a desiccator for 5 hours according to Clough and Bent (1998). The seeds were finally washed 3 times in autoclaved distilled water before being soaked in the water for 2 hours. The embryo was surgically excised off following the removal of the seed coat and the cotyledons dissected vertically into halves that were used as explants.

Plate 1. Seed material of the groundnut varieties used for regeneration.

3.2.1.2 Media preparation for groundnut regeneration

Shoot induction medium (SIM) comprised MS salts with vitamins (Duchefa, Biochemie, Netherlands) and 30g/l sucrose. The pH was adjusted to 5.7 using 1M sodium hydroxide/1N hydrochloric acid followed by addition of 8g/l plant agar. The media was autoclaved at 121ºC for 20 minutes, supplemented with different concentrations of 6-Benzylaminopurine (BAP) (1.5-9mg/l) and 2,4-dichlrophenoxy acetic acid (2, 4-D) (1, 1.5 and 2mg/l) and dispensed into sterile petri dishes under aseptic conditions in a laminar flow hood with a flame. Shoot elongation medium (SEM) comprised 4.4g/l MS salts with vitamins (Duchefa), 30g/l sucrose and 8g/l agar and halved levels of BAP i.e. (0.75, 1.5, 2.25 and 4.5mg/l).

3.2.1.3 Shoot induction

Six (6) explants were cultured per petri plate on the SIM in such a way that the cut edges were in contact with the medium for easy access of growth regulators by the meristematic cells. The experiment was replicated five (5 times). Plates were sealed with parafilm and incubated at 27±1ºC in the growth room under 16 hour light/8 hour dark photoperiod regime provided by cool fluorescent lamps ($100mEs^{-1}m^{-2}$). Emerging shoot buds were transferred onto shoot elongation medium after 14 days for 2 to 3 subcultures. Data on the number of explants forming shoots after the first and second subcultures was

collected and their frequencies computed using the Statistical Analysis Software (SAS) version 9.1 at 95% confidence level.

3.2.1.4 Root induction

Elongated shoots were rooted on root induction medium (RIM) comprising 4.4g/l MS salts with modified vitamins, 30g/l sucrose, and 8g/l agar at pH 5.7 supplemented with varying concentrations of Naphthalene acetic acid (NAA) (0.5, 1 and 1.5mg/l). Rooting plants were maintained on the RIM for 28 days. The number of shoots producing roots as well as the number of roots per shoot were counted and recorded. Frequencies of root formation in the two varieties were analyzed using SAS software. The number of plants that survived after transfer in the glass house was also counted and their frequencies computed in the two genotypes.

3.2.1.5 Acclimatization of regenerants

To acclimatize and harden the regenerants, plants with well-developed roots were transplanted into plastic pots (5cm diameter) containing autoclaved peat moss and maintained at 25ºC in a glasshouse. After ten days on peat moss, they were then transferred to larger pots (30cm diameter) with sand-soil (1:1 w/v) mixture and hardened for 10 days with irrigation using tap water. Surviving plants were allowed to grow to maturity with regular watering. Mature groundnut plants (after 3 months of growth) were uprooted and kernels collected, dried, shelled and the seeds stored.

3.3 Results

3.3.1 Groundnut regeneration

Groundnut regeneration via direct organogenesis was achieved from cotyledon explants in both varieties under study. The stages of *in vitro* plant regeneration in the two genotypes under this study are outlined in figure 3. When cotyledons from mature groundnut seeds were cultured on MS medium with vitamins and different combinations of BAP and 2,4-D, they showed direct shoot formation within 14 days. The shoot buds

formed at the proximal cut ends of the explants in all media treatments used. Within 3-7 days of culture initiation, the explants considerably enlarged in size and turned green (Plate 2A and B). This was later followed by multiple shoot bud differentiation in all treatments. Proliferation of multiple shoots was observed 3 days after first subculture in both genotypes although, in some cases, shoots could emerge before the first subculture (Plate 2C and D).

Among the various BAP and 2,4-D combinations tested, the highest shoot induction frequency (93.33%) was obtained when explants were cultured on MS medium supplemented with 4.5mg/L BAP and 1mg/L 2,4-D for CG2. The lowest frequency was 38.23%, obtained when cotyledons were cultured on media supplemented with 9mg/L BAP and 1.5mg/L 2,4-D. In ICGV-CG2, 4.5mg/L BAP along with 1.5mg/L 2,4-D produced the highest frequency of shoot induction of 90.33% while 9mg/L BAP and 1mg/L 2,4-D recorded the lowest frequency of shoot formation (Figure 2). A large white callus that did not regenerate into plantlets was also observed in both genotypes. This was seen in cultures of media supplemented with 2mg/L 2,4-D. The calli formed on the explants in the regions away from where the embryo was excised (Plate 2C and D). The average number of shoots recovered from each explant ranged from 3 to 10 although more shoots could be obtained if the explants were transferred and maintained on SEM for two or more extra sub-cultures (Table 1).

The number of shoots recorded per explant was highest on PGR regimes that produced the highest frequencies of regeneration with both varieties having a maximum of 10 shoots recovered per explant. Shoots became distinct and could be counted with ease after the second sub-culture at 28 days. Shoot buds transferred onto SEM comprising MS salts supplemented with reduced levels of BAP elongated within 28 days. Genotype CG2 shoot buds transferred to 2.75mg/L BAP had the shortest elongation period of 21 days and were well developed (Plate 2E and F).

Successful rooting in the elongated shoots was achieved on RIM supplemented with NAA. Root buds were first observed after 14 days of culture on the RIM and were fully developed within 25 days (Plate 2H and I). On average, 96.7% of the shoots in ICGV-CG2 recorded roots when the media was supplemented with 1mg/L NAA which was the highest. The lowest frequency of root formation in this variety was 66.7%, obtained when 0.5mg/L NAA was added to RIM. Similarly, CG2 produced high frequencies of root formation. In this genotype, 93.3% was the highest frequency obtained when the media was supplemented with 1mg/L NAA (Table 2). One interesting observation, however, was the *in vitro* development of flowers during shoot elongation in CG2 (Plate 2G). Rooted plants hardened well in the glasshouse on peat moss for 10 days and later developed to maturity in bigger pots in the greenhouse. High frequencies of survival were obtained following transfer of regenerants to the glasshouse (Table 3). The shoots were healthy, appeared phenotypically normal and later produced many pods (Plate 2K and L).

Plate 2. Developmental stages of regeneration in groundnuts through direct shoot bud formation from cotyledons. Swelling and greening of explants 7 days after initiation on SIM in CG2 and **(A)** and ICGV-CG2 **(B).** Differentiation of shoot buds (red arrow) at the proximal cut end of the cotyledon as seen under the light microscope in genotype CG2 **(C)** and ICGV-CG2 **(D).** Presence of the white callus that did not aid in regeneration is indicated with the white arrow. **E** and **F.** Multiple shoots for genotype ICGV-CG2 and CG2 respectively developing on shoot elongation medium after 28 days. **G.** *In vitro* development of flowers in CG2 on shoot elongation medium. Fully developed roots in genotype CG2 (**H**) and ICGV-CG2 (**I**) on root induction media supplemented with NAA. **J.** Acclimatization and hardening of developed shoots on peat moss using a pot and a plastic bag. Mature groundnut with many fully developed pods of genotype CG2 (**K**) and ICGV-CG2 (**L)**.

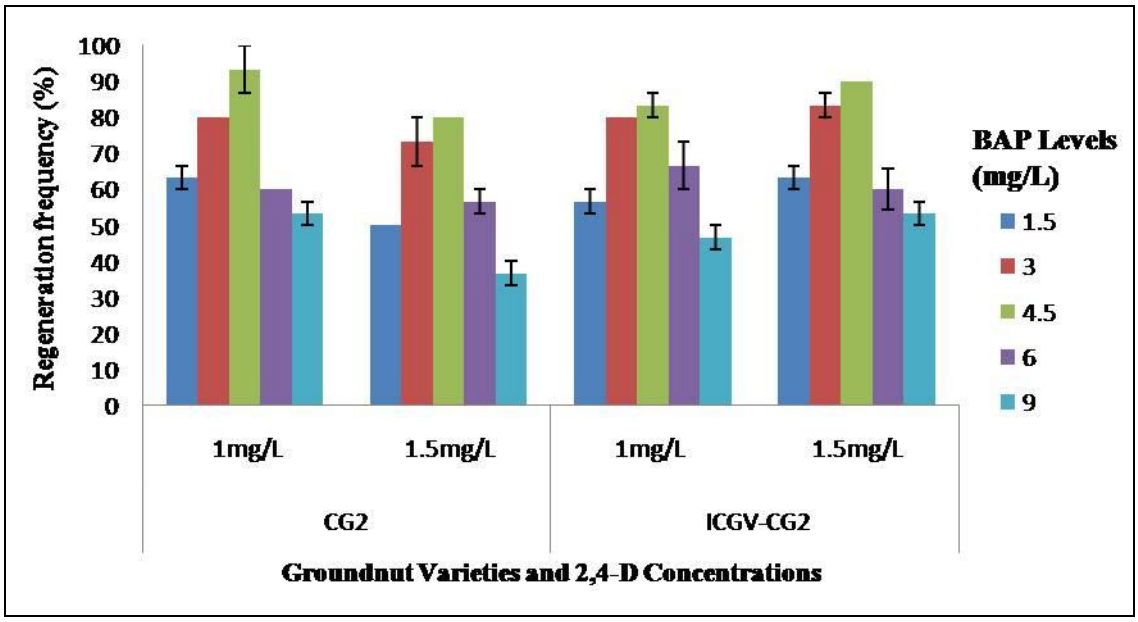

Figure 2. Effect of varying concentrations of BAP and 2, 4-D on frequency of regeneration in groundnuts. Data presented is the mean of 3 replicates of 30 explants each. Vertical bars indicate standard errors of the means according to Tukey's test at P≤0.05.

Table 1. Effect of varied 2, 4-D and BAP concentrations on the average number of shoots per explant in groundnut varieties at 35 days after culture on SIM

		Average no. of shoots per explant of different groundnut genotypes			
		1mg/L 2, 4-D		1.5mg/L 2, 4-D	
BAP Conc. (mg/L)	No of explants	CG2	ICGV-CG2	CG2	ICGV-CG2
1.5	30	1.33±0.33^{b¶}	1.33±0.33^{a¶}	1.33±0.33^{a¶}	1.33±0.33^{b¶}
3	30	2.33±0.33^{ab}	2.67±0.33^{a}	1.67±0.33^{a}	2.67±0.33^{ab}
4.5	30	3.33±0.33^{a}	3.00±0.58^{a}	2.33±0.33^{a}	3.33±0.33^{a}
6	30	1.67±0.67^{ab}	2.33±0.67^{a}	1.33±0.67^{a}	1.67±0.33^{b}
9	30	1.33±0.33^{b}	1.00±0.58^{a}	1.00±0.00^{a}	1.33±0.33^{b}

Means followed by the same letter in each column are not significantly different according to Tukey's test at *P≤0.05*.

Table 2. Effect of NAA on rooting frequency and on number of roots per shoot in groundnuts at 28 days after culture on RIM

NAA (mg/L)	Rooting frequency (%)		Average no. of roots per shoot
	CG2	ICGV-CG2	
0.5	63.3±3.3^{b¶}	66.7±6.7^{b¶}	4.7±0.6^{bc}
1.0	93.3±8.2^{a}	96.7±3.3^{a}	10.1±0.3^{a}
1.5	76.7±3.3^{ab}	83.3±3.3^{ab}	5.8±0.3^{b}

Data presented is the mean of 3 replicates of 10 explants each. ¶: Means followed by the same letter in each column are not significantly different according to Tukey's test at *P≤0.05*.

Table 3. Survival rates of groundnut genotypes regenerated

Genotype	No of plants acclimatized	Survival rate (%)
ICGV-CG2	23	95.33±2.33^{a¶}
CG2	28	91.47±6.7^{a}

¶: Means followed by the same letter in each column are not significantly different according to Tukey's test at *P≤0.05*.

3.4 Discussion

An efficient groundnut regeneration protocol for genotypes ICGV-CG2 and CG2 was successfully optimized using cotyledons derived from mature seeds. Various explants including cotyledonary nodes, epicotyls, hypocotyls and embryos have been used in the past to regenerate groundnuts (Tiwari & Tuli, 2008; Srinivasan *et al*., 2010; Iqbal *et al.*, 2011). The use of excised cotyledons in groundnut transformation has an advantage over other explants in that cells that are susceptible to *Agrobacterium* infection are easily accessible (Anuradha *et al*., 2006). The rapid regeneration of explants within 2-3 weeks and the high efficiency of shoot bud formation reported here may be ideal for any transformation process. Just like in this research, Iqbal *et al* (2011) were able to regenerate groundnuts using a combination of a cytokinin (BAP) and an auxin (picloram).

The effect of cytokinins in shoot bud formation has also been extensively reported in regeneration of various plant species including maize (Ombori *et al.*, 2008), cassava (Rossin and Rey 2011) and pigeon pea (Geetha *et al.*, 1999). However, it has been shown that combining an auxin and a cytokinin at an appropriate concentration is essential for shoot induction (Kakani *et al.*, 2009). Data on shoot bud formation from the 2 genotypes of groundnuts in this study showed significant variation across the treatments, demonstrating the important role played by genotypes as well as the concentrations of PGRs in regeneration. These results further indicated that supplementing RIM with NAA leads to differentiation and elongation of roots and is consistent with earlier studies (Verma 2009; Geng *et al*., 2011; Iqbal *et al*., 2011). The use of peat moss and plastic bags ensured effective acclimatization of plants in the glasshouse where they grew well to maturity with normal flowering and seed set. *In vitro* development of flowers during shoot elongation in CG2 was a rare occurrence that could have been due to somaclonal variation as a result of growth regulators and tissue culture conditions. Similar observations have been made in maize with Omer *et al* (2008) reporting dwarfism and formation of tassel seeds during regeneration.

3.5 Conclusions

These results demonstrate that groundnut genotypes ICGV-CG2 and CG2 adapted to East Africa are regenerable using the tested *in vitro* procedure on cotyledons. The optimum PGR level for shooting in genotype CG2 was 4.5mg/L BAP in combination with 1mg/L 2,4-D on MS media. The optimum PGR level for shooting in genotype ICGV-CG2 was 4.5mg/L BAP in combination with 1.5mg/L 2,4-D. Both groundnut genotypes had optimum rooting using MS media with 1mg/L NAA.

3.6. Recommendation

The regeneration protocol optimized during this study sets up a platform for the genetic manipulation of the crop against biotic and abiotic stresses affecting these groundnut genotypes or their derivatives.

CHAPTER FOUR

EFFECT OF DOWNREGULATION OF TRANSCRIPTION FACTOR AFLR ON AFLATOXIN BIOSYNTHESIS AND ACCUMULATION IN MAIZE AND GROUNDNUTS

4.1 Introduction

Accumulation of aflatoxins in grains like maize and groundnuts is a major food safety concern in sub Sahara Africa (Lewis *et al*., 2005; Mutegi *et al*., 2009). Management of these toxins has therefore become a priority in the region. Recently, targeted down regulation of gene expression by small interfering RNAs (siRNAs) has been successful in engineering crops against viruses (Duan *et al*., 2012), insects (Huvenne and Smagghe 2009), nematodes (Niu *et al.*, 2006), parasitic plants (Alakonya *et al*., 2012) and fungi (Nunes & Dean 2012). These biotechnological breakthroughs have opened up a platform for application of host induced gene silencing technique in developing crops against other stresses like aflatoxin biosynthesis and accumulation (Alakonya and Monda, 2013). This hypothesis is more realistic given that a plant-fungal cellular interface has also been proposed to have potential to enable host-fungal siRNA translocation (Voegele & Mendgen, 2003; Micali *et al*., 2011) and that the aflatoxin biosynthetic pathway is currently well characterized and understood (Bennet & Klich 2003; Yu *et al.*, 2004).

The aflatoxin biosynthestic pathway involves 25 genes clustered on a 70Kb span of chromosome III of the fungal genome and is regulated by a DNA binding protein (AflR) (Payne *et al*., 1993; Cary *et al*., 2000a). *In vitro* studies have shown that aflatoxin biosynthesis is conserved among the producers and that disruption of this gene thwarts the expression of other aflatoxin pathway genes and prevents aflatoxin biosynthesis (Payne *et al*., 1993; Cary *et al*., 2000b; Yu *et al*., 2004). More importantly, PTGS mediated by 21-24nt dsRNA is present and functions innately in fungi (Hamilton and Baulcombe, 1999; Macdonald *et al.*, 2005; Koch & Kogel 2014). *A. flavus and A. parasiticus* are the two leading fungal producers of aflatoxins worldwide (Yu *et al*., 2004). Further, there are reports that siRNAs can move from plants to fungi through a

plant-fungal cellular interface (Voegele & Mendgen, 2003; Micali *et al*., 2011). Guided by this understanding, the aim of this study was to generate and characterize transgenic maize and groundnut plants transformed with HIGS constructs against aflatoxin accumulation. This chapter reports transformation of maize and groundnuts targeting the conserved aflatoxin biosynthesis pathway transcription factor *aflR.*

4.2 Materials and methods

4.2.1 Preparation of the *aflR* silencing construct

4.2.1.1 Isolation of fungal genomic DNA from *Aspergillus flavus*

This work was done at the plant transformation laboratory at Kenyatta University, Nairobi, Kenya. First, fungal genomic DNA was extracted from a highly aflatoxigenic *A. flavus* isolate from Machakos (MCKII), an area endemic to aflatoxin outbreaks in Kenya, as described by Dehghan *et al* (2008). Briefly, 7 day-old fungal mycelia were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The mycelial powder was then re-suspended into DNA extraction buffer containing: 50M Tris-HCL, (pH 8.0), 50mM EDTA, 3% SDS, 1% ßmercaptoethanol and 50µl of 20mg/ml Proteinase-K. The suspension was then incubated at 65 ºC for 1 hour and the cellular debris removed by centrifugation at 13000rpm for 15 minutes. After addition of 25µl of 10mg/ml RNase A, the suspension was incubated at 37ºC for 30 minutes, extracted once with phenol-chloroform-isomyl alcohol (25:24:1) and once with chloroform-isomyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of ethanol and 3M sodium acetate, followed by centrifugation at 13000rpm for 30 minutes. The DNA pellet was rinsed with 70% ethanol and re-suspended in 50µL of RNase-free water and stored at -20ºC for PCR amplification.

4.2.1.2 PCR amplification of transcription factor *aflR*

The *aflR* specific primers were designed based on the NCBI gene bank sequence (accession number gi/19908787) and used to amplify the *aflR* gene via polymerase chain

reaction (PCR). The oligonucleotide primers were *aflR*-F AACCGCATCCACAATCTCAT and *aflR*-R GGTGCAGTTCGCTCAGAACA, purchased from the International Livestock Research Institute (ILRI) (Nairobi, Kenya). Polymerase chain reaction (PCR) analysis was carried in a 25µL reaction mixture using a thermocycler (Eppendorf, Hamburg, Germany). The PCR reaction mix components were as shown in Table 4. For PCR conditions, the master mix was pre-heated at 94ºC for 5 minutes (to give a hot start) and then subjected to 30 cycles consisting of denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 1 minute. A final 10 minute extension step at 72ºC was also included. The PCR product was electrophoresed on 1% agarose gel in TAE buffer stained with 1µl SYBR™ green and marked with DNA loading dye. The product was visualized under UV light in a trans-illuminator after running the gel at 80 volts for 1 hour.

Table 4. Contents of the PCR master mix used in amplification of *aflR* gene from genomic DNA isolated from *A. flavus*

Components	**Final Conc.**	**Vol. 1rxn (µl)**
PCR buffer (×10)	×1	2.5
dNTPs (10mM)	1mM	2.5
aflR Fwd 10µM	0.25µM	0.625
aflR Rev 10µM	0.25µM	0.625
Taq (5units/µL)	1 unit/rxn	0.2
Template DNA	10ng/µl	1
PCR Water		17.55
Total		**25**

4.2.1.3 Gel extraction and purification of the fragment

The amplified *aflR* product was recovered from the gel through gel extraction and purification of the fragment using Qiaquick gel extraction and purification kit (Qiagen, Maryland, USA) according to the protocol described in the user manual. Summarily, a clean sharp scalpel was used to excise about 300mg slice of the amplified fragment from

the gel into a 2ml tube. Then, 900µl of buffer QG was added to the gel slice in a 2ml tube and the contents incubated at 50ºC for 10 minutes with shaking after every 3 minutes. Once the color of the mixture was yellow, similar to that of the buffer, 300µl of isopropanol was added and the contents mixed thoroughly to precipitate the DNA. The DNA was then bound onto a membrane by applying 750µl of the sample to a Qiaquick spin column on a 2ml collection tube and centrifuged for 1 minute at 13000rpm. Traces of agarose were removed by adding 500µl of buffer QG to the column and spinning again for 1 minute. The bound DNA was washed using 750µl buffer PE (containing ethanol) with 1 minute spinning. An additional 1 minute spinning was included to remove traces of ethanol and the column placed in a clean 1.5ml centrifuge tube after which 50µl of elution buffer (10mM Tris-Hcl; pH 8.5) was added to the center of the membrane with 1 minute spinning at 13000rpm. The pure DNA was kept at -20ºC in a freezer for subsequent experiments.

4.2.1.4 Preparation of chemically competent *Escherichia coli* cells

Disarmed empty *E. coli* cells, strain DH5α were used in cloning. A protocol for preparation of chemically competent *E. coli* cells was modified from Tu *et al* (2005). According to the protocol, a single DH5α colony was inoculated into 20ml of liquid Luria and Bertani (LB) medium without antibiotics and then incubated overnight at 37ºC on a rotary shaker. Then, 2ml of the overnight cell culture was pipetted into 18ml of fresh LB medium and the contents incubated for 1 hour on a shaker at 37ºC. A 0.1M solution of $CaCl_2$ was prepared and then chilled on ice. The cells were centrifuged for 5 minutes at 13000rpm at 4ºC and the pellet transferred into 2ml centrifuge tubes chilled on ice. The pellet was re-suspended by gently pipetting 1ml of the ice cold $CaCl_2$ into the tubes. The cells were chilled on ice for 1 hour followed by centrifugation. After carefully discarding the supernatant, the pellet was re-suspended in 250µl of sterile ice cold $CaCl_2$. The now competent cells were chilled on ice for use in transformation as will be described in section 4.2.1.5.

4.2.1.5 TOPO cloning and *E. coli* transformation

Entry vector PCR8/GW/TOPO TA (Invitrogen Corp. Carlsbad CA, USA) (Figure 3) and the *aflR* PCR product purified in section 4.2.1.3 above were used in this step. A TOPO TA cloning reaction was set up using the reagents in Table 5 which are also described in the TOPO TA 8.0 cloning kit user manual (Invitrogen, CA USA). Summarily, the reagents were mixed gently and incubated for 5 minutes at room temperature. The mixture was then placed on ice before being used to transform *E. coli*.

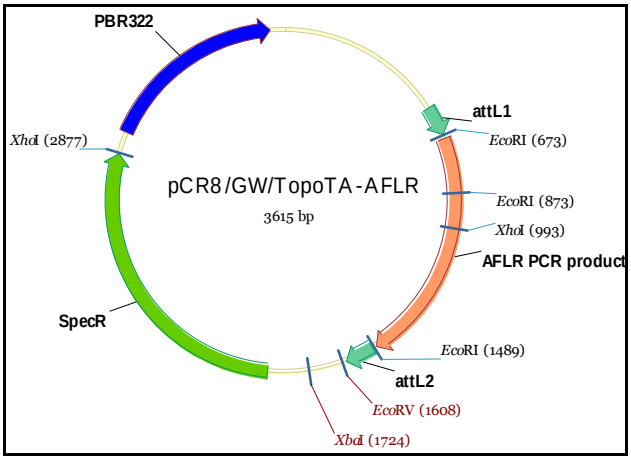

Figure 3. Map of PCR 8/GW/TOPO-TA entry vector

For *E. coli* transformation, one vial (50µl) of the prepared chemically competent cells was thawed on ice after which 2µl of the TOPO cloning reaction was added and mixed gently by finger flicking. This mixture was incubated on ice for 30 minutes. Cells were then heat shocked in a water bath at 42ºC for 30 seconds and the tubes immediately transferred to ice. Then, 250µl of LB broth medium was added followed by 1 hour incubation on a rotary shaker (37ºC) for the cells to grow. A sterile glass rod spreader was then used to spread 50µl of the bacterial culture on pre-warmed plates of solid LB medium containing 100mg/L spectinomycin and the plates incubated overnight at 37ºC. A negative control comprising non-transformed competent cells was also included.

Table 5. Reaction mix for cloning of the *aflR* PCR product into PCR8/GW/TOPO TA

Reagent	Vol/1 reaction (µl)
PCR product	2
Salt solution	0.5
TOPO vector	0.5
Total volume	3

4.2.1.6 Confirmation of transformed *E coli* cells

To verify whether transformed *E. coli* cells harbored the TOPO vector with *aflR*, a PCR using *aflR* primers was carried out. A 20µL reaction on the colonies likely transformed with PCR8/GW/TOPOTA/*aflR* was set up (Table 6). The PCR mixture was heated at 95ºC for 5 minutes and then subjected to 30 cycles consisting of denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 1 minute. A final 7 minute extension step at 72ºC was also included.

Table 6. Master Mix preparation for PCR amplification on likely transformed *E. coli* colonies

Component	Volume/reaction (µl)
Kapa Taq Ready mix ×1	10
aflR-Forward (10µM)	1
aflR-Reverse (10µM)	1
PCR water	8
Template DNA	Touch the colony And inoculate in mixture
Total	**20µl**

4.2.1.7 Restriction digestion of PCR8/GW/TOPO TA/*aflR* and sequencing

To further confirm ligation of of the *aflR* insert in the vector, a restriction digest was carried out using EcoRI enzyme. Here, a 30µl reaction comprising a master mix of 5µl of template DNA, 3µl buffer, 21µl of PCR water, 1µlof EcoRI (New England Bio-labs Inc., MA, USA) was set up. After aliquoting the master mix into PCR tubes, 5µl of the

template DNA was added to each tube and the mixture incubated for one hour at 37 ºC. The fragments released were confirmation by gel electrophoresis. This was done on a 1% agarose gel, run at for one hour at 110 volts. Two clones of the gene were taken to BeCA hub, ILRI, for sequencing. Sequences were retrieved and aligned with the Genebank sequence and only those clones perfectly matching the NCBI sequence were selected for further experiments.

4.2.1.8 Sub-cloning of *aflR* into binary vectors

To clone the *aflR* fragment into the expression cassettes pHellgate and pStargate, PCR8/TOPOTA/*aflR* was linearized through digestion with EcoRV restriction enzyme. It was then cleaned using a PCR cleaning kit (Qiagen, USA) and stored at -20ºC. The pHellsgate and pStargate vectors (Figure 4) are tailored for expression into dicots and monocots respectively. An LR Clonase™ reaction was set up using 3µl of the linearized clean PCR8/TOPOTA/*aflR*, 1µl of the binary vector (either pHellgate and pStargate) and 1µl of the clonase enzyme and the mixture incubated for 1 hour at room temperature according to the LR cloning kit user manual (Invitrogen Corp. Carlsbad CA, USA).

Then, 0.5µl of proteinase K was added to the reaction followed by 10 minutes of incubation at 37ºC. After the reaction, 2µl of the reaction was used to transform 30µl of *E. coli* chemically competent cells (prepared in section 4.2.1.4). Confirmation for the presence of the *aflR* insert in binary vectors was done via colony PCR targeting the *aflR* transgene. Briefly, a PCR reaction mix (without template DNA) was set up using Taq polymerase and *aflR* primers as earlier described. The reaction mixture was then aliquoted into 200µl tubes. Template DNA was added by touching a bacterial colony, using a sterile pipette tip, and re-suspending the bacterium into the reaction mixture. Finally, the PCR conditions described (section 4.2.1.6) were used for amplification and later confirmed on agarose gel. To verify orientation of the transgene in the vectors, a restriction digestion reaction was set up using EcoRI (New England Bio-labs Inc., MA USA). A 60µl reaction comprising Buffer 4 (X10), X100 BSA buffer with 1 unit/reaction EcoRI enzyme and 22µl of water was set up according to the

manufacturer's instructions. After aliquoting the master mix into PCR tubes, 30µl of the template DNA was added to each tube and the mixture incubated for 2 hours at 37ºC. A positive control from a vector with *aflR* was also included. EcoRI cuts the vector at 3 restriction sites and it is therefore expected to release 2 fragments and a backbone. One colony of the clone with the expected fragments was picked from which minipreps were prepared and used for *Agrobacerium* transformation.

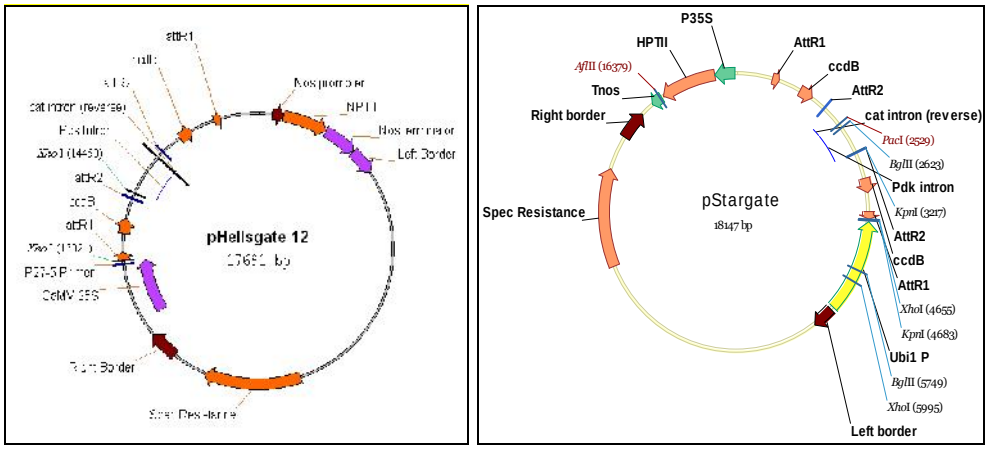

Figure 4. Plant expression vectors pHellsgate and pStargate used for expressing *aflR* precursors in groundnuts and maize respectively.

4.3 *Agrobacterium tumefaciens* transformation

To deliver the cloned constructs into groundnuts and maize, disarmed *A. tumefaciens*, strain EHA 101, was used. The bacterium was first initiated from glycerol stocks and maintained on solid LB medium supplemented with 100mg/l kanamycin. Chemically competent cells were prepared according to Tu *et al*. (2005). To transform EHA 101, one vial of the competent cells was thawed on ice and 1µl of plasmid DNA containing the recombined binary vectors with *aflR* added separately with gentle mixing. The mixture was frozen by immersing and maintaining the tubes in liquid nitrogen for 10 minutes. They were then removed and thawed at 37ºC in a water bath for 5 minutes. After transformation, 1ml of room temperature LB broth medium was added and the culture incubated in darkness at 28ºC for 3 hours on a shaker. Then, 50µl of the bacterial

culture was spread on LB agar plates containing 100mg/l spectinomycin and 50mg/l kanamycin for selection of the construct and bacteria respectively. The plates were wrapped with parafilm and incubated in darkness for 3 days at 28ºC. Emerging colonies were screened for presence of the hairpin *aflR* (hp-*aflR*) cassette via PCR using *aflR* primers. One positive colony was selected for each construct, pHellsgate and pStargate, and used to transform groundnuts and maize respectively.

4.3 Production of transgenic plants

4.3.1 Groundnut transformation

4.3.1.1 Infection and co-cultivation

A protocol for groundnut transformation using cotyledons was adopted from Anuradha *et al.* (2006). Briefly, a loopful of *Agrobacterium* EHA 101 carrying the pHellsgate/*aflR* from a three-day old single colony was inoculated into 20ml liquid LB medium supplemented with 50mg/l kanamycin and 100mg/l spectinomycin and grown overnight at 28°C. Bacterial suspension (5ml) was pelleted by centrifugation for 10 minutes at 5000rpm and the cells re-suspended in 25ml of filter sterilized infection medium comprising half strength MS with modified vitamins, 30g/l sucrose, 8g/l agar, supplemented with 100µM acetosyringone. A small volume of the bacterial suspension (2-3ml) was poured into a sterile petri plate and sterile freshly excised cotyledon explants (prepared as earlier explained in section 3.2.1.1 above) immersed with the cut edges into the bacterial suspension for 30 seconds.

Excess bacterial suspension was blotted off the cotyledons using sterile filter paper and the explants immediately cultured on groundnut SIM containing100µM acetosyringone, with the cut ends in contact with the media. Just like in the regeneration experiment in section 3.2.1.3, six explants were placed per plate before wrapping with parafilm followed by incubation in the growth room at 28°C with a 16-hour light/8 hour dark

photoperiod provided by cool fluorescent tubes. The cotyledons were co-cultivated with the *Agrobacterium* for 72 hours.

4.3.1.2 Shoot induction and selection of putative transformants

After co-cultivation, responding explants were washed with sterile water supplemented with 500mg/L cefotaxime to inhibit *Agrobacterium* growth. Excess water was blotted off using sterile Whatman filter paper before transfer of the explants to groundnut SIM supplemented with 4.5mg/l BAP and 1mg/l 2,4-D and filter-sterilized 250mg/l cefotaxime for two weeks with transfer to fresh medium once a week. Explants bearing shoots were transferred to medium containing 250mg/l cefotaxime and 125mg/l kanamycin to initiate selection for two subcultures of two weeks each. Explants bearing shoots were excised and transferred to SEM containing 175mg/l kanamycin for another two sub-cultures. The elongated shoots (3-4cm long) were transferred to groundnut RIM without any antibiotics and monitored for root development.

4.3.2 Maize transformation

4.3.2.1 Growth of explants in the field

Maize inbred line CML 144 from the International Maize and Wheat Improvement Center (CIMMYT) was used for production of explants for transformation. Seeds were first planted and maintained in a farm at JKUAT with regular watering. At silking, the maize plants were self pollinated and covered with a plastic bag for effective fertilization and prevent any cross pollination. Maize ears were harvested between 10 to 14 days after pollination and stored at 4ºC to await transformation.

4.3.2.2 Surface sterilization of maize ears and embryo excision

A protocol by Ishida *et al.* (2007) was adopted during maize transformation. Briefly, maize ears with embryos between 1-2mm long were harvested from the farm and stored at 4ºC. After removal of the husks, the ears were surface sterilized using 3.85% (v/v) NaOCl in Jik (bleach), together with 2 drops of tween 20 for 20 minutes. They were then

rinsed 3 times with autoclaved distilled water. A sterile scalpel blade was used to chop off the top of the kernels while they were still attached to the cob and an excisor used to isolate the immature embryos aseptically. Embryos were suspended in 5ml of filter sterilized infection medium (pH 5.2) in a sterile petri plate.

The infection medium comprised 4.4g/l MS with vitamins, 34.25g/l sucrose, 18g/l glucose, 1.5mg/l 2,4-D, 1mg/l casein hydrolysate and 100µM acetosyringone. Prior to embryo isolation, a loopful of *A. tumefaciens* with the pStargate/*aflR* construct was picked from a 3 day old plate grown on LB medium supplemented with 100mg/l spectinomycin and 50mg/l kanamycin and then suspended in 20ml of infection medium containing 100µM acetosyringone in sterile 50ml centrifuge tubes. The cultures were incubated at 28ºC in darkness for 3 hours on a shaker. This was followed by addition of 1ml of the *Agrobacterium* suspension to the embryos and the plate covered with aluminum foil to provide darkness and left for 5 minutes for efficient infection before co-cultivation.

4.3.2.3 Co-cultivation and callus induction

To initiate maize callus formation, infected embryos were transferred onto co-cultivation medium comprising 4.4g/l MS with vitamins, 20g/l sucrose, 10g/l glucose, 0.7g/l proline, 1.5mg/l 2,4-D, 100mM $CuSO_4$, 0.5g/l MES monohydrate and 8g/l agar. Excess *Agrobacterium* suspension was aspirated off using a sterile pipette tip and the embryos oriented to ensure that scutella were facing upwards on the surface of the medium. The plates were sealed with parafilm and later incubated at 22ºC in the dark for 3 days as described by Ishida *et al*. (2007). After 72 hours of culture, responding embryos were transferred onto resting medium (4.4g/l MS with vitamins, 20g/l sucrose, 0.7g/l proline, 1.5mg/l 2,4-D, 0.5g/l MES monohydrate and 8g/l agar, pH 5.8) supplemented with filter sterilized silver nitrate (1.6mg/l) and carbenicillin (250mg/l) and incubated at 28ºC in the dark for 10 days before taking them to selection.

4.3.2.4 Selection and regeneration of putatively transformed maize

To initiate selection of putatively transformed tissues, explants forming calli were transferred to media comprising 4.4g/l MS with vitamins, 30g/l sucrose, 0.7g/l proline, 1.5mg/l 2,4-D, 0.5g/l MES monohydrate and 8g/l agar supplemented with 250mg/l carbenicillin and 15mg/l hygromycin for 2 weeks. The cultures were covered with 2 layers of aluminum foil to provide darkness and later incubated at 28ºC. Surviving calli were transferred to second selection medium containing 30mg/l hygromycin for 28 days with a 2 week subculture. Hygromycin-resistant calli (now with somatic embryos) were transferred onto embryo maturation medium (4.4g/l MS with vitamins, 60g/l sucrose, 0.7g/l proline, 0.5g/l MES monohydrate and 8g/l agar, pH 5.8) with 250mg/l carbenicillin for 2 weeks. Calli showing presence of somatic embryos were transferred to regeneration medium comprising 4.4g/l MS with vitamins, 30g/l sucrose, 0.7g/l proline, 0.5g/l MES monohydrate and 8g/l agar, pH 5.8 and incubated in light. Regenerated plants with well-developed roots were acclimatized in the glasshouse according to Ishida *et al.* (2007).

4.3.3 Molecular analysis of putative transgenic maize plants

4.3.3.1 RNA isolation

To verify presence and expression of the hp-*aflR* cassette in putatively transformed maize plants, reverse transcriptase polymerase chain reaction (RT-PCR) analysis was carried out using 2 independent primer sets targeting the hygromycin (plant selectable marker) transgene and 18S rRNA (internal amplification control for maize) (Table 6). Briefly, total RNA was isolated from leaf tissues of each of the first generation (T1) plants using the RNeasy Plant Mini kit (Qiagen) including a step for on column DNase digestion. Summarily, approximately 20mg of ground tissue was used for total RNA extraction as per the instructions of the RNeasy® mini kit manual (Qiagen, Valencia. USA). To disrupt the tissues, 350µL of the RTL buffer was added to the 20mg of tissue and centrifuged for 3 minutes at 10000 rpm. One volume (350µL) of 70% ethanol was added to the lysate and mixed well by pipetting up and down.

Up to 700µl of the sample was transferred into an RNeasy mini spin column placed in a 2 ml collection tube and centrifuged for 30 seconds at 10000 rpm. The total RNA was subjected to DNAse treatment by adding 80µL of DNase 1 incubation mix directly to the RNeasy column membrane, and placing the component on bench top at 20-30°C for 15 minutes. Buffer RW1 (350µl) was then added to the column and centrifuged at 10000 rpm for 30 seconds. The flow through was discarded and the RNA washed by addition of 500µl of buffer RPE to the spin column followed by a 30 seconds spinning at 10000 rpm. The spin column was then placed in a new 2 ml collection tube and centrifuged at maximum speed for 1 minute to dry the membrane. The column was placed in new 1.5 ml collection tube and 30µL of RNase–free water added directly to the spin column membrane with a 1 minute spinning at 10000 rpm to elute the RNA. Total RNA was quantified using a nanodrop and 5µg used for cDNA synthesis.

4.3.3.2 Complementary DNA synthesis and RT-PCR

Complementary DNA (cDNA) synthesis was performed using the First strand Superscript™ III reverse transcriptase kit using random hexamers (Invitrogen). According to the manufacturer's instructions, 5ng of total RNA was first primed using 50ng/µl of random hexamers, 10mM dNTP mix and DEPC-treated water to 10µl volume. The reaction was first incubated at 65ºC for 5 minutes and then placed on ice for 1 minute. A cDNA synthesis mixture was then prepared by adding 2µl of 10X RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT, 1µl of 40U/µl RNaseOUT and 1µl of 200U/µl Superscript III reverse transcriptase enzyme in a separate tube. The mixture was gently mixed, and then 10µl added to the RNA/primer mixture above with gentle mixing. This reaction was then incubated at 25ºC followed by another incubation of 50ºC for 50 minutes. The reaction was stopped at 85ºC for 5 minutes and chilled on ice. Finally, 1µl of RNase H was added to the mixture and incubated for 20 minutes at 37ºC. The cDNA was then stored at -20ºC to await PCR. PCR amplification was performed in 20µl reaction mix containing 10ng cDNA, 1X KAPA Taq (containing 1.5mM $MgCl_2$ and 0.2mM of each dNTP) (www.kapabiosystems.com) and 2.5µM of each primer

(Table 7) using a DNA thermocycler (Eppendorf AG, Hamburg, Germany) at an annealing temperature of 61˚C for both primer sets.

Table 7. Primer sequences used for RT-PCR

Primer	Sequence 5'-3'
Hygro-F	GTCTGCACCATCGTCAACC
Hygro-R	GAAGTCCAGCTGCCAGAAAC
18S-F	TCCTGAGTAACGAACGAGACC
18S-R	CACGATGAAATTTCCCAAGAT

4.3.4 Bulking of transgenic maize seed

PCR positive plants were maintained in the glasshouse, regularly watered and allowed to grow. At tasselling and silking, transgenic plants were self-pollinated and covered with a plastic bag for 3 days to ensure a moist environment for effective fertilization. These plants were maintained in the glasshouse until maturity upon which they were harvested and dried in the sun. Cobs were then shelled and dried further to ensure most of the moisture was removed. The first generation (T1) kernels were then placed in storage bags and stored at 4ºC.

4.4 Evaluation of transgenic plants against aflatoxin accumulation

4.4.1 Infection of transgenic maize with aflatoxigenic *A flavus*

To determine the effect of the silencing construct on aflatoxin biosynthesis and accumulation in maize, transgenic plants were challenged with toxigenic fungi MCKII from Machakos. First, a non-wounding technique for maize infection as described by Windham and Williams, (2007) was employed. According to the protocol, a highly aflatoxigenic *A flavus* (MCKII) was first grown on potato dextrose agar (PDA) in petri plates for 5 days at 28ºC. The cultures were then flooded with 0.05% Triton X-100 and then gently rubbed with a glass rod to dislodge the fungi from the medium. Conidia were

diluted to 5×10^5 spores/ml using 0.05% Triton X-100 and sterile distilled water. The spore suspension, used as inoculum, was prepared one day before infection and stored at 4 ºC.

First generation (T1) transgenic maize seeds collected in section 4.3.4 above were planted in 15cm pots filled with soil and maintained in the glasshouse to grow. Ten (10) plants (biological replicates) from 5 independent transgenic events (technical replicate) were used in the assays with 10 wild type CML 144 plants as negative controls. The plants were inoculated 6 days after pollination by spraying each silk with 1ml *A. flavus* inoculum using a hand sprayer under controlled conditions in the glass house After inoculation, the ears were covered with a plastic bag for 3 days to maintain a humid environment so as to enhance fungal colonization. The plants were watered regularly until maturity upon which ears were harvested and dried under controlled temperature in the glasshouse. The maize was shelled upon drying and the seeds collected, wrapped in storage bags and stored at 4ºC for subsequent aflatoxin analyses.

4.4.2 Re-isolation of fungi colonizing transgenic maize

To recover the fungi used to challenge the maize above, *A. flavus* was re-isolated from the infected transgenic and wild type maize kernels collected in section 4.4.1 as described by Pitt and Hockings (1997). Summarily, seeds were first surface sterilized using 3% NaOCl for 3 minutes followed by a dip in 70% ethanol for 2 minutes. They were then washed 3 times in sterile distilled water under aseptic conditions in the lamina flow hood and then dried using sterile Whatman filter paper. Ten (10) seeds per cob from each of the 5 transgenic events and 10 wild type kernels were cultured on petri plates with modified Rose Bengal agar (MRBA) medium, wrapped with parafilm and the cultures incubated at 37ºC for 5 days. Fungal colonies were sub-cultured by touching the colony with a sterile pin and transferring a single spore onto fresh PDA plates with incubation for a further 7 days to obtain pure colonies of the fungus. *A flavus* was identified based on colony color and morphological characteristics under the microscope using an *Aspergillus* identification key by Klich and Pitt, (1988).

4.4.3 Testing for *in vitro* aflatoxin production of the re-siolated *A. flavus* isolates

The toxigenic potential of *A flavus* re-isolated from maize was first determined using neutral red desiccated coconut agar (NRDCA) medium according to Atanda *et al.* (2011). To prepare the NRDCA media, 10g of the desiccated coconut (purchased from Nakumat supermarket, Nairobi, Kenya) was soaked in 500ml of hot distilled water for 30 minutes, mixed aseptically in a blender for 10 minutes and later filtered through four layers of cheesecloth. Then, 1% bacteriological agar, 0.2% neutral red dye and 0.01% chloramphenicol were added to the filtrate followed by sterilization by autoclaving at 121ºC for 20 minutes. The media was dispensed into sterile glass plates after vigorously shaking it to a uniform mixture. Plates with NRDCA media were separately inoculated at the centre with a single spore of the re-isolated fungi from transgenic and wild type maize and other plates inoculated with MCKII (the original plate used for cloning and inoculation), sealed with parafilm and incubated at 28ºC for 5 days to grow. Plates were later examined under the long wave (365nm) U.V. light for fluorescence. Fluorescence results were scored using the plus (+) or (-) signs; where +++ indicates bright fluorescence**, ++** moderate, **+** weak and **–** no fluorescence.

4.4.4 Molecular determination of silencing of target *aflR*

To investigate silencing of target *aflR* transcripts in *A. flavus* re-isolated from transgenic maize with the hp-*aflR* construct, an RT-PCR analysis was carried out on fungal cDNA. Summarily, RNA was isolated from 5 day-old *A. flavus* cultures grown on yeast extract sucrose (YES) media. Approximately 200mg of mycelia were recovered with a sterile spatula, dried in absorbent paper and ground under liquid nitrogen in a sterile cold mortar and pestle. Total RNA was isolated using the RNeasy Plant Mini Kit with on column DNase digestion (Qiagen) according to manufacturer's instructions (in a similar fashion to plant RNA as earlier described in section 4.3.3.1). One microgram of the RNA was then subjected to cDNA synthesis using Superscript III reverse transcriptase kit with random hexamers (Invitrogen) before RT-PCR analysis.

Primers used for amplification of *aflR* were *aflR*-F CAAGTCTTTTCTGGGTTCTAAGC and *aflR*-R TTCGTTGCCTCCTACTTAGAC and were designed in such a way that the reverse primer annealed outside the cloned region to avoid amplifying the construct. B-*Tubulin* primers *Tub*-F GCTTTCTGGCAAACCATCTC and *Tub*-R GGTCGTTCATGTTGCTCTTCA were used as amplification control for fungi. Reverse transcriptase PCR was performed in 20µL reaction volumes comprising 8µL of One-Step RT-PCR Pre-Mix kit (KAPA TAQ), 0.2 µM of each primer and 1µl of template cDNA. Amplification was confirmed by electrophoresis on 1% agarose gel in TAE buffer stained with 1µl SYBR green and marked with DNA loading dye.

4.4.5 Quantification of aflatoxin production in maize kernels

Quantitative analysis of aflatoxins in maize kernels and fungal cultures was performed at the University of Nairobi via enzyme-linked immunosorbent assay (ELISA) using the RIDASCREEN® kit (R-Biopharm AG, Darmstadt, Germany). The maize kernels harvested and shelled in section 4.4.1 above were used in the analysis. Five (5) kernels each from each of the 5 independent transgenic events and 5 kernels from wild type plants (also infected with *A flavus*) were subjected to ELISA according to the manufacturer's instructions. The kernels were separately ground in a blender (with thorough cleaning using methanol/distilled water (70/30 v/v) in between samples) and then 2g of each sample weighed and transferred into a glass vial where 10ml of methanol/distilled water (70/30 v/v) was added and mixed well for 10 minutes at room temperature to extract the aflatoxins. The contents were then centrifuged for 10 minutes at 2000rpm to separate the debris. Then, 100µl of the supernatant was diluted using 600µl of distilled water.

Ten (10) micro titer wells (6 for standards and 4 for the samples with their duplicates) coated with capture antibodies were placed into a microwell holder and 50µL of aflatoxin standard solutions and sample filtrates added to them. The aflatoxin standards contain 0, 0.05, 0.15, 0.45, 1.35 and 4.05ppb aflatoxin B_1 in methanol/water. Afterwards,

50µl of enzyme conjugate and 50µl of the antibody were then respectively added to each well with gentle mixing by manually shaking the plates. The mixtures were incubated for 30 minutes at room temperature in the dark. The liquid was then poured out of the wells before tapping the microwell holder against adsorbent paper to ensure complete removal of the liquid from the wells. The wells were washed 3 times using 250µl washing buffer after which 100µl of substrate chromogen was added to each well with gentle shaking and the contents incubated for 15 minutes at room temperature in the dark. Finally, 100µl of a stop solution (containing 1N sulfuric acid) was added to stop the reaction. Aflatoxins were quantified by measuring the absorbance of samples using a microtiter spectrophotometer at 450nm (R-Biopharm AG, Darmstadt, Germany).

4.4.6 Quantification of *in vitro* aflatoxin production by fungal cultures

This analysis was also done at the Department of Veterinary Medicine, University of Nairobi, Kabete Campus. To get the quantities of aflatoxins produced by different fungal cultures from the transgenic and wild type maize plants in the florescence experiment above, the re-isolated MCKII *A. flavus* was cultured and tested for aflatoxin production on aflatoxin-inducing YES medium. The fungi was inoculated on petri plates and incubated at 28°C for 7 days in the dark. Aflatoxins were then extracted from the cultures as described by Khayoon *et al.* (2010). Briefly, 3 agar plugs were removed from one colony, placed into a 4ml vial and ground into a fine powder. Aflatoxins B1, B2, G1 and G2 standard solutions were prepared by injecting 1ml of acetonitrile (HPLC grade) into each acetonitrile:water (90:10, v/v%) with 1g of the ground *A. flavus* mycelia and shaken vigorously for 30 minutes. After filtering using a No. 4 Whatman filter paper, 1ml of the filtrate was passed through the ISOLUTE clean-up column which had been previously pre-conditioned with 5ml of methanol and 5ml acetonitrile: water (90:10, v/v %) at a flow rate of 2ml/min.

The eluate was evaporated to dryness under nitrogen in amber bottles (to provide darkness). The dry residues were then derivatized by adding 100µl trifluoroacetic acid (TFA) and 300µl n-hexane, vortexed for 30 seconds and allowed to stand for 15 minutes

in a dark place at room temperature. The mixture was then diluted with 900µl of acetonitrile: water (10:90, v/v%) and the derivatized samples vortexed for 30seconds. The two layers were allowed to separate, and the bottom layer introduced into HPLC vials. To prepare solutions for matrix matched calibrations, extracts of blank *A flavus* samples were spiked with appropriate amounts of aflatoxins working solutions after derivatization. Chromatographic analysis was carried out using a Waters Alliance HPLC system (Milford, MA, USA) with an auto sampler for fluorescence detection.

4.5 Measurement of plant growth parameters

To determine the effect of hp-*aflR* on plant growth, various growth parameters in first generation maize plants were investigated. Another set of experiments, different from those infected with *A. flavus* for aflatoxin analysis, was set up. Transgenic maize seeds, harvested in section 4.3.4 above were planted in 15cm pots filled with soil supplemented with animal manure in the glasshouse with regular watering. A total of ten (10) seeds from each of the 5 transgenic events were planted alongside 10 non-transformed seeds. Data on growth parameters (plant height, wet/dry weight and the number of kernels per cob) was collected on five (5) transgenic maize plants randomly sampled from the 5 independent transformation events and non-transformed CML 144 plants included as negative controls. Plant heights were taken upon silking (2 months) using a tape measure and their respective averages calculated. For yield determination, the number of kernels per cob was counted after harvest and their averages also computed. Wet weights of the harvested cobs were then taken using a weigh balance and recorded before drying them at 65ºC in an oven for 7 days. Upon drying, dry weights of the cobs from transgenic and wild type plants were determined using the weigh balance. Analysis of variance (ANOVA) on the plant height and weights was performed using SAS version 9.1 and comparisons drawn. Representative images of the plants were also taken during the developmental stages.

4.6 Molecular determination of *aflR* off targets in maize and groundnuts

To identify *aflR* homologs in maize and groundnuts, BLASTX searches were performed on non-redundant (NR) databases of nucleotide sequences at National Center for Biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST) and at Plant Transcription Factors Database (PTFD) (Center for Bioinformatics, Perking University, China) (http://www.planttfd.cbi.pku.edu.cn) with an E value cutoff of 1-e10. The *aflR* sequence from *A. flavus* used in cloning above was retrieved from available genomic sequences at NCBI. The *aflR* domain sequence was scored against NR maize transcription factor database using the PFAM matrix. Hits with PFAM trusted cutoffs were retrieved and considered for further analysis. Multiple sequence alignment (MSA) was carried out using Vector NTI Advance™ version 10.

The *aflR* transcript sequence was also used as a reference to query and predict potential off-targets between its siRNAs and the two ERFs identified after BLAST search on PTFD on the Si-Fi software v3.1 platform (http://labtools.ipk-gatersleben.de). The parameters used included a 50 antisense strand, starting with an A or U base, a 50 sense strand, starting with G or C, and the first seven bases from a 50 antisense strand end containing at least three to five A/U bases. Possible *aflR* siRNAs enumeration was also carried out using RNAiScan; a publicly accessible online analysis tool (http://bioinfo2.noble.org/RNAiScan.htm) according to the rules by Ui-Tei *et al.* (2004) The search included a 5' antisense strand, starting with an A or U base, a 5' sense strand, starting with G or C, and the first seven bases from a 5' antisense strand end containing at least three to five A/U bases. Another online database, pssRNAit (http://plantgrn.noble.org/pssRNAit) was then used for genome-wide off-target gene assessment by querying the cDNA/transcript libraries of *Zea mays* using the enumerated siRNAs.

4.7 Results

4.7.1 Construction of RNAi cassettes for groundnut and maize transformation

PCR amplification using *aflR* primers revealed the expected fragment (798bp) of the *aflR* gene that was used for sub-cloning (Plate 3A). *E coli* clones that revealed presence of the *aflR* insert in pHellsgate12 and pStargate vectors by PCR were analyzed further by restriction digestion using EcoRI and produced the expected bands of 616bp and 2kb on agarose gel in all the clones hence confirming the presence of the *aflR* cassette (Plate 3B). Successful preparation of the hp-*aflR* construct was also based on the perfect pairwise alignment between a 778 bp *aflR* fragment and the full cDNA sequence (accession number AF441439.1.) retrieved from the gene bank (Figure 5). Clone 6 (in Plate 3C below) met the criteria and was chosen and the plasmid DNA from the colony used to sub-clone the binary vectors (Plate 4A and B) separately. Confirmation for the presence of the constructs in the in *A tumefaciens* via PCR using *aflR* primers produced the *aflR* gene expected band of 798bp on agarose gel (Plate 4C).

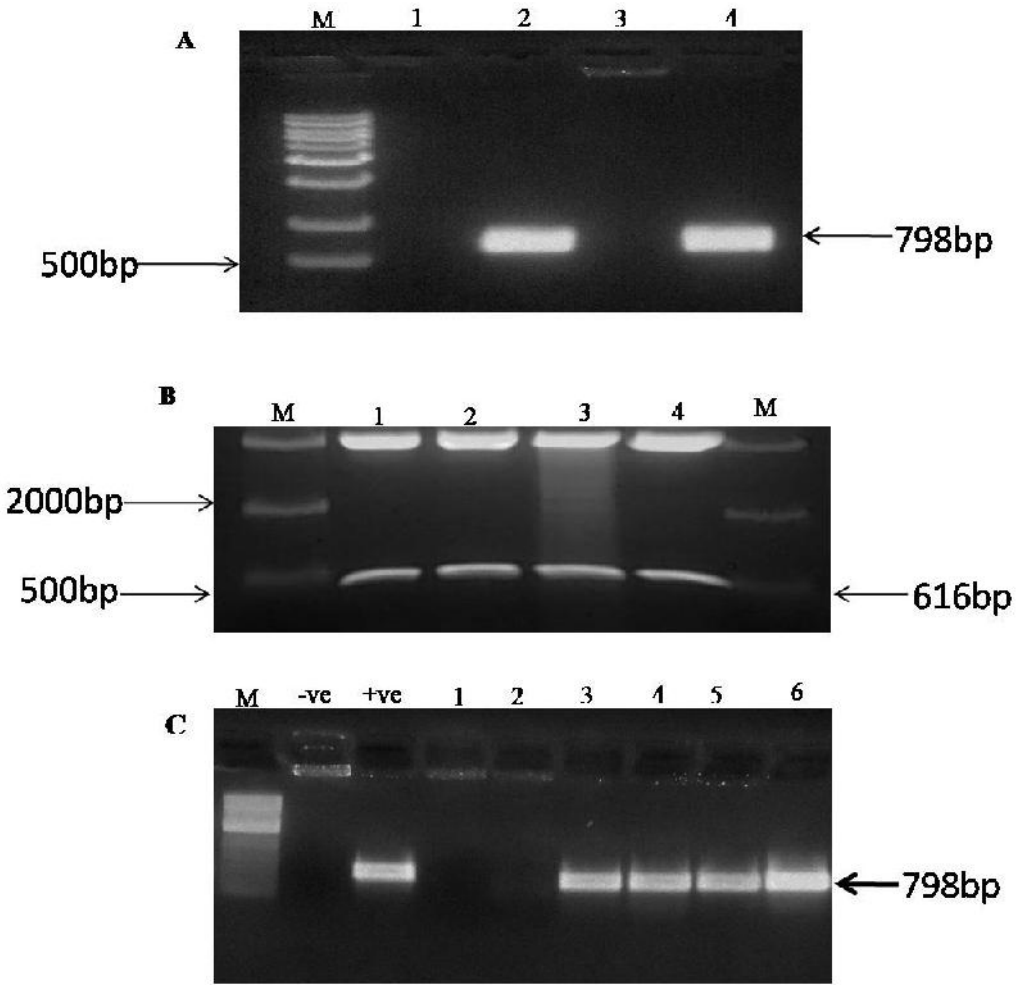

Plate 3. PCR cloning of entry vector PCR8/TOPOTA. **A.** Amplification of the *aflR* gene via PCR using *aflR* primers alongside a 1kb DNA ladder (M). Samples 2 and 4 were positively amplified revealing the expected fragment (798bp) of the *aflR* gene while no amplification occurred in 1 and 3. **B.** Restriction digestion of PCR8/TOPOTA/*aflR* vector in *E. coli* using EcoRI. Expected bands included a 616bp fragment, a smaller fragment of 200bp (not seen on the gel) and a backbone of 2815bp. Lane M was loaded with a 1kb DNA ladder. **C.** Amplification of *aflR* in *E. coli* through colony PCR using *aflR* primers alongside a positive control carrying the aflR gene. Lane M was loaded with 1kb DNA ladder.

```
                                1                                                 50
AF441439.1. Gene bank     (1) CATGGCTGAGGATAGCTCGTGAACAAGGCCCAGCGGGGAAGATATTGAAT
          hp aflR ZM      (1) --------------------------------------------------
           Consensus      (1)
                               51                                                100
AF441439.1. Gene bank    (51) TAGAAGTCCGCCGGCAAATCGCCGTGCGGTGCAAGGAGATCTATTGAACT
          hp aflR ZM      (1) --------------------------------------------------
           Consensus     (51)
                              101                                                150
AF441439.1. Gene bank   (101) TTTCATTGGATTTTAAAAGTCGCTGAGAATACGGGTGATCTGAAGAGGTT
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (101)
                              151                                                200
AF441439.1. Gene bank   (151) TTGGATTTGACCAGTGTAGTCCTTCCTCTGCGTCAAGGAGTGCTGATCTG
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (151)
                              201                                                250
AF441439.1. Gene bank   (201) CAAGCCGGGTACTATCCGCCGACACGTACTTTTGATCTTTAGCAAGCCAT
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (201)
                              251                                                300
AF441439.1. Gene bank   (251) CGTTAGCAAGGCAGGCATATCTATGTCCCATTCTTAGAATAGCTTCGCAG
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (251)
                              301                                                350
AF441439.1. Gene bank   (301) GGTGGTTTATCAACACCCCAACAGGACGGACCCAGGGCTCCCTGGCGCTC
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (301)
                              351                                                400
AF441439.1. Gene bank   (351) ATGCAGGTGCCAAAGATCTAGCTTGCAGGAAACAAGTCTTTTCTGGGTTC
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (351)
                              401                                                450
AF441439.1. Gene bank   (401) TCAGCCCGCCCATGACGGACTACGTTATCTTGAGCCCGAGGCATGCATGC
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (401)
                              451                                                500
AF441439.1. Gene bank   (451) GGGCGGGCCAGCTAGCTGAACATTATTTGTTATTCTTGGTTTGATTCGTT
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (451)
                              501                                                550
AF441439.1. Gene bank   (501) AAACCGATCCCGTAGTTCTCTGGTCACCCGGTTTCAGCCTCGGTATGTAA
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (501)
                              551                                                600
AF441439.1. Gene bank   (551) ACAAGGAATGCACAGCTAGACAATCCTTAGGTCAAGTCAGAACCCCTCAG
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (551)
                              601                                                650
AF441439.1. Gene bank   (601) CTAGTGACGTTTGTGTACATACATTTAGACCTCAGTGCGAGGCAACGAAA
          hp aflR ZM      (1) --------------------------------------------------
           Consensus    (601)
```

```
                                651                                                 700
AF441439.1. Gene bank   (651) AGGGCAGGCTACTCTCCAGGAGAAAGCCTTCACATTGTGTGTTTTCTTTC
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (651)
                                701                                                 750
AF441439.1. Gene bank   (701) CCGCTTTCAATTGAAAATTATACTGAATTCCTTCCTCAACTCCACGATGG
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (701)
                                751                                                 800
AF441439.1. Gene bank   (751) TTGACCATATTTCCCCCCGGGCATCTCCCGGGCCGATGCGTTCCTCCCAG
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (751)
                                801                                                 850
AF441439.1. Gene bank   (801) ACTCGCCGCGCTCGAAAGCTCCGGGATAGCTGTACGAGTTGTGCCAGTTC
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (801)
                                851                                                 900
AF441439.1. Gene bank   (851) AAAAGTGCGATGCACCAAGGAGAAACCGGCCTGTGCTCGGTGTATCGAAC
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (851)
                                901                                                 950
AF441439.1. Gene bank   (901) GTGGTCTTGCCTGTCAATACATGGTCTCCAAGCGGATGGGCCGCAATCCG
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (901)
                                951                                                1000
AF441439.1. Gene bank   (951) CGCGCTCCCAGTCCCCTTGATTCAACTCGGCGACCATCAGAGAGTCTTCC
           hp aflR ZM     (1) --------------------------------------------------
            Consensus   (951)
                               1001                                                1050
AF441439.1. Gene bank  (1001) TTCAGCCGGATCGGAACAGGGACATCCGGCGCATAACACGTATTCAACTC
           hp aflR ZM     (1) --------------------------------------------------
            Consensus  (1001)
                               1051                                                1100
AF441439.1. Gene bank  (1051) CTCATGCTCATCCCCAACCGCATCCACAATCTCATCCTCAATCGAATCAA
           hp aflR ZM     (1) ------------------------------------------------ATCAA
            Consensus  (1051)                                                    ATCAA
                               1101                                                1150
AF441439.1. Gene bank  (1101) CCACCACACGCTCTGCCCACCCCCAATGGTAGCAGTGGTGTCTCCGCCAT
           hp aflR ZM     (6) CCACCACACGCTCTGCCCACCCCCAATGGTAGCAGTGGTGTCTCCGCCAT
            Consensus  (1101) CCACCACACGCTCTGCCCACCCCCAATGGTAGCAGTGGTGTCTCCGCCAT
                               1151                                                1200
AF441439.1. Gene bank  (1151) CTTTTCTCATCAGAGTCCCCCGCCACCCGTGGAGACCCAGGGCCTTGGAG
           hp aflR ZM    (56) CTTTTCTCATCAGAGTCCCCCGCCACCCGTGGAGACCCAGGGCCTTGGAG
            Consensus  (1151) CTTTTCTCATCAGAGTCCCCCGCCACCCGTGGAGACCCAGGGCCTTGGAG
                               1201                                                1250
AF441439.1. Gene bank  (1201) GAGATTTGGCCGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAATCGAT
           hp aflR ZM   (106) GAGATTTGGCCGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAATCGAT
            Consensus  (1201) GAGATTTGGCCGGTCAGGAGCAAAGCACCCTGTCTTCCCTAACAATCGAT
                               1251                                                1300
AF441439.1. Gene bank  (1251) TCGGAATTTGGGGGCTCTTTGCAGTCCATGGAACACGGAAACCATGCCGA
           hp aflR ZM   (156) TCGGAATTTGGGGGCTCTTTGCAGTCCATGGAACACGGAAACCATGCCGA
            Consensus  (1251) TCGGAATTTGGGGGCTCTTTGCAGTCCATGGAACACGGAAACCATGCCGA
                               1301                                                1350
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AF441439.1. Gene bank  (1301) TTTCTTGGCTGAGCCGACGGGAAGTCTTTTCGACGCGTTTTTGGAAGTGG
           hp aflR ZM   (206) TTTCTTGGCTGAGCCGACGGGAAGTCTTTTCGACGCGTTTTTGGAAGTGG
            Consensus  (1301) TTTCTTGGCTGAGCCGACGGGAAGTCTTTTCGACGCGTTTTTGGAAGTGG
                              1351                                            1400
AF441439.1. Gene bank  (1351) GGACCCCCATGATCGACCCGCTCCTCGAGTCGGCCCCACTGCCACCGTTT
           hp aflR ZM   (256) GGACCCCCATGATCGACCCGCTCCTCGAGTCGGCCCCACTGCCACCGTTT
            Consensus  (1351) GGACCCCCATGATCGACCCGCTCCTCGAGTCGGCCCCACTGCCACCGTTT
                              1401                                            1450
AF441439.1. Gene bank  (1401) CAGGCGCGCTATTGCTGCTTTTCGCTTGCACTACAAACACTGACCCACCT
           hp aflR ZM   (306) CAGGCGCGCTATTGCTGCTTTTCGCTTGCACTACAAACACTGACCCACCT
            Consensus  (1401) CAGGCGCGCTATTGCTGCTTTTCGCTTGCACTACAAACACTGACCCACCT
                              1451                                            1500
AF441439.1. Gene bank  (1451) CTTCCCCCACGCCCCGCTGGGCTGTCAGCTGCGGCTGACAGACGGCGAGG
           hp aflR ZM   (356) CTTCCCCCACGCCCCGCTGGGCTGTCAGCTGCGGCTGACAGACGGCGAGG
            Consensus  (1451) CTTCCCCCACGCCCCGCTGGGCTGTCAGCTGCGGCTGACAGACGGCGAGG
                              1501                                            1550
AF441439.1. Gene bank  (1501) ACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGGGAACAAG
           hp aflR ZM   (406) ACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGGGAACAAG
            Consensus  (1501) ACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTCGGGGAACAAG
                              1551                                            1600
AF441439.1. Gene bank  (1551) AAGGCTACAGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGA
           hp aflR ZM   (456) AAGGCTACAGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGA
            Consensus  (1551) AAGGCTACAGATGCGGTCCGGAAGATCCTCGGGTGTTCGTGCGCGCAGGA
                              1601                                            1650
AF441439.1. Gene bank  (1601) TGGCTACTTGCTGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCGT
           hp aflR ZM   (506) TGGCTACTTGCTGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCGT
            Consensus  (1601) TGGCTACTTGCTGAGCATGGTCGTCCTTATCGTTCTCAAGGTGCTGGCGT
                              1651                                            1700
AF441439.1. Gene bank  (1651) GGTATGCAGCGGCAGCAGGCACCCAGTGTACCTCGACGGCGGTGGGGGGA
           hp aflR ZM   (556) GGTATGCAGCGGCAGCAGGCACCCAGTGTACCTCGACGGCGGTGGGGGGA
            Consensus  (1651) GGTATGCAGCGGCAGCAGGCACCCAGTGTACCTCGACGGCGGTGGGGGGA
                              1701                                            1750
AF441439.1. Gene bank  (1701) GAAACCAGCAGTGGCAGCTGTGGCAACAGTCCCGGCACCGTGTCCAGTGG
           hp aflR ZM   (606) GAAACCAGCAGTGGCAGCTGTGGCAACAGTCCCGGCACCGTGTCCAGTGG
            Consensus  (1701) GAAACCAGCAGTGGCAGCTGTGGCAACAGTCCCGGCACCGTGTCCAGTGG
                              1751                                            1800
AF441439.1. Gene bank  (1751) CTGTCTGTCGGAAGAGCGCGTGCTGCACCTGCCAAGTATGGTGGGCGAGG
           hp aflR ZM   (656) CTGTCTGTCGGAAGAGCGCGTGCTGCACCTGCCAAGTATGGTGGGCGAGG
            Consensus  (1751) CTGTCTGTCGGAAGAGCGCGTGCTGCACCTGCCAAGTATGGTGGGCGAGG
                              1801                                            1850
AF441439.1. Gene bank  (1801) ATTGTGTGGATGAGGAAGACCACCCGCGAGTGGCGGCACAGCTCGTTCTG
           hp aflR ZM   (706) ATTGTGTGGATGAGGAAGACCACCCGCGAGTGGCGGCACAGCTCGTTCTG
            Consensus  (1801) ATTGTGTGGATGAGGAAGACCACCCGCGAGTGGCGGCACAGCTCGTTCTG
                              1851                                            1900
AF441439.1. Gene bank  (1851) AGTGAACTGCACCGAGTACAGTCGCTGGTGAATCTATTGGCCAAGCGCCT
           hp aflR ZM   (756) AGTGAACTGCACCGAGTACAGTC-------------------------
            Consensus  (1851) AGTGAACTGCACCGAGTACAGTC
                              1901                                            1950
AF441439.1. Gene bank  (1901) GCAAGAAGGCGGAGACGATGCAGCATGGATACCGACGCACCATCCAGCAT
           hp aflR ZM   (779) -------------------------------------------------
            Consensus  (1901)
                              1951                                            2000
AF441439.1. Gene bank  (1951) CCCCTATCTCACTACTCGGATTTAGCGGCCTCGAAGCAAATCTCCGTCAC
```

```
           hp aflR ZM   (779) ------------------------------------------------
            Consensus  (1951)
                                2001                                        2050
AF441439.1. Gene bank  (2001) CGCTTGCGCGCCGTGTCCTCTGACATTATTGATTACCTGCATCGAGAATG
           hp aflR ZM   (779) ------------------------------------------------
            Consensus  (2001)
```

Figure 5. Pairwise sequence alignment between full cDNA of transcription factor *aflR* from *Aspergillus flavus* (AF441439.1. Gene bank) with the cloned fragment (hp-*aflR* ZM). Alignment was done using Vector NTI Advance™ version 10 and it revealed 100% similarity between the 778bp fragment cloned into pStargate vector and the full cDNA. The region marked yellow shows perfect alignment.

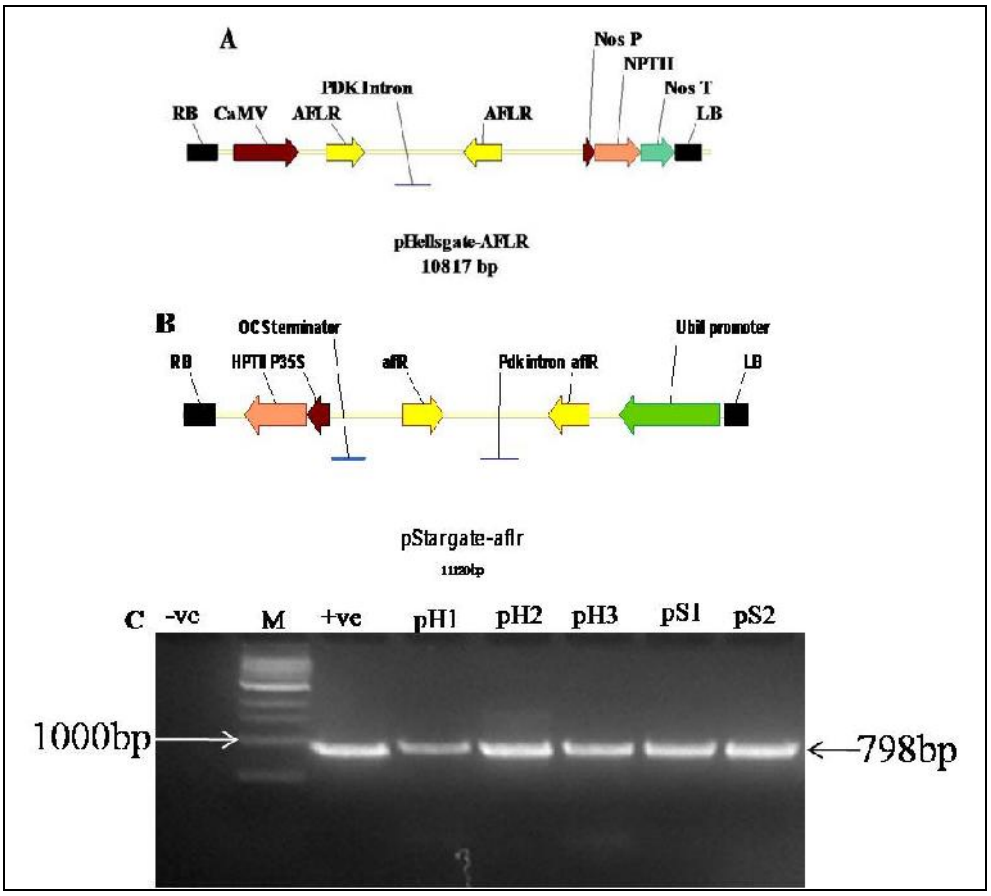

Plate 4. Linearized maps for pHellsgate/*aflR* and pStargate/*aflR* vectors forexpression into groundnut and maize respectively. **A.** Schematic representation of the T-DNA region of the binary vector pHellsgate with the *aflR* sequences in sense and antisense orientation. The aflR genes are driven by a 35S CaMV promoter and an OCS terminator. It also carries a kanamycin sulfate *(nptII)* resistance gene for plant selection driven by Nos promoter and terminator. **B.** Schematic representation of the T-DNA region of the binary vector pStargate with the *aflR* sequences in sense and antisense orientation. From the RB-right border is the hygromycin phosphotransferase (*HPTII*) resistance gene for plant selection driven by the 35S cauliflower mosaic virus promoter and terminator at the downstream of the plant selection marker. The silencing sequences are driven by the maize ubiquitin promoter while aflR sense and antisense sequences are separated by pdk intron with an octopine synthase terminator downstream. **C.** Confirmation by PCR amplification of the *aflR* gene cloned in pHellsgate/*aflR* (pH) and pStargate/*aflR* (pS) in *A. tumefaciens* strain EHA 101 colonies via PCR using *aflR* primers. Lane M was loaded with 1kb DNA ladder.

4.7.2 Transformation

When groundnut cotyledons were co-cultivated with the *A. tumefaciens* harboring binary vector pHellsgate/*aflR*, they exhibited greening and later developed shoots on SIM **(**Plate 5A**).** Explants forming shoots, when transferred onto first selection medium with 125mg/l kanamycin, showed browning after 7 days of culture due to the effect of kanamycin sulfate (Plate 5B and C). This was observed in both putatively transformed shoots as well as the non-transformed groundnuts used as controls. Some of the shoots that survived first selection underwent severe browning and eventually died off when maintained on second selection medium containing 175mg/L kanamycin.

Attempts to further multiply and root the putative transformants *in vitro* encountered difficulties. It was observed that shoots could not elongate further even after maintaining them on media for 4 weeks with subculture after every 2 weeks. Both varieties exhibited plants that had small and poorly formed leaves with very short stems (Plate 5D and E). Elongating the time of culture on SEM did not result in any observable difference in both varieties. Two (2) elongated shoots from both varieties were successfully rooted on media supplemented with 1mg/L NAA but without antibiotics (Plate 5F). However, these plants were found to be negative after PCR with kanamycin primers. Analysis of variance (ANOVA) according to Tukey's HSD test at $P \leq 0.05$ revealed no significant differences in both transformation and regeneration frequencies between the groundnut varieties tested (Figure 6).

Transformation of maize embryos using the protocol by Ishida *et al.* (2007) resulted in trangenics as outlined in Plate 6. Selection of putative hp-*aflR* maize transformants was achieved using hygromycin **(**Plate 6A). Compact calli surviving hygromycin action (Plate 6B) was obtained during the process. These calli later regenerated into whole plants (Plate 6C) with successful hardening and acclimatization on peat moss (Plate 6D). A transformation frequency of 15.12% was obtained from the total number of calli surviving the second selection (30mg/L hygromycin) as a percentage of the total number

of embryos co-cultivated with EHA 101 harboring the RNAi construct. The regeneration frequency was 43.11% expressed as the total number of calli producing at least one shoot as a percentage of the total number of calli surviving second selection. A total of 41 transgenic plants were regenerated from the 5 independent transformation events in the study (Table 8).

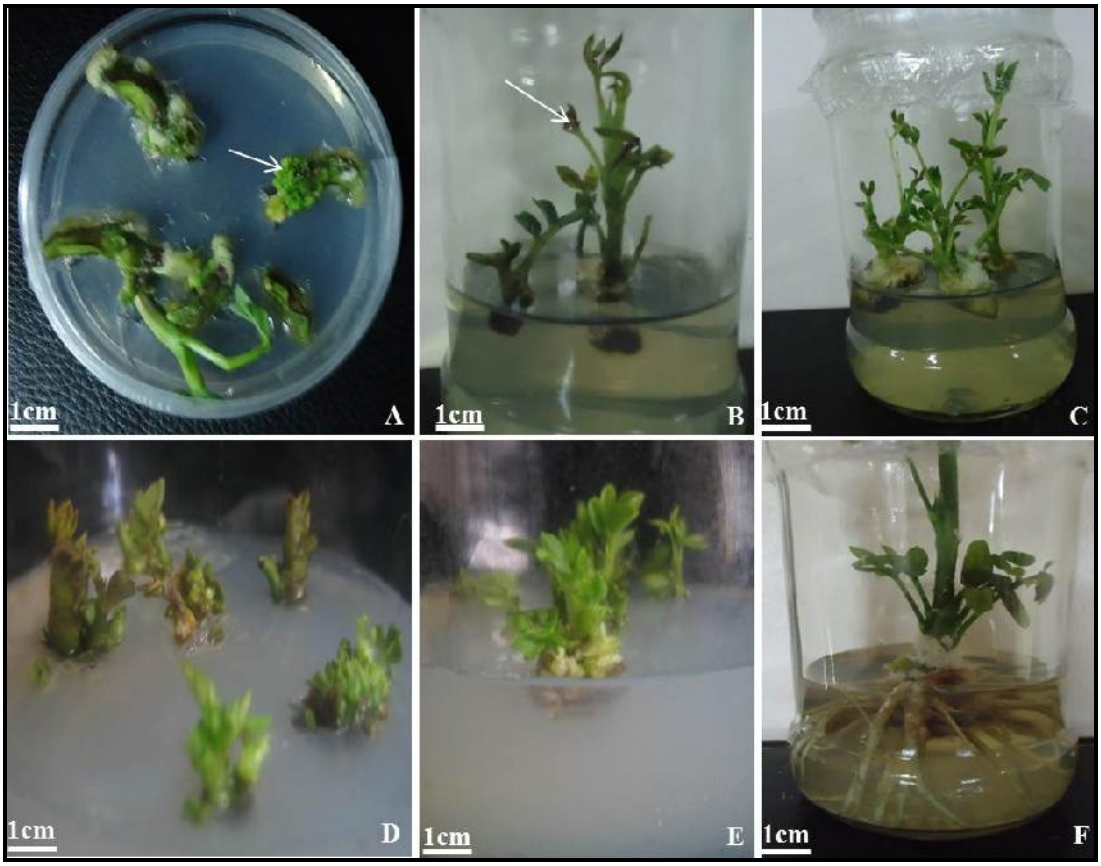

Plate 5. Stages in transformation and regeneration of putative transgenic groundnuts. **A.** Proliferation of multiple shoot buds in groundnuts genotype CG2 after 2 weeks of culture on SIM. **B.** First selection media with 125mg/l kanamycin showing browning of the leaves. Arrows show browning leaves. **C.** Groundnut shoots on second selection media with 175 mg/l kanamycin. **D.** Appearance of putatively transformed CG2 shoots that survived selection in (C) after excision for multiplication. **E.** A stunted shoot in genotype ICGV-CG2 after 8 weeks of culture on SEM. The shoots could not elongate further. **F.** A putatively transformed groundnut plant that survived selection, elongated and rooted well on RIM with 1mg/l NAA. This plant was found to be non-transformed after PCR.

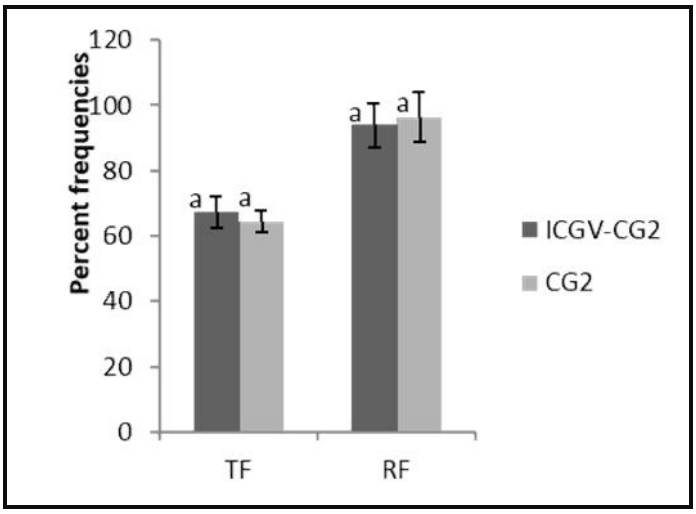

Figure 2. Transformation (TF) and regeneration (RF) frequencies for groundnuts. Data presented is the mean of 3 replicates of 30 explants each. Error bars indicate standard deviations of the respective frequencies according to Tukey's HSD test at *P≤0.05*.

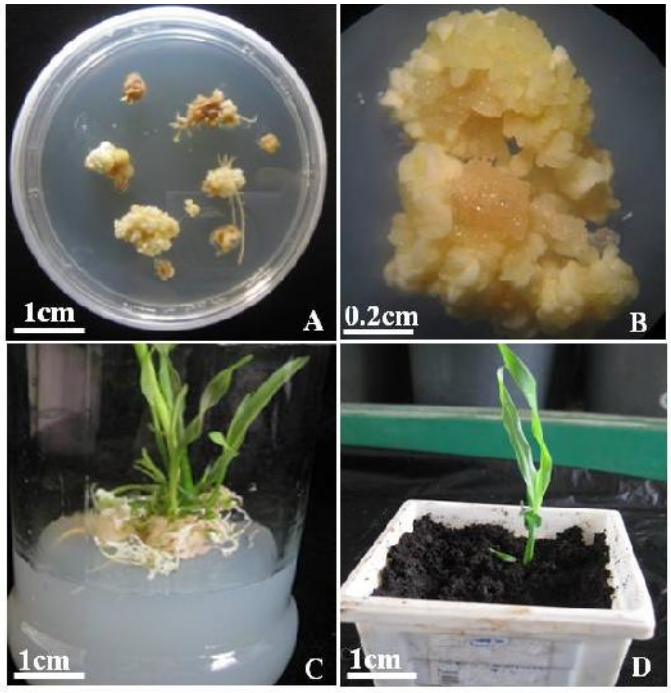

Plate 6. Stages in transformation and regeneration of putative transgenic maize plants. **A.** Putatively transformed maize calli maintained on MS medium with 30mg/l hygromycin for selection. **B.** Compact callus obtained after second selection showing presence of somatic embryos. **C.** Multiple maize shoots on regeneration medium. **D.** Acclimatization of a maize plantlet on peat moss in the glass house.

Table 8. Number of plants regenerated from immature embryos of CML 144 transformed with *hp-aflR*

Transformation experiment	Number of embryos infected	Number of plants regenerated
1	101	9
2	98	7
3	100	5
4	97	11
5	109	9
Total	**505**	**41**

4.7.3 Expression of hp-*aflR* construct in transgenic maize

RT-PCR analysis of T_1 maize plants for the transgene revealed that 28 out of the 41 transformants screened were positive, producing the expected 861bp fragment of the hygromycin gene on the agarose gel. The band only amplified in the transgenic lines but not in the wild type (Plate 7A). The 18S ribosomal RNA gene used as the internal amplification control was also amplified with the expected fragment (324bp) produced on the gel (Plate 7B).

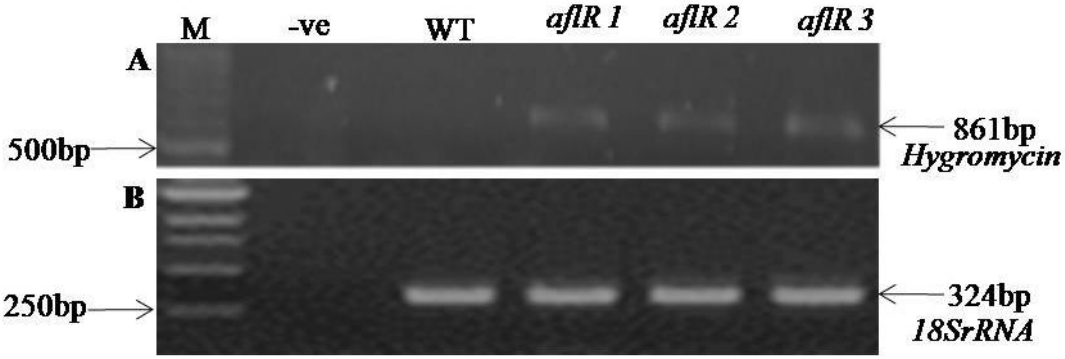

Plate 7. Analysis of transgenic maize through RT-PCR. Lane M in panel **A** was loaded with a 1kb DNA ladder while that in panel **B** was loaded with a 100bp ladder, -ve is the negative control with water as the template, WT is cDNA from wild type maize while *aflr* represents amplification obtained in cDNA from transgenic maize.

4.7.4 Evaluation of silencing of target transcripts in *A flavus* and aflatoxin accumulation in maize

MCKII fungi used for infection and that re-isolated from wild type maize showed a bright blue fluorescence on neutral red desiccated coconut agar media, characteristic of high aflatoxigenicity. On the other hand, moderately bright blue fluorescence was observed in fungal cultures re-isolated from maize transformed with hp-*aflR* transgene (Plate 8A). At the molecular level, RT-PCR result showed reduced signals in *A. flavus* cultures recovered from transgenic maize as compared to those from wild type plants and the culture used for infection (Plate 8B). Similarly, ELISA results revealed that transgenic maize kernels accumulated an average of 10ppb total (Figure 7A). These aflatoxin levels were significantly lower compared with those obtained from the wild type maize (141ppb), also infected with *A. flavus*. Higher levels of total aflatoxins were again recorded from the fungal cultures re-isolated from transgenic maize and the MCKII culture used for infection. On average, the culture used for infection accumulated 70.3ppb total aflatoxins while those from transgenic maize (*aflR)* had 35.9µg/kg and 43.6µg/kg (Figure 7B). It was also interesting to note that *A flavus* cultures re-isolated from wild type maize also infected with *A. flavus* recorded a higher level of total aflatoxins (an average of 117ppb) that the other treatments in this experiment.

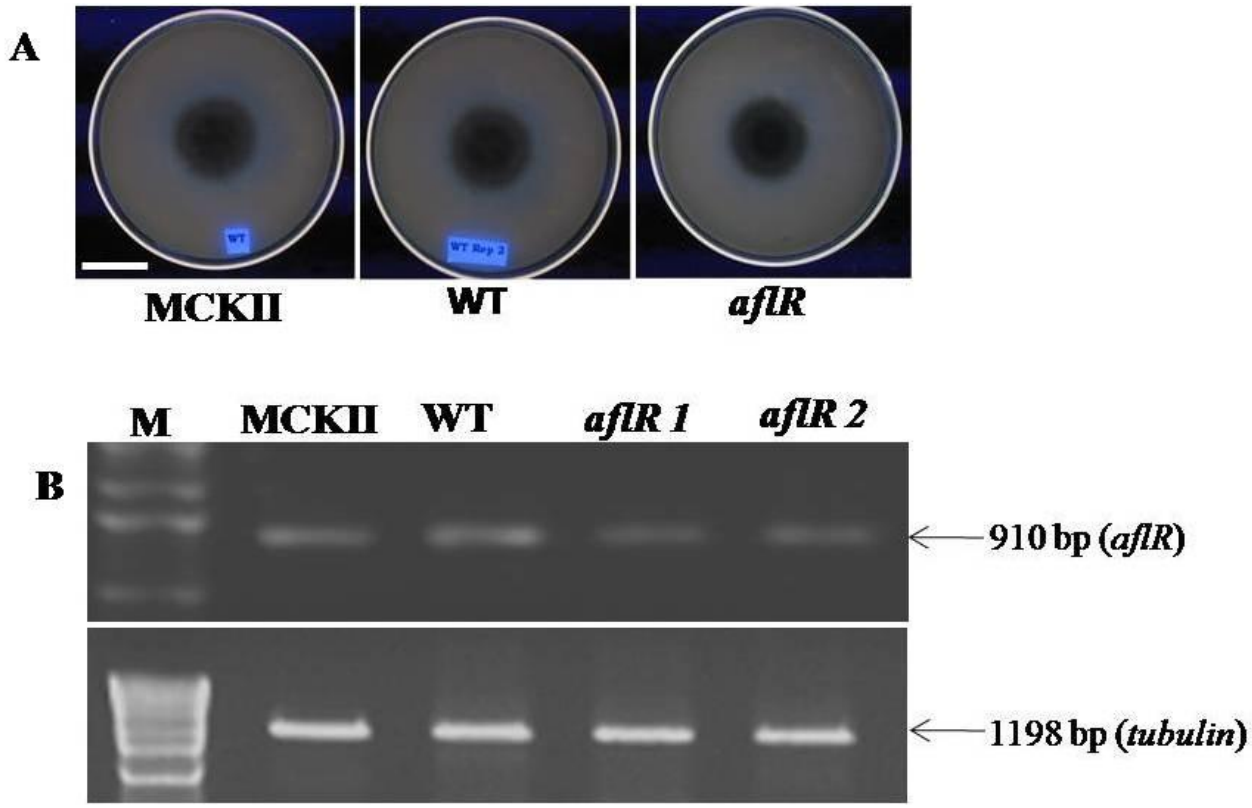

Plate 8. Determination of the effect of hp-*aflR* construct in maize on *in vitro* aflatoxin production and *aflR* gene expression in *A. flavus*. **A.** Screening for in vitro production of aflatoxin on neutral red desiccated coconut agar media**. B.** Silencing of the *aflR* gene in A. flavus cultures colonizing transgenic and non-transformed (WT) maize. Lane M was loaded with a 1kb DNA ladder. MCKII represents amplification on cDNA from the fungal culture used for infection. WT is amplification on cDNA from fungi infecting wild type maize while *aflR* represent amplification in cDNA from fungi from transgenic maize.

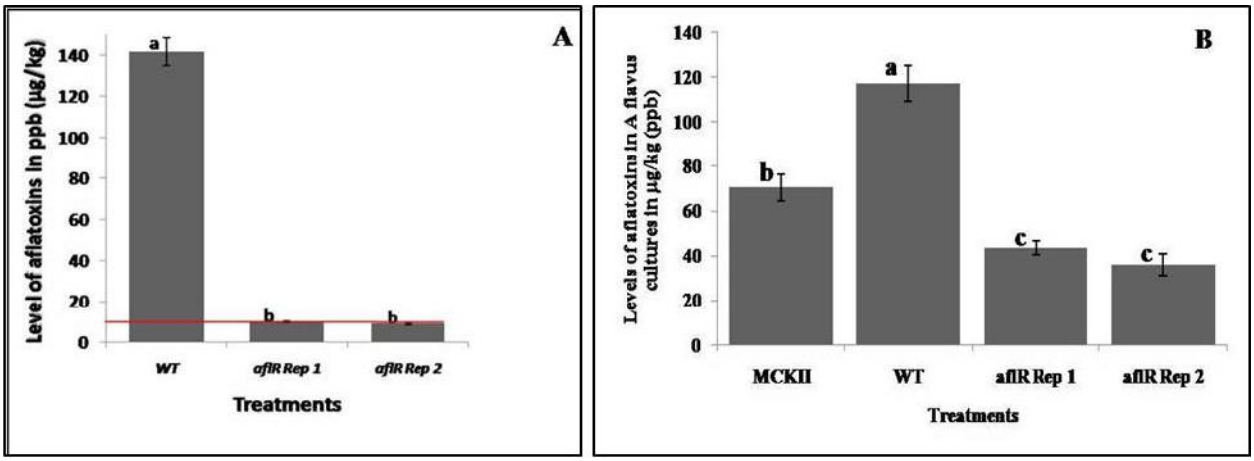

Figure 7. Levels of total aflatoxins. **A.** Total aflatoxins accumulated in non-transformed maize (WT) and transgenic (*aflR*) maize transformed with an hp-*aflR* construct. The red line indicates the maximum limit of total aflatoxins in foodstuffs permitted by the Kenya Bureau of Standards (KEBS). **B.** Total aflatoxins in *A. flavus* cultures re-isolated from non-transformed maize (WT) and from maize with hp-*aflR* construct (aflR Rep 1 and aflR Rep 2) alongside the culture used in infection (MCKII). Values on the Y-axis are an average of spectrophotometer readings for 5 randomly selected maize samples from 5 independent transformation events.

4.7.5 Effect of the hp-*aflR* transgene on maize plant architecture and yield

A significant decrease in plant height and cob weight was observed in maize plants expressing the *hp-aflR* construct when compared to the non-transformed (WT) plants **(**Plate 8 A-D). On average, transgenic maize recorded a height of 100cm that represents a significant 2.5 fold reduction in relation to the corresponding wild type (Plate 8 E). A reduction in the weight of cobs at harvest was also observed in transgenic maize when compared with the wild type. The 5 independent transgenic events analyzed exhibited a large decrease in fresh weights of the cobs (mean of 85% lower than the controls). As expected, a similar significant decrease was recorded after drying the cobs to a moisture content of <13% (Plate 8F). The hp-*aflR* transgenic maize plants produced cobs that exhibited relatively lower number of kernels per cob than the corresponding wild type. Generally, transgenic maize exhibited small cobs that were poorly filled. Analyzed cobs from the 5 independent transgenic events recorded an average of less than 90 kernels per cob, which represents a significant reduction from the corresponding wild type plants.

Interestingly, also, was the observation that some of the cobs from transgenic events had no kernels even after self pollination (Plate 8G).

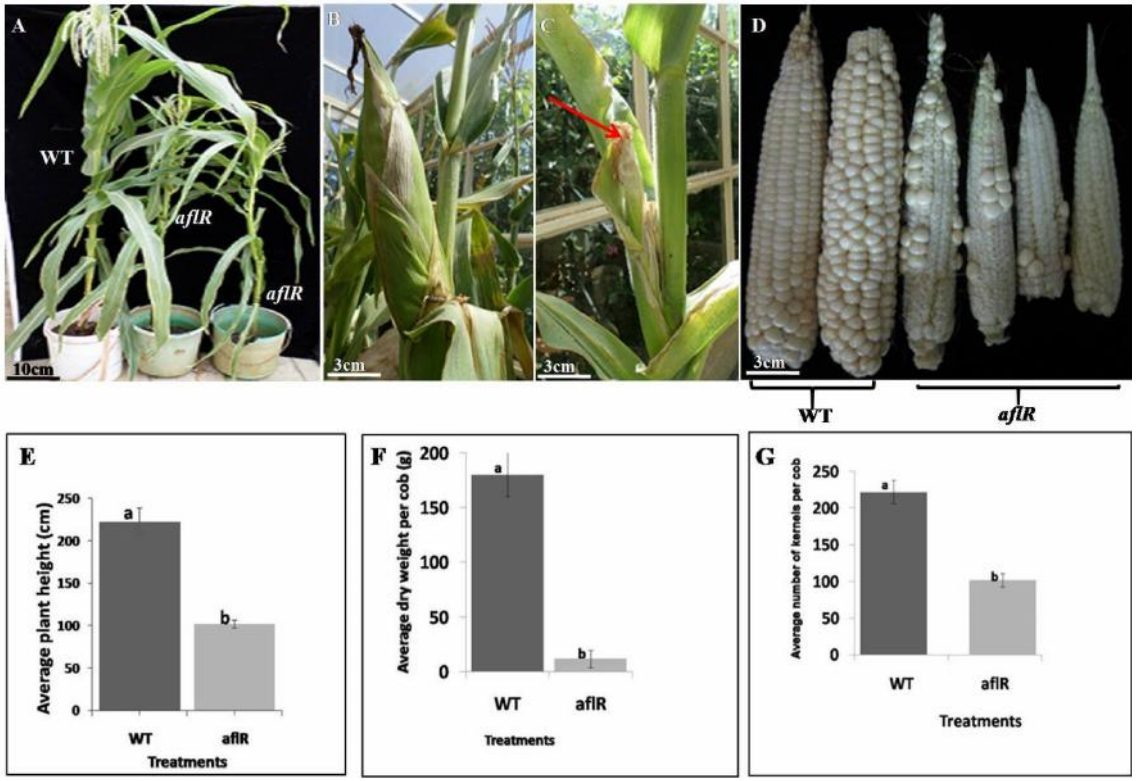

Plate 8. Determination of the effect of introduction of the hp-*aflR* transgene on growth and yield in maize. **A.** A comparative appearance of whole transgenic plants (*aflR*) with controls (WT) at silking stage. **B.** Appearance of maturing non-transformed ear cobs after pollination. (**C**) Appearance of maturing transgenic ears after pollination. **D.** Kernel placement in transgenic (*aflR*) and non-transformed (WT) plants. **E.** The average plant height reduced in transgenics expressing the hp-aflR transgene (*aflR*) as compared to non-transformed controls (WT). **F.** Average dry weight of transgenic maize cobs alongside the wild type. **G.** The mean number of kernels per cob in transgenic (*aflR*) alongside the WT plants.Vertical bars represent error bars. Significant differences were determined using Tukey's HSD test at P≤0.05.

4.7.6 Molecular analysis of potential off targets

BLAST searches using the *aflR* sequence at NCBI revealed a number of sequences, mostly from *Aspergillus spp*. No homologous sequences could however be identified in plants. BLAST results at the PTFD did not reveal any hits using the recommended cutoff value (1-e10). Raising the cutoff value, however, revealed two hits of ethylene responsive factors (ERF) transcription factors in maize. The obtained ERFs represent a bZIP family protein (accession number GRMZM2G136266_P01) and a GATA family protein (accession number GRMZM2G464037_P01) and show sequence similarities of 31 and 21 %, respectively to the *aflR* query. The two coding sequences are involved in diverse functions in cellular processes in maize, such as DNA-dependent regulation of transcription as well as regulation of sequence-specific DNA-binding activity. They are also involved in control of various essential stages including shoot apex development, tassel and ear inflorescence and pollen development stages.

To investigate presence of conserved motifs between the retrieved sequences and the *aflR* transcription factor, nucleotide sequences of the ERFs were mapped to the *aflR* query for a multiple sequence alignment using Vector NTI Advance and no perfect alignment was found (Figure 8). The possible siRNAs from the cloned *aflR* fragment are as outlined in figure 18. Analysis using Si-Fi tool did not reveal any potential off-targets between *aflR* siRNAs and the two ERFs. Similarly, no potential off targets were found in the maize genome upon analysis of the effective siRNAs (Figure 9) using pssRNAit.

```
                          1                                                  50
GRMZM2G136266_P01     (1) ATGTATGACACGCACCACCCGCACCGCACCCACCCACCAACCCGACCTTC
GRMZM2G172936_P01     (1) ATGGACGACGCTCAGGAGCCTC-CCTC-CCCTCCCTCCCCCTCGCAGTCA
       hp aflR ZM     (1) ATCAACCAC-CACACGCTCTGC-CCACCCCCAATGGTAGCAGTGGTGTCT
        Consensus     (1) ATG ACGAC C CACGA CCGC CC C CCCACCC CC CC CG  GTC
                         51                                                 100
GRMZM2G136266_P01    (51) CGCTGCTCACACACACACACACACACACACACACACACTC--TACACGCGAG
GRMZM2G172936_P01    (49) TCCTCCTC---CTCTTCCTCGTCGTCCTCCGTGGCCCC--GA-ACCCGAA
       hp aflR ZM    (49) CCGCCATC--TTTTCTCATCAGAGTCCCCCGCCACCCGTGGAGACCCAGG
        Consensus    (51) CCCTCCTC   CTC TCCTCA AGTCCCCCGC  CCCC  GA ACCCGAG
                        101                                                150
GRMZM2G136266_P01    (99) CGAGACCACGCGCCG-GACCGC-CGCAAGCCG-GCCGCCGCCG--CCGCG
GRMZM2G172936_P01    (93) GAAGCGGGCGCGCAA-GGACGG-C---AGCCG-GCACCCGACGTACCGCG
       hp aflR ZM    (97) GCCTTGGAGGAGATTTGGCCGGTCAGGAGCAAAGCACCCTGTCTTCCCTA
        Consensus   (101) G AG GGACGCGC   GGCCGG C   AGCCG GCACCCG CGT CCGCG
                        151                                                200
GRMZM2G136266_P01   (144) ACCTGGAGCTTGCG-AGCTAGCTCTGGCCTCGCTGCCTGCGCCGACTGAG
GRMZM2G172936_P01   (137) GCGTGC-GCATGCGGAGCTGGGGCAAG--TGGGTGTCGGAGATCAGGGAG
       hp aflR ZM   (147) ACAATC-GATTCGGAATTTGGGGGCTCTTTGCAGTCCATGGAACACGGAA
        Consensus   (151) AC TGC GCTTGCG AGCTGGGGC  G  TGG TGCC G GA CACGGAG
                        201                                                250
GRMZM2G136266_P01   (193) ATGGACCCGCGCTTTCCGC--TGCCGGCCGC-GGCGGCGCCGTC-CACGG
GRMZM2G172936_P01   (184) CCCCGCAAGAAGTCTC-GC--ATCTGGCTG--GGCACCTTCG-C-CACCG
       hp aflR ZM   (196) ACCATGCCGATTTCTTGGCTGAGCCGACGGGAAGTCTTTTCGACGCGTTT
        Consensus   (201) ACC  CCCGA  TCTC GC  AGCCGGC G  GGC  CTTCG C CAC G
                        251                                                300
GRMZM2G136266_P01   (239) GCGGCGGCGGCGGCGCCA-------GAGGGCACCACCGGC-GGGCCCACT
GRMZM2G172936_P01   (227) -CGGAGATGGCGGCGC---------GCGCGCACGAC--GT-GGCCGCGCT
       hp aflR ZM   (246) TTGGAAGTGGGGACCCCCATGATCGACCCGCTCCTCGAGTCGGCCCCACT
        Consensus   (251)  CGGAGGTGGCGGCGCC        GCGCGCACCAC  GT GGCCCCACT
                        301                                                350
GRMZM2G136266_P01   (281) CGGAGACGTTCCTCAGCTTCCACGACGCGGACCTGCTCCTCGACCCGGAC
GRMZM2G172936_P01   (264) CG----CCATCAAGGGCGCGCGCGACGCGCACCTCAACTTC--CCGGGCC
       hp aflR ZM   (296) GCCACCGTTTCAGGCGCGCTATTGCTGCTTTTCGCTTGCACTACAAACAC
        Consensus   (301) CG A  C TTCA G GCGC C CGACGCG ACCTC TCCTC ACC GGAC
                        351                                                400
GRMZM2G136266_P01   (331) GGCGACTTCT--TCTCCGAC-CTCGACT------TCCCCTCCCTCTC---
GRMZM2G172936_P01   (308) TCGCGCACCT--GCTCCCGCGCCCGGCC------ACCGCGGCGCC-----
       hp aflR ZM   (346) TGACCCACCTCTTCCCCCACGCCCCGCTGGGCTGTCAGCTGCGGCTGACA
        Consensus   (351) TG C CACCT  TCTCCCACGCCCGGCT      TCCGCTGCG CT
                        401                                                450
GRMZM2G136266_P01   (369) -----CGACGAC-----TCTCCGGCCGCCTCCGA-------CCCCACCCC
GRMZM2G172936_P01   (345) -----CAAGGAC-----GTGCAGGCCGCGGCCGC-------GCTCGCCGC
       hp aflR ZM   (396) GACGGCGAGGACAGTTCGTGCAACCTGATGACGACTGATATGGTCATCTC
        Consensus   (401)      CGAGGAC     GTGCAGGCCGC GCCGA       GCTCACC C
                        451                                                500
GRMZM2G136266_P01   (402) G-CCGCCGCATCCGCAGCAGCAGCCG-CCGCAGGCTTCCCGGCCCCACG
GRMZM2G172936_P01   (378) GGCCGCCGACTTCCCT-CTGCCGCCGTCCCCCGGCGGCGCCAATGCCAAG
       hp aflR ZM   (446) GGGGAACAAGAAGGCTACAGATGCGGTCCGGAAGATCCTCGGGTGTT-CG
        Consensus   (451) GGCCGCCGA T CGCT CAGC GCCGTCCGCAGGCT C CCGGTGCCACG
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                          501                                                 550
GRMZM2G136266_P01   (450) TCCGCCCGGCGGGACCCACACGCGGAGCCTCTCCCTC-GACGCCGCCTTC
GRMZM2G172936_P01   (427) A--GCCC--CGAGAGCAGCA-GCTCCGGCTCCGCCTCCGCCTCCGCCTCC
       hp aflR ZM   (495) TGCGCGCA-GGATGGCTACTTGCTGAGCATGGTCGTCCTTATCGTTCTC-
        Consensus   (501) T CGCCC  CGAGAGC ACA GCTGAGCCTC  CCTCCG CTCCGCCTCC
                          551                                                 600
GRMZM2G136266_P01   (499) TTCGAGGGCCTCGCGTTTCAGGGACCCAGTGGGAGCGCCGGGGGTGGAGG
GRMZM2G172936_P01   (472) GACGACGCCAGCGCCGCCTCG----CCGGCGGCGCCGCAGGACGCCGAGC
       hp aflR ZM   (543) -AAGGTGCTGGCGTGGTATGCAGCGGCAGCAGGCACCCAGTGTACCTCGA
        Consensus   (551)  ACGA GCC GCGCGGT T G G  CCAGCGGG  CGCAGGG GCCGAG
                          601                                                 650
GRMZM2G136266_P01   (549) TGGGCCCGGCCACA-AGAGGAGCGGCTCGATGGATGGGGACA--CCTCGC
GRMZM2G172936_P01   (518) CCAACCCGGACC-----GACGCGCTGTTCGACCTCCCGG---A--CCT-GC
       hp aflR ZM   (592) CGGCGGTGGGGGGAGAAACCAGCAGTGGCAGCTGTGGCACACAGTCCCGGC
        Consensus   (601) CGG CCCGG C  A AGACGAGC GTTCGA CT TGGG ACA  CCT GC
                          651                                                 700
GRMZM2G136266_P01   (596) CGTTCGAGGGCGAGTCG-GCGCTCTCCAGCGGCCTGCCGGACTACGCCAA
GRMZM2G172936_P01   (557) TTCTGGACCT-GAGATG-CTGCGGGCCGTCGTCGTG--GGCCGTCGAC--
       hp aflR ZM   (642) ACCGTGTCCAGTGGCTGTCTGTCGGAAGAGCGCGTGCTGCACCTGCCAAG
        Consensus   (651)   CT GACC  GAG TG CTGC GGCCG CGGCGTGC GGAC TCGCCA
                          701                                                 750
GRMZM2G136266_P01   (645) ----GAAGGCCATGCCCGCCGAGAGGATCGCCGAGCTCGCGCTCATCGAC
GRMZM2G172936_P01   (601) ----GACGACGTGGCCGGCGG-------CGGCGCGTTCCGGATCATCGAG
       hp aflR ZM   (692) TATGGTGGGCGAGGATTGTGTGGATGA-GGAAGACCACCCGCGAGTGGCG
        Consensus   (701)     GA GGCGAGGCC GCGG GA GA CG CGAGCTCCCGCTCATCGAG
                          751                                                 800
GRMZM2G136266_P01   (691) CCCAAGCGCGCAAAGAGGATTCTGGCGAACAGGCAGTCGGCAGCGAGGTC
GRMZM2G172936_P01   (640) GAGCCGCT-GCTGTGGGAGTACTGA------------------------
       hp aflR ZM   (741) GCACAGCTCGTTCTGAGTGAACTGCACCGAGTACAGTC------------
        Consensus   (751) GC CAGCTCGCT TGAG GTACTG         CAGTC
                          801                                                 850
GRMZM2G136266_P01   (741) AAAGGAGAGGAAGATCATGTACACTAGTGAACTGGAGAAGAAGGTCCAGA
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus   (801)
                          851                                                 900
GRMZM2G136266_P01   (791) CTCTGCAAACGGAGGCCACTACGCTGTCAGCACAGCTGACCCTGCTCCAG
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus   (851)
                          901                                                 950
GRMZM2G136266_P01   (841) AGGGATACAACTGGTTTAACTGCCGAAAACAGAGAGCTCAAGCTTCGGTT
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus   (901)
                          951                                                1000
GRMZM2G136266_P01   (891) GCAGTCCATGGAAGAGCAAGCTAAACTGCGGGACGCTTTGAATGAAGCCC
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus   (951)
                         1001                                                1050
GRMZM2G136266_P01   (941) TGCGAGAAGAAGTCCAGCGACTTAAGATAGCCGCAGGACAAGTCGGGAAC
GRMZM2G172936_P01   (664) --------------------------------------------------
```

```
       hp aflR ZM   (779) --------------------------------------------------
        Consensus  (1001)
                            1051                                         1100
GRMZM2G136266_P01   (991) ATGAATGGGAACCCCTTCAATGGTGGACTCCAGCAGCAGATTCCATCCTA
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus  (1051)
                            1101                                         1150
GRMZM2G136266_P01  (1041) TTTCGTGCAGCAGCAGCAGCAGCAGACACCGTACTTTGGTGGCCACC
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus  (1101)
                            1151                                         1200
GRMZM2G136266_P01  (1091) AGGCTCAGCTTCACAATCAAAATCAAAACCATCGTCACCAGATCTCGTCA
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus  (1151)
                            1201                                         1250
GRMZM2G136266_P01  (1141) AATGTTGGGCAGACGCTCAGTGGTCAGTCCCTAAACGACTCCATGGATTT
GRMZM2G172936_P01   (664) --------------------------------------------------
       hp aflR ZM   (779) --------------------------------------------------
        Consensus  (1201)
                            1251
GRMZM2G136266_P01  (1191) CATGTGA
GRMZM2G172936_P01   (664) -------
       hp aflR ZM   (779) -------
        Consensus  (1251)
```

Figure 8. Multiple sequence alignment of the 2 ERFs alongside the *aflR* transcription factor demonstrating a lack of perfect alignment.

Target dataset = Zea mays (maize) DFCI Gene Index (ZMGI) version 19 release on 8/12/2010

Query sequence fragments do not have hits in target dataset

Predicted efficient siRNAs

Number	Fragment	siRNA AS (3' - 5')	Hits
1	aflR (16 - 36)	GAGACGGGUGGGGGUUACCAU	0
2	aflR (40 - 60)	CAUCGCAGAGGCGGUAGAAAA	0
3	aflR (44 - 64)	GCAGAGGCGGUAGAAAAGAGU	0
4	aflR (45 - 65)	CAGAGGCGGUAGAAAAGAGUA	0
5	aflR (131 - 151)	GUGGGACAGAAGGGAUUGUCA	0
6	aflR (164 - 184)	GCCCCCGAGAAACGUCAGUUA	0
7	aflR (168 - 188)	CCGAGAAACGUCAGUUACCUU	0
8	aflR (191 - 211)	GCCUUUGGUACAGCUAAAGAA	0
9	aflR (214 - 234)	GGCUCAGCUGCCCCUCAGAAA	0
10	aflR (215 - 235)	GCUCAGCUGCCCCUCAGAAAA	0
11	aflR (218 - 238)	CAGCUGCCCCUCAGAAAAGCU	0
12	aflR (227 - 247)	CUCAGAAAAGCUGCGCAAAAA	0
13	aflR (231 - 251)	GAAAAGCUGCGCAAAAACCUU	0
14	aflR (302 - 322)	CAAAGUCCGCAGUCGCAUUUA	0
15	aflR (306 - 326)	GUCCGCAGUCGCAUUUACUAA	0
16	aflR (328 - 348)	CGGGGAAGUCGCGCGUCUUAU	0
17	aflR (329 - 349)	GGGGAAGUCGCGCGUCUUAUA	0
18	aflR (331 - 351)	GGAAGUCGCGCGUCUUAUAGU	0
19	aflR (332 - 352)	GAAGUCGCGCGUCUUAUAGUU	0
20	aflR (335 - 355)	GUCGCGCGUCUUAUAGUUUGU	0
21	aflR (377 - 397)	GGGUGGGUAGGCCGGGUUUUA	0
22	aflR (387 - 407)	GCCGGGUUUUAGGGUGGAAAA	0
23	aflR (389 - 409)	CGGGUUUUAGGGUGGAAAAGA	0
24	aflR (398 - 418)	GGGUGGAAAAGACGUGGUAUU	0
25	aflR (399 - 419)	GGUGGAAAAGACGUGGUAUUU	0
26	aflR (414 - 434)	GUAUUUCACGUAGGGAAAGUA	0
27	aflR (461 - 481)	GGCAGAGUCUGCGGGUCUAUU	0
28	aflR (462 - 482)	GCAGAGUCUGCGGGUCUAUUA	0
29	aflR (463 - 483)	CAGAGUCUGCGGGUCUAUUAU	0
30	aflR (467 - 487)	GUCUGCGGGUCUAUUAUGGAU	0
31	aflR (472 - 492)	CGGGUCUAUUAUGGAUGGAAA	0
32	aflR (488 - 508)	GGAAACCGUACGCCAACCAAA	0
33	aflR (489 - 509)	GAAACCGUACGCCAACCAAAA	0
34	aflR (501 - 521)	CAACCAAAAAUUGCCUACUAU	0
35	aflR (540 - 560)	GUGGUGAACGCAUGUGGAUUU	0
36	aflR (543 - 563)	GUGAACGCAUGUGGAUUUCGU	0
37	aflR (561 - 581)	CGUUGGCGCUGGUGUGUUAGU	0
38	aflR (562 - 582)	GUUGGCGCUGGUGUGUUAGUA	0
39	aflR (580 - 600)	GUAGUGGUAUCGAGUCUGAAU	0
40	aflR (590 - 610)	CGAGUCUGAAUCGACCUAUAA	0
41	aflR (591 - 611)	GAGUCUGAAUCGACCUAUAAU	0
42	aflR (595 - 615)	CUGAAUCGACCUAUAAUAGGU	0
43	aflR (597 - 617)	GAAUCGACCUAUAAUAGGUUU	0
44	aflR (637 - 657)	GUUCACGUAGCGGCACAUUGA	0
45	aflR (690 - 710)	CUGCGACCGGAUAGUGCUAAU	0
46	aflR (692 - 712)	GCGACCGGAUAGUGCUAAUAU	0

Figure 3. Effective siRNA sequences from the *aflR* fragment cloned into binary vextors and transformed in maize and groundnuts. The siRNAs were querried against a maize genome database and revealed no potential off targets in this index.

4.8 Discussion

4.8.1 Groundnut transformation

The 72-hour period of co-cultivation of groundnut explants with *Agrobacterium* was an important factor in enhancing the transformation frequencies obtained in both varieties. This allowed ample time for the bacteria to infect explant tissues for effective T-DNA integration (Anuradha *et al.,* 2006). Furthermore, excision of the cotyledon explants prior to infection also increased the number of competent cells at the wounded site that enhanced chances of *Agrobacterium* uptake for efficient infection. The junction between the cotyledon and the embryo axes contains axillary meristematic cells that are highly regenerable and therefore are useful as a potential target for gene delivery (Sharma & Anjaiah, 2000). Recently, reports have indicated that the type of explant used is a major determining factor in genetic transformation of groundnuts and other crops (Ishida *et al.,* 2007; Sharma & Anjaiah, 2000; Venkatachalam *et al.,* 2000; Olhoft *et al.,* 2003).

Selection of putative transformants is vital in ensuring that only tissues with the gene of interest are used for subsequent experiments. Kanamycin sulfate proved effective in visually selecting putatively transformed shoots based on the bleaching of leaves and stems which is consistent with other studies (Sharma & Anjaiah, 2000; Anuradha *et al.,* 2006). This was however not enough and other molecular tests had to be done to prove the transgenic status of these plants. Regeneration frequencies in both groundnut varieties were relatively high but the putative transformants had little or no elongation after the second subculture on fresh media and had problems rooting as compared to their non-transformed counterparts. These findings indicate that the transgene may be involved in altering plant growth and development including rooting. On the other hand, the lack of significant differences between transformation frequencies shows that transformation of these two groundnut genotypes using *A. tumefaciens* and this protocol was genotype independent. Similarly, their regeneration frequencies were also not significantly

different which is consistent with the earlier data obtained from the optimized protocol for regeneration of these 2 groundnut varieties.

4.8.2 Effect of hp-*aflR* construct on aflatoxin biosynthesis in transgenic maize

Host induced gene silencing has been successfully used in management of various stresses caused by fungi in plants (Nunes & Dean, 2012). The recent proofs of concept for HIGS through expression of hairpin cassettes in plants for control of pathogenic *Fusarium verticilloides* (Tinoco *et al.,* 2010), mycotoxin-producing *Fusarium graminearum* (Koch *et al.,* 2013), leaf rust fungus *Puccina striiformis* (Yin *et al.,* 2011) and powdery mildew fungus *Blumeria graminis* (Nowara *et al.,* 2010) opened up a platform for the use of this mechanism in controlling other phytopathogenic fungi such as aflatoxin-producing *A. flavus*. The results in this study showed that hairpin-*aflR* cassette transformed into tropical maize line CML 144 not only downregulated *aflR* transcripts in *A. flavus* cultures colonizing transgenic maize but also reduced aflatoxin biosynthesis and accumulation in infected kernels. In the past, reports have shown success in disruption of aflatoxin biosynthesis *in vitro* through knockdown/knockout techniques targeting various genes that control rate limiting steps in the pathway including *aflR* (Brown *et al.,* 1996; Flaherty & Payne, 1997; Keller *et al*., 2000).

The *aflR* transcription factor, which has also been targeted in previous experiments for *in vitro* disruption of aflatoxin biosynthesis, is a positive acting regulatory factor that is involved in transcription activation of all the structural genes in the aflatoxin biosynthetic pathway (Carry *et al*., 2000b; Yu *et al*., 2004; Yu, 2012). Previous experiments have implicated *aflR* inverted repeat transgenes in aflatoxin suppression in *in vitro* cultures of *A. flavus* and *A. parasiticus* (McDonald *et al.,* 2005) making it an ideal candidate for HIGS to control aflatoxin accumulation in plants. The effect of hp-*aflR* on aflatoxin biosynthesis and accumulation in maize was notable. It is logical that the result in reduced accumulation of aflatoxins in transgenic maize plants was because these transgenic maize synthesized primary siRNAs that were complementary to the *aflR*

transcription factor in *A. flavus* from the double stranded hp-*aflR* constructs transformed in maize. Upon colonization, the primary siRNAs resulting from the hp-*aflR* construct then trafficked into infecting fungi, possibly through the recently proposed plant-fungal cellular interface (Micali *et al*., 2011; Voegele & Mendgen, 2003). On reaching the fungi, the siRNA signal was amplified through Dicer-dependent RNA silencing mechanism and chopped the complementary mRNA transcripts most likely in a homology-dependent manner as proposed by Hamilton and Baulicome (1999) resulting in gene expression downregulation. This could even be more true given many studies that have shown the existsnce of RNA ineterence mechanisms in many organism including fungi (Niu *et al.,* 2006; Huvenne & Smagghe 2009; Alakonya *et al*., 2012; Duan *et al*., 2012; Nunes & Dean 2012).

The low levels of functional *aflR*, as revealed by the RT-PCR data was a result of post transcriptional gene silencing mediated by 21-24nt dsRNA that is present and functions innately in fungi (Hamilton & Baulcombe, 1999; Koch & Kogel, 2014; Macdonald *et al.*, 2005). The result of this silencing may have led to the disruption of the optimal transcription of other downstream aflatoxin biosynthesis pathway genes and subsequently interrupted aflatoxin biosynthesis. These results support those by Abdel-Hadi *et al.* (2011) who demonstrated that aflatoxin production is disrupted if any step in the biosynthetic pathway is blocked. This work provides a proof of concept that aflatoxin biosynthesis can indeed be down-regulated in *planta* through silencing of genes controlling the biosynthetic pathway as earlier proposed by Alakonya and Monda (2013).

The detrimental alteration of plant architecture observed in both transgenic maize and groundnuts transformed with the RNAi cassette are a pitfall to the use of HIGS for controlling aflatoxin in maize and groundnuts by targeting *aflR.* Effects on essential physiological processes including plant elongation, synchronized silking and tassel formation, kernel setting and their placement on the cobs in maize, as well as negative effects on shoot elongation and rooting in groundnuts point towards a possible “off target” effect of the transgene on the plants. Off-target silencing effects occur when

siRNAs are processed by RISC and downregulate unintended target mRNAs (Xu *et al*., 2006). Such targeting has been shown to lead to changes in development in targeted organisms with effects on the phenotype in both plants and animals (Jackson *et al*., 2003; Xu *et al.,* 2006). Reports have also demonstrated that there exists a high risk of off-target gene silencing during PTGS in plants due to a cross reaction of siRNAs with targets of non-specific nature or those of limited sequence similarity. Theoretically, siRNAs derived from cleavage of dsRNAs by Dicer are responsible for high efficiency RNA silencing of target sequences in host plants. However, studies have also implicated these short nucleotides sequences to the suppression of unintended genes containing identical sequences to some of the siRNAs (Jackson *et al.,* 2003; 2004, Snove *et al.,* 2004; Xu *et al*., 2006). Further it has been shown that single base pair mismatches between siRNAs and the target transcripts is enough to alter siRNA function (Saxen *et al.,* 2003).

In the current study, negative impacts to plant physiology could have been due to a cross reaction between siRNAs with unintended sequences in maize and groundnuts although this remains to be investigated. The *aflR* binding motifs are conserved in the zinc-finger DNA binding domains in fungi but the transcription factor has also been shown to bind to deviated sequences rather than the typical motifs (Yu, 2012). This has however only been demonstrated in fungi. At the mRNA level, no *aflR* homologs were identified in maize and groundnuts using bioinformatics tools such as BLAST searches with the recommended parameters. The 2 identified ethylene responsive factors (ERFs) at the PTFD could not be trusted since the cut off (e-value) was higher than the recommended hence their homology to *aflR* could only have occurred by chance. Furthermore, use of tools such as Si-Fi and pssRNAit, that cleave the construct sequence into all theoretically possible siRNAs and looks for off targets for these siRNAs, did not reveal any potential off target sequences in maize and groundnuts. From a research standpoint, such negative impacts to plant physiology are a shortcoming to the application of these biotechnological techniques in plant improvement.

4.9 Conclusions

This study successfully demonstrated the downregulation of aflatoxin biosynthesis and accumulation in maize using the *aflR* hairpin constructs. This study further emphasises the critical role played by *aflR* in regulating aflatoxin biosynthesis. The level of aflatoxins accumulating in transgenic plants was just at the threshold for the limit permitted in food by KEBS and way below the 20ppb set by the American Food and Drug administration. Although the gene targeted resulted into other non-intended effects on the host plant, it opens a novel frontier where other regions of *aflR* or other genes in the aflatoxin biosynthetic pathway could be targeted using the HIGS-based technology as will be elucidated in chapter 5 of this thesis.

4.10 Recommendation

The off-target effects on growth and development of transgenic maize and groundnuts need further investigation to help elucidate the interaction of the transcription factor with other plant proteins, particularly the ethylene responsive factors (ERFs) and how this interaction affects physiological function.

CHAPTER FIVE

HOST INDUCED GENE SILENCING OF THE AFLATOXIN RATE LIMITING ENZYMES (*STCA* AND *STCJ)* FOR REDUCED AFLATOXIN ACCUMULATION IN MAIZE

5.1 Introduction

Mycotoxin contamination in feeds and food is a major concern worldwide (Van Egmond, 2007). Among the magement strategies employed in aflatoxin management are chemical treatments, breeding of resistant germplasm, biological control and genetic engineering (Koch *et al.,* 2013). More importantly these management strategies have certain disadvantages. For instance, application of synthetic fungicides to control phytopathogenic fungi has environmental implications. Furthermore, there are cases of reduced sensitivity and resistance by the fungi to the chemicals leading to reduced success of this technique (Koch *et al.,* 2013). To a certain degree, pre-harvest control of aflatoxins in crops has been achieved through development of resistant germplasm (Cleveland, 2003). While this strategy has been successful in developing maize inbred lines with increased resistance to *A. flavus* infection and aflatoxin contamination, attempts to further develop commercial varieties with similar resistance have encountered difficulties (Cary *et al.,* 2011).

This is because resistance to aflatoxin contamination by plants is a polygenic process that takes years of research to produce a resistant variety and the lack of biomarkers to facilitate transfer of resistance genes into other varieties makes it a slow process (Cary *et al.,* 2011). HIGS could offer a solution to this problem through targeting of non-plant genes that are directly involved in biosynthesis of the toxin. Aflatoxin biosynthesis is controlled by 30 pathway genes clustered within a 75kb region of the fungal genome (Trail *et al*., 1995; Yi *et al*., 1995; Yu *et al*., 2004). Studies have indicated that the biosynthetic pathway involves 2 rate limiting steps catalyzed by 3 enzymes. First, synthesis of a hexanoate starter (hexanoyl CoA) from an acetate and malonyl-coA

involves action of 2 genes (*stcJ* and *stcK*) that encode α and β subunits of the fatty acid synthase in the sterigmatocystin and aflatoxin gene cluster (Mahanti *et al*., 1996). The next step entails conversion of hexanoyl-coA to norsoloronic acid (NOR), the first stable intermediate, through a series of extensions. This is catalyzed by a polyketide synthase (pksA) gene termed *stcA* (Yu, 2012). Norsoloronic acid then undergoes approximately 12-17 enzymatic reactions through a series of pathway intermediates to form aflatoxins (Sweeney & Dobson, 1999). Earlier *in vitro* reports have indicated that disruption of *stcJ* and *stcK* in *Aspergillus spp.* results in mutants that do not produce aflatoxins (Brown *et al.,* 1996). The ability of these mutants to synthesize sterigmatocystin and aflatoxins was however restored upon addition of hexanoic acid to growth media. This understanding, coupled with reports that RNA silencing mechanism exists in fungi and that it could result in development of aflatoxin resistant germplasm (Alakonya and Monda, 2013), justified the need to explore HIGS with the enzymes cotrolling two key steps as targets. The aim of this study was to transform maize with HIGS constsructs targeting the *stcA* and *stcJ* genes in the aflatoxin biosynthetic pathway and further characterize transgenic for *in planta* aflatoxin accumulation.

5.2 Materials and methods

5.2.1 Silencing constructs and explant preparation

Silencing constructs hp-*stcA* and hp-*stcJ* (figure 10A and B) reported herein and used for maize transformation were kindly provided by Dr. Amos Alakonya of (IBR, JKUAT). Binary vector pStargate (Invitrogen Corp. Carlsbad CA, USA), with ubiquitin promoter driving the genes of interest and a hygromycin gene for selection of transformed tissues, was used (see section 4.2.1.8 above). To verify presence of the transgene sequences in the silencing vectors, a pairwise sequence alignment, between each cloned hairpin sequence and the genebank sequence from NCBI, was carried out using Vector NTI. Tropical maize CML 144 (CIMMYT) was used for production of explants. Seeds were first planted and maintained at the JKUAT farm with regular watering. Plants were later

self-pollinated and immature embryos collected at 10-14 days post pollination for use as explants in the transformation process according to Ishida et al. (2007) as in chapter four above.

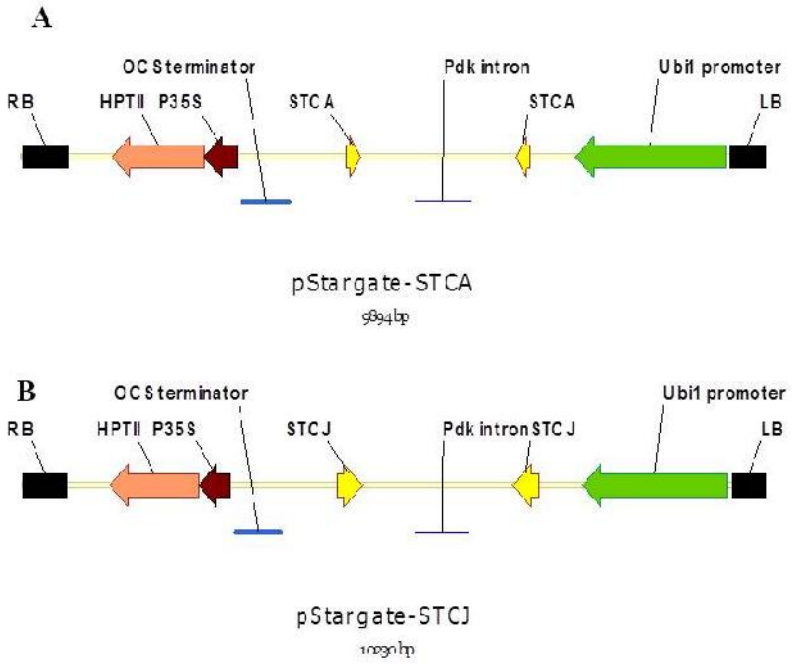

Figure 10. Schematic representation of the T-DNA region of the binary vector pStargate with *stcA* (A) *and stcJ* (B) sequences in sense and antisense orientation. From the RB-right border is the hygromycin phosphotransferase resistance gene for plant selection driven by the CaMV 35S promoter and OCS terminator. The silencing sequences are driven by the maize ubiquitin promoter with separated by pdk intron separating the inverted repeats.

5.2.2 Production of transgenic maize

Transgenic maize was generated as described in section 4.3.2.2 above. Briefly, immature zygotic embryos harvested from maize ears, between 10 to 14 days after pollination, were infected and co-cultivated with EHA 101 harboring the silencing cassettes. After 10 days of resting on MS media with 2mg/L 2,4-D and supplemented with 250mg/L carbenicillin, selection of putative transformants was initiated by transfer of the calli onto media containing 15mg/L hygromycin for 2 weeks. A second 3-week selection phase with 30mg/L hygromycin was also included. Putatively transformed plantlets with well-

developed roots were acclimatized and hardened on peat moss then later transferred to potting soil in the glasshouse where they grew to maturity.

5.2.3 Molecular characterization of transgenic plants

Presence of the construct in putatively transformed maize was confirmed via PCR using primers targeting the selectable marker gene hygromycin. Genomic DNA from the putative transformants was extracted as described by Zidani *et al*. (2005). Briefly, leaf samples from each of the putative transgenic plants was collected, quickly wrapped in aluminium foil and immediately chilled in liquid nitrogen where they remained until DNA extraction was done. Tissues were removed from the liquid nitrogen and immediately crushed to a fine powder using a mortar and pestle under liquid nitrogen. The powder was transferred to a 10ml centrifuge tube containing 4ml of CTAB extraction buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA and 1% β-mecarptoethanol) and incubated for 30 minutes in a water bath at 65°C. Then, 4ml of chloroform isoamyl-alcohol (24:1) was added to the samples and inverted several times to mix.

The mixture was centrifuged at 13000rpm for 10 minutes and later 1ml of the supernatant was transferred to a new centrifuge tube. DNA was precipitated by addition of 0.7ml ice-cold isopropanol to each tube and gently inverted to mix. The tubes were chilled at -20°C for 30 minutes and then centrifuged at 13000rpm for 30 minutes. The supernatant was discarded, 1ml of 70% ethanol added to wash the DNA pellet and centrifuged for 10 minutes. The supernatant was carefully discarded and the DNA pellet air dried after which 50µl of de-ionized water was added to dissolve the pellet. The DNA was stored at 4°C for PCR analysis. Hygromycin primers Hygro F-CGCGTCTGCTGCTCCATACAAG and Hygro R-TTCGATGTAGGAGGGCGTGGAT amplifying the hygromycin gene were used. PCR analysis was carried out using DNA thermocycler (Eppendorf AG, Hamburg, Germany) with amplification performed in 20µl reaction mixture containing 10ng genomic DNA, 1X Kapa Taq enzyme mix and 2.5µM of each primer. PCR mixture was heated at 94ºC for 1 minute then subjected to 30 cycles

consisting denaturation at 94ºC for 30 seconds, annealing at 61ºC for 1 minute 30 seconds. An initial and final extension steps of 72ºC each for 1 minute 40 seconds and 10 minutes respectively were also included. The PCR product was electrophoressed on 1% agarose gel and visualized under U.V light.

5.2.4 Bulking of transgenic seeds

Plants that were positive for the hygromycin selectable marker after PCR were maintained in the glasshouse in potted soil, supplemented with animal manure, with regular watering and allowed to grow. At silking stage, the plants were self-pollinated and covered with a plastic bag for 3 days to ensure a moist environment for effective fertilization and prevent cross pollination. These plants were maintained in the glasshouse until maturity, ears harvested and later dried in the sun. Cobs were then shelled and dried further to ensure stable moisture content was achieved. The first generation (T1) kernels from individual transgenic events were then collected in storage bags, labeled and stored at 4ºC for further analysis.

5.2.5 Evaluation of transgenic plants against aflatoxin biosynthesis and accumulation

To determine whether transgenic maize expressing the RNAi precursors inhibit aflatoxin production by fungi and its accumulation in maize, toxin assays were carried out as earlier described in section 4.4 above. Summarily, a non-wounding technique for maize infection (Windham and Williams, 2007) was used to achieve colonization of maize by aflatoxigenic *Aflavus*. Ten (10) T_1 seeds from five (5) independent transgenic events (for each silencing cassette) and 5 wild type (WT) plants were maintained in the greenhouse until they reached the silking stage after which inoculation using an aflatoxigenic *A. flavus* isolate MCKII was done. Upon maize maturity, *A. flavus* was re-isolated from the infected transgenic and WT kernels on MRBA medium as described by Pitt and Hockings, (1997) and sub-cultured on PDA until pure colonies of the fungus were obtained. Approximately 30 kernels from each transgenic event were cultured.

Identification of *A. flavus* from the rest of the fungi was according to Klich and Pitt, (1988).

5.2.6 Fungal RNA isolation and RT-PCR

To compare the expression levels of *stcA* and *stcJ* in *A. flavus* cultures re-isolated from transgenic and WT maize, RT-PCR was performed. Summarily, total RNA was isolated from 5 day-old *A. flavus* cultures grown on aflatoxin-inducing YES medium. About 200mg of mycelia were first recovered using a sterile spatula, dried in absorbent paper and ground with liquid nitrogen in a sterile, cold mortar and pestle. Total RNA was then isolated using the RNeasy Plant Mini Kit according to the user manual (Qiagen, Maryland, USA) including a step for genomic DNA digestion using RNase-free DNase as described in section 4.3.3.1. Complementary DNA synthesis was performed using the Superscript III reverse transcriptase kit (Invitrogen) with random hexamers according to the manufacturer's instructions (see section 4.3.3.2). Gene specific primers used for amplification of the transcripts are as shown in table 8 with *Tubulin* primers also used as the internal amplification control for fungi. PCR was performed in 20µL reaction comprising 10µL PCR KAPA TAQ (Pre-Mix kit), 2.5µM of each primer (Table 9) and 10ng of template cDNA.

Table 9. Primer sequences used for RT-PCR amplification of *stcA* and *stcJ* transcripts

Primer name	Primer sequence 5"-3"
stcA F	ATCTAGACAGCGTAAATGATTTGACC
stcA R	AGTCG ACGCATGCCAAAGGTAGGT
stcJ F	ACTCGAGTGCATTGCCGTGTAACTG
stcJ R	ACTGCAGCAACTACAGAGCCATCGCAG
Tub-F	GCTTTCTGGCAAACCATCTC
Tub-R	GGTCGTTCATGTTGCTCTTCA

5.2.7 Determination and quantification of aflatoxins *in vitro* and in *planta*

To determine aflatoxigenecity of *A. flavus* re-isolated from the maize *in vitro*, NRDCA medium, which enables rapid detection of aflatoxigenic fungi and visual determination of aflatoxins, was used as earlier described in section 4.4.3 above. To determine the quantities of aflatoxins accumulating in the maize kernels, an ELISA was carried out as described in section 4.4.5 above with aflatoxins quantified by measuring the sample absorbance using a microtiter spectrophotometer at 450nm (R-Biopharm AG, Darmstadt, Germany). Levels of aflatoxins produced by the different cultures from hp-*stcA* and hp-*stcJ* treatments in the florescent experiment above were determined using HPLC. The re-isolated *A. flavus* was cultured on YES medium. Then, the methodology by Khayoon *et al.* (2012) was employed as described in section 4.4.6. Briefly, 3 agar plugs were removed from one colony, and placed into a 4ml vial, where 1 ml of methanol was added to extract total aflatoxins. After 60 min, the extract was filtered by 0.45µm filters and analyzed via HPLC at the Department of Veterinary Medicine, University of Nairobi, Kabete Campus. The results were compared to those from analysis of the maize kernels and fluorescence experiments for comparison.

5.2.8 Prediction of possible siRNA off-targets in maize cDNA library

The complete gene cluster sequence of *A. flavus* isolate used in cloning and infection assays was downloaded from NCBI (http://www.ncbi.nlm.nih.gov). Analysis of siRNAs was done using RNAiScan (http://bioinfo2.noble.org/RNAiScan.htm). These siRNAs were then enumerated for each of the transcripts (*stcA* and *stcJ*) according to the rules by Ui-Tei *et al.* (2004) including a 5' antisense strand, starting with an A or U base, a 5' sense strand, starting with G or C, and the first seven bases from a 5' antisense strand end containing at least three to five A/U bases. Upon determination of siRNAs, patterns of potential off-targets were obtained by querying the cDNA/transcript libraries of *Zea mays* using another online database, pssRNAit (http://plantgrn.noble.org/pssRNAit) for genome-wide off-target gene assessment.

5.2.9 Plant growth measurements

To examine any possible effect of hairpin constructs on architecture and yield, plant height and dry biomass of transgenic T1 plants were measured and compared with non-transformed counterparts. Seeds were first sowed in potted soil in the glasshouse with regular watering using tap water. Transgenic status of the plants was confirmed via regular PCR after which top dressing was done using di-ammonium phosphate (DAP). Plant growth measurements were only done on PCR-positive plants maintained in the glasshouse under the following conditions; 12 h light/12 h darkness photoperiod, temperatures maintained between 26 and 28ºC and a 60% relative humidity. For each construct, plant height was taken at silking stage using a tape measure on 10 transgenic plants in at least 2 independent experiments (replicated 3 times). Ten (10) wild type plants were also included as controls. After physiological maturation, grain yield was estimated by harvesting the cobs before drying them in an oven (65ºC). The biomass was determined by calculating the mean weight of the dried cobs (with kernels) and comparing with wild type plants. Analysis of variance was done using SAS software at 95% confidence interval.

5.3 Results

5.3.1 Development of putatively transformed maize calli

Confirmation of the transgenes in silencing vectors was achieved following a perfect pairwise alignment between each cloned hairpin sequence and the genebank sequences as shown in figures 11 and 12. Generally, there were no visual differences between callus cultures infected with hp-*stcA* and hp-*stcJ* cassettes shown in Plate 9. A slight increase in size was observed when infected maize zygotic embryos were maintained on co-cultivation medium for 3 days (Plate 9A). Upon transfer onto resting medium, embryos significantly increased in size by the third day of culture and formation of calli followed. Both experiments set were observed to initiate calli that grew well. Most of the explants formed calli that were white in color, dry and compact although some were observed to

form calli that were brown with watery sections on the surfaces. Some calli were observed to form roots while on resting medium although these were not found to affect their development (Plate 9B).

5.3.2 Selection of transformed maize calli using hygromycin

Embryos responding to callus formation, when transferred and maintained on MS medium with 15mg/l hygromycin, increased in size and grew well. Some calli showed browning and eventually died off within 2 weeks of culture on selection media due to the effect of hygromycin sulphate (Plate 10A). Calli surviving first selection, when transferred and maintained on second selection medium containing 30mg/l hygromycin, showed presence of somatic embryos (Plate 10B). However, some of these calli underwent browning and eventually died off (Plate 10C).

5.3.3 Regeneration and hardening of putatively transformed maize plantlets

Calli surviving second selection showed presence of somatic embryos that turned green within 2-5 days of exposure to light on regeneration medium (Plate 10D). Shoots later formed after 7 days in most of the cultures although the frequency varied across the two experiments transformed with hp-*stcA* and hp-*stcJ*. Multiple shoots were recovered from independent transformation events and their transfer onto hormone-free media resulted in recovery of single plantlets that grew well (Plate 10E). Root formation was successfully achieved on regeneration medium after the second subculture although most of the calli formed shoots and roots within the first two weeks of transfer to regeneration media.

The two experiments, hp-*stcA* and hp-*stcJ,* produced putative plants with relatively high transformation frequencies. Maize transformed with the hp-*stcA* cassette had the highest transformation frequency (13.7%) followed by hp-*stcJ* (12.1%). These frequencies were however not significantly different according to Tukey's test at $P \leq 0.05$ (Table 10). Regeneration was achieved in both experiments with maize transformed with hp-*stcA* showing the highest regeneration frequency of 44% while hp-*stcJ* had 37.5% according to Tukey's test (Table 10). Shoots developed roots within 21 days of culture on

regeneration media and became fully developed after the 28th day. Putatively transformed plantlets were successfully acclimatized and hardened in the glasshouse on autoclaved peat moss (Plate 10F). When transplanted into pots containing peat moss, the plantlets showed signs of wilting but recovered after 5 days of covering using a plastic bag. The plants successfully hardened in pots containing soil with regular watering (Plate 10G and H). These plants grew well under these conditions to maturity where they produced normally following self pollination (Plate 10I).

```
                        1
50
AY510452.1stcAfull     (1) ATCAACGTAAAATGATTAGCACCGTCCGCTCGGACAATGTCAAACGAGGC
      hp stcA .txt     (1) --------------------------------------------------
         Consensus     (1)
                           51                                              100
AY510452.1stcAfull    (51) CCCGGGCATGATCGTATCCCACCCATCGGGACCAAATTCCGTCCGCTTCT
      hp stcA .txt     (1) -----------------------------------TTCCGTCCGCTTCT
         Consensus    (51)                                    TTCCGTCCGCTTCT
                          101                                              150
AY510452.1stcAfull   (101) GAATCATGAAATGCATTCCTTTCATCTTGGGAGCATCCCGCTCGTCCATG
      hp stcA .txt    (15) GAATCATGAAATGCATTCCTTTCATCTTGGGAGCATCCCGCTCGTCCATG
         Consensus   (101) GAATCATGAAATGCATTCCTTTCATCTTGGGAGCATCCCGCTCGTCCATG
                          151                                              200
AY510452.1stcAfull   (151) ACTGTCTCTGCCGCCCAGACGATGCCGACCTTGGGCATCCGGCGCGTATG
      hp stcA .txt    (65) ACTGTCTCTGCCGCCCAGACGATGCCGACCTTGGGCATCCGGCGCGTATG
         Consensus   (151) ACTGTCTCTGCCGCCCAGACGATGCCGACCTTGGGCATCCGGCGCGTATG
                          201                                              250
AY510452.1stcAfull   (201) CAACGGGGCCAGCTTGTAATCCAGCATCACATCCACCACAGCGGTAAAGT
      hp stcA .txt   (115) CAACGGGGCCAGCTTGTAATCCAGCATCACATCCACCACAGCGGTAAAGT
         Consensus   (201) CAACGGGGCCAGCTTGTAATCCAGCATCACATCCACCACAGCGGTAAAGT
                          251                                              300
AY510452.1stcAfull   (251) GTGGGATCAAGTAGGACGGAGGCTCAGTCGAGCCGTCCGGACTAGCCCCC
      hp stcA .txt   (165) GTGGGATCAAGTAGGACGGAGGCTCAGTCGAGCCGTCCGGACTAGCCCCC
         Consensus   (251) GTGGGATCAAGTAGGACGGAGGCTCAGTCGAGCCGTCCGGACTAGCCCCC
                          301                                              350
AY510452.1stcAfull   (301) GGCTGGGTAGCGAACAATCCAATGCTATTACAGTGCTCGTAAAAGGCTCG
      hp stcA .txt   (215) GGCTGGGTAGCGAACAATCCAATGCTATTACAGTGCTCG-----------
         Consensus   (301) GGCTGGGTAGCGAACAATCCAATGCTATTACAGTGCTCG
                          351                                              400
AY510452.1stcAfull   (351) GGGGAGTTGTTCCATGGCTTGTGGAATAGGCGCATCAATAATGATTAACG
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (351)
                          401                                              450
AY510452.1stcAfull   (401) AATCCACCTCCTCGCCTTGGTTAACAAGTGCCTCGGCCACGACATAAGCG
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (401)
                          451                                              500
AY510452.1stcAfull   (451) AATGCACCACCGGACGACCAGCCGCCCAAATGATAGGGGCCCCGTGGCTG
```

```
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (451)
                           501                                             550
AY510452.1stcAfull   (501) TCGCCGCCGGATCTCATTGCAAAAGCTCTCAATCATAGCTCCATGTGTGC
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (501)
                           551                                             600
AY510452.1stcAfull   (551) AGTTCATGTTCTCGGGATCCCGAGCATAAGGGCAATTCAGGCCCACAACG
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (551)
                           601                                             650
AY510452.1stcAfull   (601) GCAGTATCTGATTTGAGGCGCGGCAGGGAGGCGTAGGAGAAGGCAGACCC
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (601)
                           651                                             700
AY510452.1stcAfull   (651) TCCGCCATCAGGGAGCATGAATAGAGTTTTCCGCGCCACCATAGGGAGAC
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (651)
                           701                                             750
AY510452.1stcAfull   (701) CTTGTAGGACGACAGAAGTTGAGGGGCGGCAGTAGGGCTTCAGATCAAGT
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (701)
                           751                                             800
AY510452.1stcAfull   (751) GCAGGCGGACTTCCAGTGTCGGCGCCGGACTCACTCGAATGACCGTGATC
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (751)
                           801                                             850
AY510452.1stcAfull   (801) TTCGCTGGCGAAAATAGAGTCGGAGGCGCTACTGGACCAATCGGTTTCGG
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (801)
                           851                                             900
AY510452.1stcAfull   (851) GGTTGATGCCGGGAATAGCTGATGAAGGAGAATCTTCTATATTGCAGCCA
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (851)
                           901                                             950
AY510452.1stcAfull   (901) CTGCCAGCACCCCCACTTCCCAACATGAAGTCTTTCAAGGCACGCACGGT
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (901)
                           951                                            1000
AY510452.1stcAfull   (951) AGTGCAATCAATGAAAAGAGAAAACTCAGGCCCCAGGTCCAGCCCCAGGT
      hp stcA .txt   (254) --------------------------------------------------
         Consensus   (951)
                          1001                                            1050
AY510452.1stcAfull  (1001) CCTCTCTGAAGCGGCTCCCAATGACCATTGAACTCAGAGAGTCGATGCCC
      hp stcA .txt   (254) --------------------------------------------------
         Consensus  (1001)
                          1051                      1080
AY510452.1stcAfull  (1051) ATGTCAGCAAAGTTGCTGTCATCGGTGAGC
      hp stcA .txt   (254) ----------------------------
         Consensus  (1051)
```

Figure 11. Pairwise sequence alignment between full cDNA of stcA from *Aspergillus flavus* (AY510452.1. Gene bank) with the cloned fragment (hp stcA). Alignment was

done using Vector NTI Advance™ version 10 and it revealed 100% similarity between the 253bp fragment cloned into pStargate vector and the full cDNA. The yellow region indicate a perfect alignment.

```
                          1                                                  50
AY510452.1 stcJfull     (1) GATCCTGGGAGATACGTGCCAAGGGATCGAGAAAAATGCGGGGGGCATCC
       hp stcJ .txt     (1) --------------------------------------------------
          Consensus     (1)
                         51                                                 100
AY510452.1 stcJfull    (51) TGCTCGTCGTACGGGTTTTGCGTCTGGATCTTCACCACCGCCTGCGACAT
       hp stcJ .txt     (1) --------------------------------------------------
          Consensus    (51)
                        101                                                 150
AY510452.1 stcJfull   (101) GACCGCCGCGGCGTAGGCGCGGTCACCCGCCTCGGTTCGAACCTTCACCT
       hp stcJ .txt     (1) ----------------------------------GAACCTTCACCT
          Consensus   (101)                                    GAACCTTCACCT
                        151                                                 200
AY510452.1 stcJfull   (151) TGCGATAGTAGCCCTCAACCTGGGGGCGGGGGAGCGTCGCCAAGAAGTAT
       hp stcJ .txt    (13) TGCGATAGTAGCCCTCAACCTGGGGGCGGGGGAGCGTCGCCAAGAAGTAT
          Consensus   (151) TGCGATAGTAGCCCTCAACCTGGGGGCGGGGGAGCGTCGCCAAGAAGTAT
                        201                                                 250
AY510452.1 stcJfull   (201) TTGGGGGCCACGCCAACGACTTGGCCCCCCTTCTGTCCGAAGCCAAATGA
       hp stcJ .txt    (63) TTGGGGGCCACGCCAACGACTTGGCCCCCCTTCTGTCCGAAGCCAAATGA
          Consensus   (201) TTGGGGGCCACGCCAACGACTTGGCCCCCCTTCTGTCCGAAGCCAAATGA
                        251                                                 300
AY510452.1 stcJfull   (251) GGTCAGCAGGAATGCCTTGACCTCACGTAGCTGCACGGTGCGCGTCGGGA
       hp stcJ .txt   (113) GGTCAGCAGGAATGCCTTGACCTCACGTAGCTGCACGGTGCGCGTCGGGA
          Consensus   (251) GGTCAGCAGGAATGCCTTGACCTCACGTAGCTGCACGGTGCGCGTCGGGA
                        301                                                 350
AY510452.1 stcJfull   (301) AGCAGAGATGAGACGCGCTGCTCAAGGCCTCGTCCAGCGTGTCCAGATTG
       hp stcJ .txt   (163) AGCAGAGATGAGACGCGCTGCTCAAGGCCTCGTCCAGCGTGTCCAGATTG
          Consensus   (301) AGCAGAGATGAGACGCGCTGCTCAAGGCCTCGTCCAGCGTGTCCAGATTG
                        351                                                 400
AY510452.1 stcJfull   (351) CGGTTGCCCGGCACCAACCCCGAGTCCAGTACTTGCAGGCATCCATTGAG
       hp stcJ .txt   (213) CGGTTGCCCGGCACCAACCCCGAGTCCAGTACTTGCAGGCATCCATTGAG
          Consensus   (351) CGGTTGCCCGGCACCAACCCCGAGTCCAGTACTTGCAGGCATCCATTGAG
                        401                                                 450
AY510452.1 stcJfull   (401) CATCCATGCGGCCGCTGGGGCTTTAGGGTGTCCCGTCACCGACTTTTGGC
       hp stcJ .txt   (263) CATCCATGCGGCCGCTGGGGCTTTAGGGTGTCCCGTCACCGACTTTTGGC
          Consensus   (401) CATCCATGCGGCCGCTGGGGCTTTAGGGTGTCCCGTCACCGACTTTTGGC
                        451                                                 500
AY510452.1 stcJfull   (451) AGATGGCCCACAGGGGGCGGCCAGGAGTGCGACCTAAATGGCGCATCTGC
       hp stcJ .txt   (313) AGATGGCCCACAGGGGGCGGCCAGGAGTGCGACCTAAAT-----------
          Consensus   (451) AGATGGCCCACAGGGGGCGGCCAGGAGTGCGACCTAAAT
                        501                                                 550
AY510452.1 stcJfull   (501) GTCTCGATCACCTCGGGCTCATTGAGATCGTTAGCGCGCGTCGAGGTGCC
       hp stcJ .txt   (352) --------------------------------------------------
          Consensus   (501)
                        551                                                 600
AY510452.1 stcJfull   (551) GTGCAATGAGGCCACATCCAGGTCGTCGATAGTTAGCCCCCAAGTCGCAA
       hp stcJ .txt   (352) --------------------------------------------------
          Consensus   (551)
                        601                                                 650
```

```
AY510452.1 stcJfull  (601) GTGCTGCTCGCAGCGGAGCTATATCAGTGGTGGATCGGGCGCTTTGTGTA
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (601)
                             651                                               700
AY510452.1 stcJfull  (651) CGAGGAGTGGGATTGCTGATTGAGGTGATTGAGGTCAGGGATGATTTGTC
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (651)
                             701                                               750
AY510452.1 stcJfull  (701) CGAGACTTCAGATGATGTGCTGCTACGGCTACTCGGGCGCGACGTGACGG
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (701)
                             751                                               800
AY510452.1 stcJfull  (751) ATATCATACTCGATCGAGCGCGCTCACGTGAGAAGCTTAGAATGCCCTGG
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (751)
                             801                                               850
AY510452.1 stcJfull  (801) CCCGGTGCTGGCACCGAGGAACCAATCTTGTCGGCTGCCATGGCTGAGCT
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (801)
                             851                                               900
AY510452.1 stcJfull  (851) GGCAATGACCGCATAGATAGGAAGGCCCATTTGCAGGGCGATGTCACCTC
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (851)
                             901                                               950
AY510452.1 stcJfull  (901) GACACAGCAGCTGCACACCGCAGCCATGCGCCTCGACAAAGCCAGCACGA
       hp stcJ .txt  (352) ----------------------------------------------------
          Consensus  (901)
                             951                          982
AY510452.1 stcJfull  (951) CTCTCAGCCATGGGGCGCGACATCTCCGAGGG
       hp stcJ .txt  (352) --------------------------------
          Consensus  (951)
```

Figure 12. Pairwise sequence alignment between full cDNA of stcJ from *Aspergillus flavus* (AY510452.1.) Gene bank) with the cloned fragment (hp stcJ). Alignment was done using Vector NTI Advance™ version 10 and it revealed 100% similarity between the 351bp fragment cloned into pStargate vector and the full cDNA. The yellow region indicate a perfect alignment.

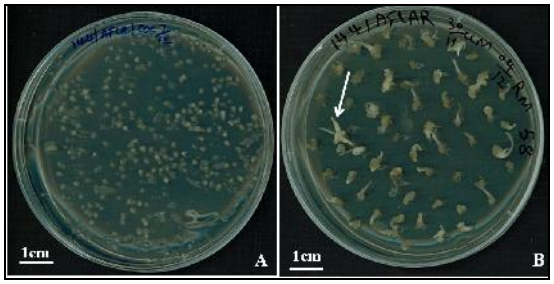

Plate 9. Developmental stages of maize embryos after infection with *A. tumefaciens.* **A**. Co-cultivation of immature zygotic embryos on MS medium supplemented with 2mg/L 2, 4-D. **B**. Callus formation in embryos on resting medium. The arrow indicates presence of roots in some of the embryos at this stage.

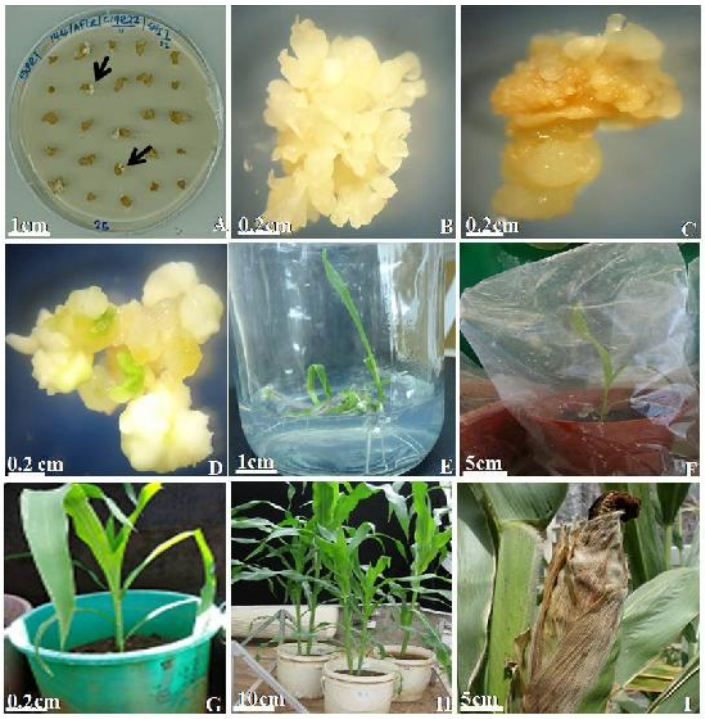

Plate 10. The different **s**tages involved in development of transgenic maize transformed with hp-*stcA* and hp-*stcJ*. **A.** Transformed maize calli maintained on MS medium supplemented with 30mg/l hygromycin for selection. Arrows indicate calli that survived hygomycin action. **B.** A compact callus obtained after 2 weeks of culture on second selection medium showing presence of somatic embryos. **C.** A brown callus on selection medium that eventually died off due to hygromycin action. **D.** An embryogenic callus after transfer onto embryo maturation medium and exposure to light. **E**. Multiple maize shoots that emerged after exposure of mature embryos to light on regeneration medium. **F.** Acclimatization of a maize plantlet on peat moss using a pot and plastic bag in the glass house. **G.** Hardening of the putative transformants in soil. **H.** Established putative transformed plants in the glasshouse. **I.** A mature transgenic maize cob in maize transformed with hp-*stcA*.

Table 10. Transformation and regeneration frequencies in maize putatively transformed with hairpin cassettes

Experiment/ Event	No. of embryos infected	Transformation frequency (%)	Regeneration frequency (%)
hp-*stcA*	470	13.74±4.67[a]	44.02±2.09[a]
hp-*stcJ*	378	12.31±2.75[a]	37.74±1.24[ab]

Values are means of 3 replications followed by their standard errors. Means in each column followed by the same letter are not significantly different according to Tukey's test at $P \leq 0.05$.

5.3.4 Molecular analysis of putatively transformed maize plants

PCR analysis of the hygromycin selectable marker gene on genomic DNA extracted from putative transgenic maize using *hptII* primers showed positive results in almost all putative plants. The expected fragment (816bp) of the hygromycin gene was amplified on 1% agarose gel (Plate 11). The fragment represents the hygromycin selectable marker used for recovery of the transformants in both expression cassettes.

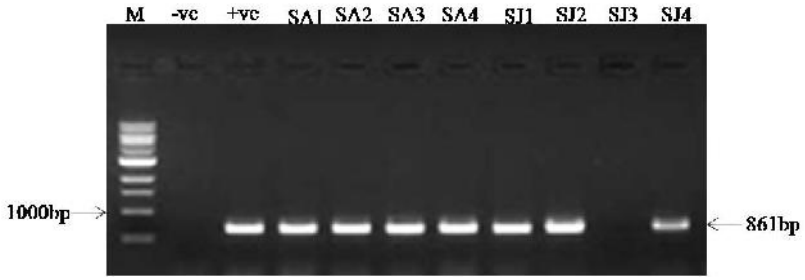

Plate 11. PCR analysis of hygromycin transgene in maize plants putatively transformed with hp-*stcA* and hp-*stcJ*. Lane M was loaded with a 1kb DNA ladder. SA represents putative plants transformed with hp-*stcA* while SJ are plants transformed with hp-*stcJ*. –ve represents non-transformed maize genomic DNA while +ve was (a positive control) plasmid DNA from E coli harboring pStargate vector with a hygromycin gene.

5.3.5 Infection of transgenic maize plants with *A. flavus* and collection of seed

Fungal colonization of transgenic maize was observed 3 days after inoculating the plants with *A. flavus*. Moulds formed on the inoculated silks in all treatments (non-transformed, hp-*stcA* and hp-*stcJ* maize) and were visible after removal of the plastic bag (Plate 11A). All maize plants grew normally despite the fungal colonization although some kernels showed massive infection. Some of the kernels turned to a yellowish-brown color while others remained white (Plate 11B). Moulds were detected in most of the cobs across treatments although this was not necessarily considered an effect of *A. flavus*. Maize kernels from each event and infection treatments were collected, dried and stored at 4ºC for aflatoxin analysis.

5.3.6 *A. flavus* re-isolation from infected kernels and *in vitro* aflatoxin determination

Fungal growth from maize kernels was observed 3 days after culture of kernels on MRBA. The fungus was seen growing from the kernels onto the medium with colonies becoming distinct by color after the fifth day of culture. Several types of fungi emerged from the maize kernels but *A. flavus* was distinguished from the rest on the basis of morphological characteristics; mainly the colony color and conidia morphology as seen under the light microscope as described by Klich and Pitt, (1988). Isolates showing a yellow to green coloration with smooth conidia were selected as *A. flavus*. Pure colonies of *A. flavus* were successfully obtained after transferring a single spore from the colony onto fresh media (Plate 11C).

Fungal growth on NRDCA medium was observed after 3 days of culture. Fluorescence was detected upon visualization under UV light (365nm) after the third and fifth days of incubation on NRDCA medium. MCKII fungi used for infection and that re-isolated from wild type maize showed a bright blue fluorescence characteristic of high aflatoxigenecity. This was scored with +++ (Table 11). Moderately bright to weak fluorescence was observed in fungal cultures re-isolated from maize transformed with *stcA* (recorded as ++). Cultures re-isolated from maize transformed with hp-*stcJ* cassette

exhibited either a weak or no blue fluorescence under UV light at 3 and 5 days (Plate 13). The no florescence was taken as non aflatoxigenic *A. flavus*.

It was interesting to note that a reduction in frequency of aflatoxigenic fungi was recorded in cultures from transgenic maize events. Moderately low frequencies (<45%) of occurrence of *A. flavus* were generally obtained in all treatments after culture of the kernels on PDA media (Table 12). Upon screening of the re-isolated *A. flavus* for aflatoxin production, it was found that 99% of the fungal cultures re-isolated from non-transformed maize plants (WT) showed presence of aflatoxins after visualization under U.V light. A high frequency of aflatoxigenic fungi (85.3%) was also recorded in *A. flavus* recovered from maize events with hp-*stcA.* However, a very low frequency of aflatoxigenic *A. flavus* was recorded in events with hp-*stcJ* which was significantly different from the others according to Tukey's HSD test at $P \leq 0.05$ (Table 12).

Figure 4. Infection of maize with and recovery of *A. flavus in vitro*. **A.** Growth of fungi on maize silk three days after inoculation with *A. flavus*. **B.** An infected maize cob at maturity showing fungal colonization. Appearance of molds on the cob doesn't necessarily indicate A. flavus colonization. **C**. A pure *A. flavus* colony on PDA.

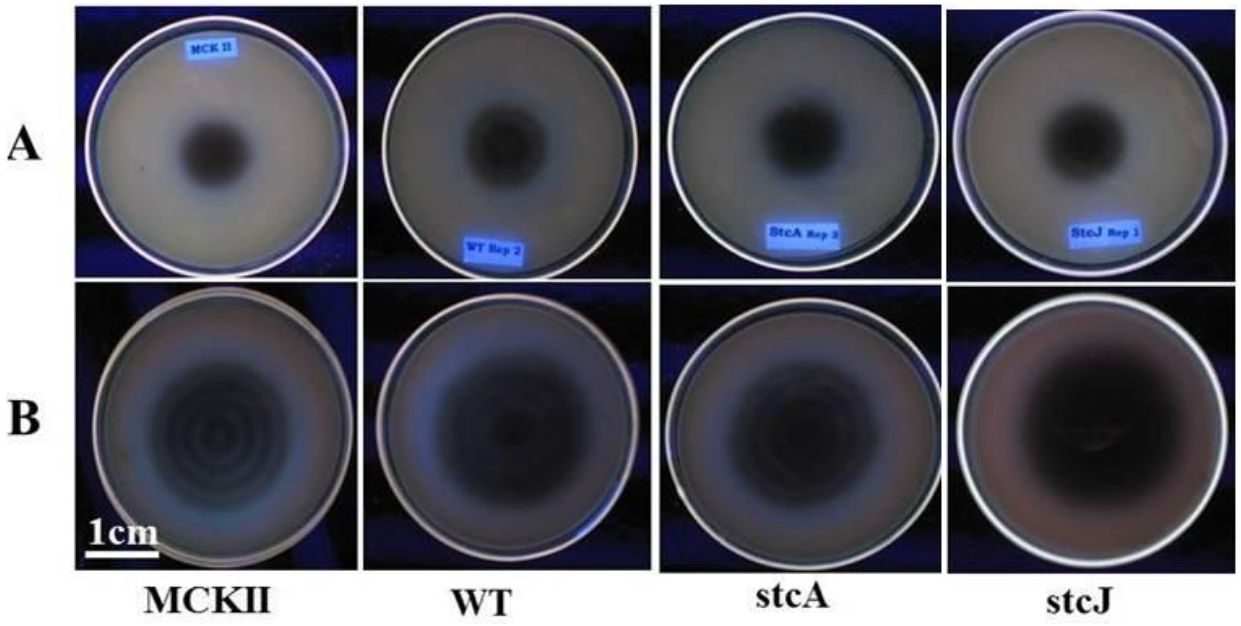

Plate 13. Determination of aflatoxin production after culture on NRDCA medium. Presence of a blue fluorescence around the isolate indicates aflatoxin production. Panel (**A**) shows detection of aflatoxins after 3 days of culture while panel (**B**) is detection after 5 days. MCKII is the original *A. flavus* culture used in infection. WT-fungal cultures from non-transformed maize while *stcA* and *stcJ* are cultures from transgenic maize.

Table 11. Fluorescence intensity of fungal cultures isolated from maize infected with aflatoxigenic *A. flavus* on NRDCA medium after visualization under U.V light.

Identity/ Treatment	**Fluorescence intensity**
MCKII	+++
WT	+++
WT	+++
WT	+++
stcA	++
stcA	++
stcA	++
stcJ	+
stcJ	+
stcJ	-

+++Bright fluorescence**;** ++Moderate; +Weak and **–** No fluorescence

Table 12. Occurrence of *A. flavus* re-isolated from maize and the frequency of aflatoxigenic fungi after visualization under UV light

Treatment	*A. flavus* occurrence on MRBA (%)	Frequency of aflatoxigenic *A flavus* on NRDCA (%)
WT	42.67±1.03[a]	99.03±1.00[a]
stcA	40.33±2.40[a]	85.33±2.33[b]
stcJ	44.67±1.45[a]	38.67±2.03[c]

NRDCA-neutral red desiccated coconut agar, MRBA-modified rose Bengal agar, WT-wild type. Values followed by the same letter in each column are not significantly different according to Tukey's test at P≤*0.05*.

5.3.7 Molecular determination of silencing of target transcripts in *A. flavus*

Reverse transcription polymerase chain reaction analysis on cDNA from fungal cultures showed a diverse pattern of gene expression across the treatments. There was a uniformly strong expression signal across all cultures for *β-tubulin*, used as an internal control (Plate 14). A clear reduction in the signal was however noted upon amplification of *stcA* and *stcJ* on cDNA from fungal isolates colonizing transgenic and non-transgenic plants. The *stcJ* transcript showed very low signal bands (Plate 14). However, a high signal band was observed on cDNA from *A flavus* colonizing non-transformed maize and that from MCKII isolate (also grown on culture media) (Plate 14).

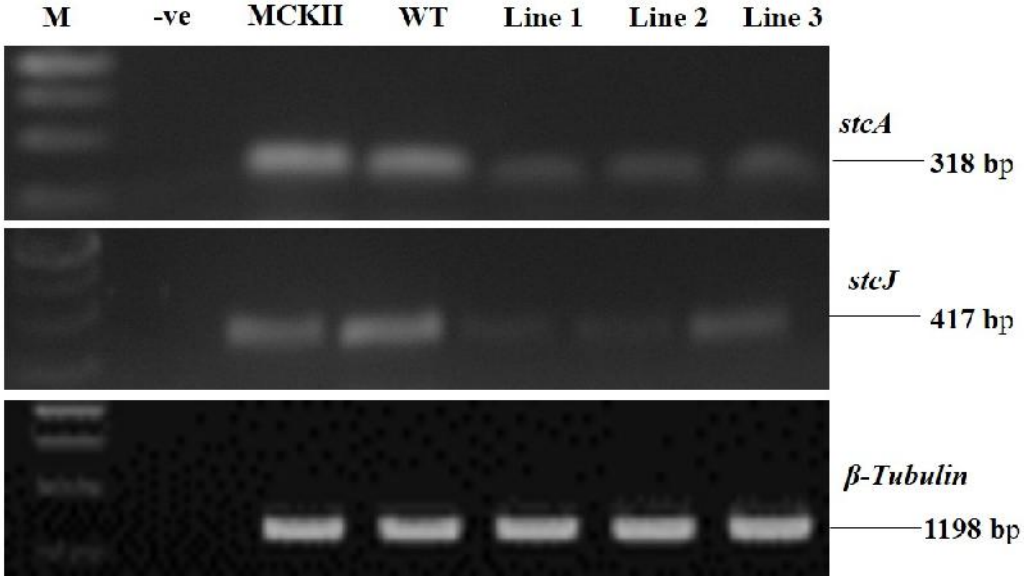

Figure 14. Reverse transcription polymerase chain reaction analysis of expression of target *stcA* and *stcJ* transcripts in *A. flavus* colonizing transgenic and non-transformed maize. Expression was done with reference to the expression of *β-Tubulin* rRNA in fungi. Lane M was loaded with a 1kb DNA ladder (Bioline) while –ve is a negative PCR control. MCKII is amplification on cDNA from fungi used for cloning but not infecting any maize. WT represents amplification on cDNA from fungi re-isolated from non-transformed maize while Line1-Line 3 is amplification in representative fungi from each of the 3 independent lines.

5.3.8 Quantification of aflatoxins in maize kernels and re-isolated fungal cultures

ELISA results revealed very high levels of aflatoxins in maize kernels from non-transformed maize plants infected with the aflatoxigenic *A. flavus*. These kernels recorded total aflatoxins of >141.75ppb which is the highest detection limit on the spectrophotometer. Maize samples transformed with hp-*stcA* silencing cassette resulted in lower levels of total aflatoxins, when compared to the wild type maize, resulting in 12.4 and 10ppb total aflatoxins (Figure 13A). On the other hand, events transformed with hp-*stcJ* recorded the lowest level of total aflatoxins accumulation. This event had total aflatoxins of <1.75ppb which is the lowest test detection limit (Figure 13B). HPLC results for total aflatoxins in fungal cultures showed a correlation to the levels of the total aflatoxins obtained earlier in the maize kernels screened via ELISA. On average, *A. flavus*

cultures re-isolated from wild type maize again recorded the highest level of total aflatoxins at 117ppb followed by the MCKII culture, used for infection and cloning but not colonizing any maize, which recorded 70.3ppb (Figure 13C). The cultures from events transformed with hp-*stcJ* silencing construct had the lowest levels, average <1.75ppb, of total aflatoxins (Figure 13D).

5.3.9 Effect of *stcA* and *stcJ* silencing cassettes on plant height and yield

Expression of the 2 silencing cassettes in this chapter had no observable differences on plant heights and cob sizes (Plate 15 A and B). There were no significant differences in the average plant heights in maize expressing the silencing constructs as compared with non-transformed ones under the present conditions (Plate 15C). On average, transgenic maize recorded a mean height of 204.2 cm (*stcA)* and 215.6 cm (*stcJ)* at silking stage (Plate 15C-D). Similarly, no significant differences were obtained upon determination of yield. Here, transgenic maize produced average dry biomass of 234 and 226g per cob in *stcA* and *stcJ* respectively. This was slightly lower but not significantly different from the dry biomass from non-transformed maize which had an average dry biomass of 245g (Plate 15D). The effective siRNA candidates generated by the hairpin construct and expressed in maize are as outlined in Figures 14 (*stcJ*) and 15 (*stcA*). Querrying these siRNAs against the maize genome did not reveal any off targets in this plant (maize).

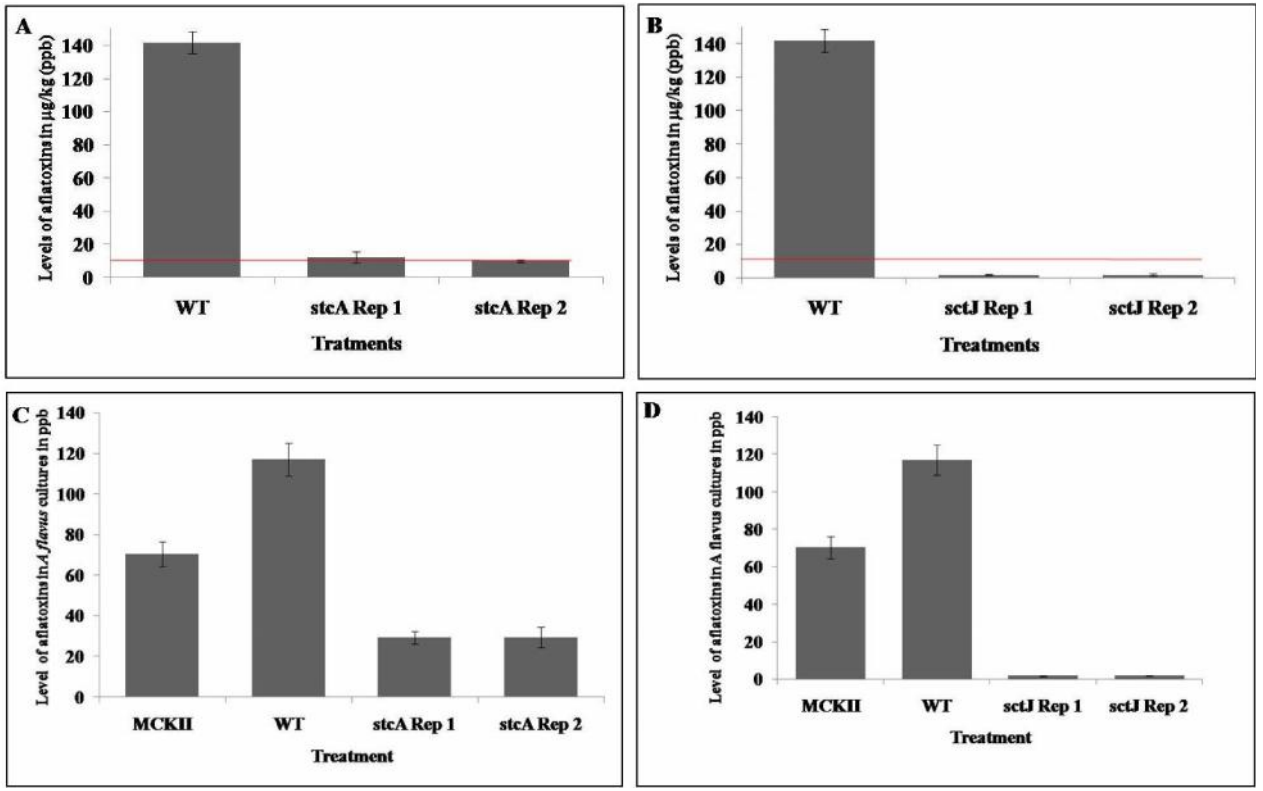

Figure 13. Determination of total aflatoxin accumulation in maize and fungal cultures. **A.** Levels of total aflatoxins accumulating in transgenic maize transformed with with hp-*stcA.* B. Levels of aflatoxins accumulating in transgenic maize with hp-*stcJ.* The red line indicates the maximum limit of total aflatoxins in foodstuffs permitted by KEBS. WT represents maize kernels from non-transformed plants. *stcA* and *stcJ* are transgenic maize harboring hp-*stcA* and hp-*stcJ* cassettes respectively. Values are average spectrophotometer readings quantified via ELISA. C. Total aflatoxins in fungal cultures re-isolated from transgenic maize expressing hp-*stcA.* D. Total aflatoxins in cultures from transgenic maize with hp-*stcA.* Values are average spectrophotometer readings quantified via HPLC alongside total aflatoxin levels in fungal cultures re-isolated from wild type maize (WT) and the culture used for infection (MCKII). Vertical bars indicate the standard errors of the mean. Means with same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$).

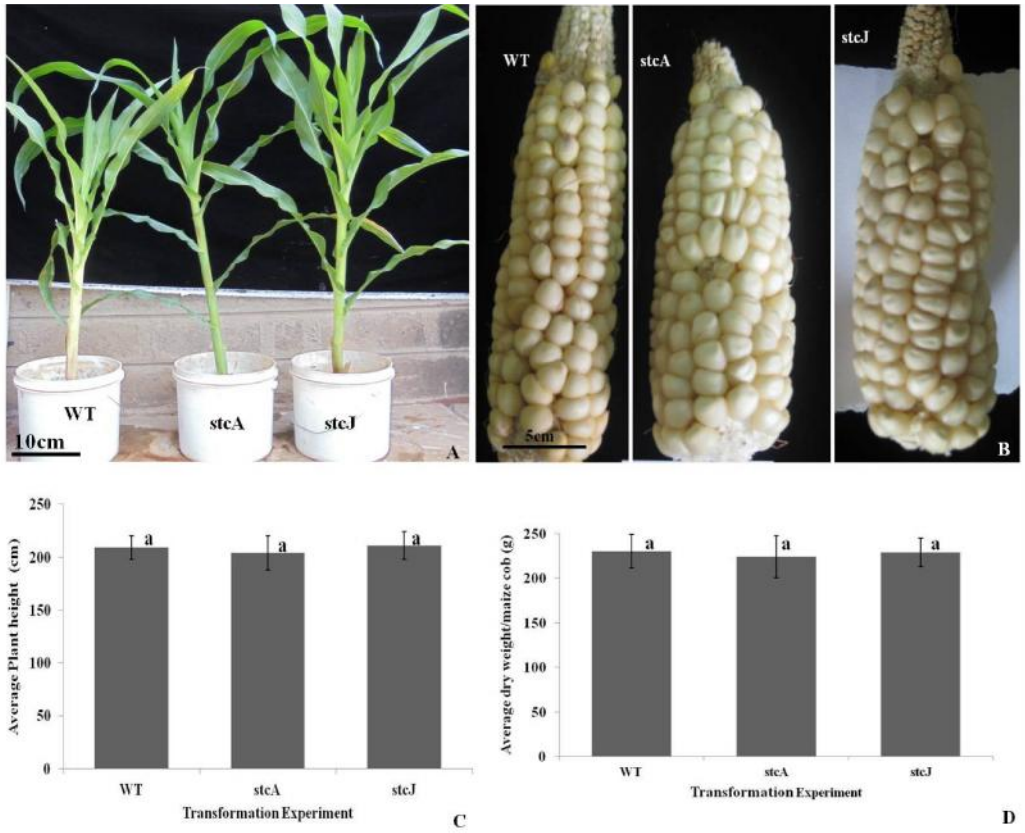

Plate 15. Determination of the effect of silencing cassesttes on growth and yield in maize. **A** Representative images of transgenic plants (*stcA* and *stcJ*) alongside controls (WT) at silking stage. **B.** Appearance of maize cobs from transgenic (*stcA* and *stcJ*) and non-transformed (WT) maize. **C.** Average plant height in transgenics expressing hairpin constructs (stcA and stcJ) as compared to the corresponding non-transformed controls (WT). **D.** Average dry weight of transgenic maize cobs alongside the wild types. Vertical bars indicate the standard errors of the mean. Means with same letter are not significantly different according to Tukey's HSD test ($P \leq 0.05$).

Target dataset = Zea mays (maize) DFCI Gene Index (ZMGI) version 19 release on 8/12/2010

Query sequence fragments do not have hits in target dataset

Predicted efficient siRNAs

Number	Fragment	siRNA AS (3' - 5')	Hits
1	stcJ (15 - 36)	GCACGGUUCCCUAGCUCUUUUU	0
2	stcJ (16 - 37)	CACGGUUCCCUAGCUCUUUUUA	0
3	stcJ (63 - 84)	CCCAAAACGCAGACCUAGAAGU	0
4	stcJ (139 - 160)	CUUGGAAGUGGAACGCUAUCAU	0
5	stcJ (177 - 198)	GCCCCCUCGCAGCGGUUCUUCA	0
6	stcJ (178 - 199)	CCCCCUCGCAGCGGUUCUUCAU	0
7	stcJ (179 - 200)	CCCCUCGCAGCGGUUCUUCAUA	0
8	stcJ (180 - 201)	CCCUCGCAGCGGUUCUUCAUAA	0
9	stcJ (181 - 202)	CCUCGCAGCGGUUCUUCAUAAA	0
10	stcJ (229 - 250)	GGAAGACAGGCUUCGGUUUACU	0
11	stcJ (376 - 397)	GGUCAUGAACGUCCGUAGGUAA	0
12	stcJ (426 - 447)	CCCACAGGGCAGUGGCUGAAAA	0
13	stcJ (467 - 488)	CGCCGGUCCUCACGCUGGAUUU	0
14	stcJ (468 - 489)	GCCGGUCCUCACGCUGGAUUUA	0
15	stcJ (506 - 527)	CUAGUGGAGCCCGAGUAACUCU	0
16	stcJ (512 - 533)	GAGCCCGAGUAACUCUAGCAAU	0
17	stcJ (563 - 584)	GUGUAGGUCCAGCAGCUAUCAA	0
18	stcJ (603 - 624)	CGACGAGCGUCGCCUCGAUAUA	0
19	stcJ (607 - 628)	GAGCGUCGCCUCGAUAUAGUCA	0
20	stcJ (629 - 650)	CCACCUAGCCCGCGAAACACAU	0
21	stcJ (652 - 673)	CUCCUCACCCUAACGACUAACU	0
22	stcJ (661 - 682)	CUAACGACUAACUCCACUAACU	0
23	stcJ (675 - 696)	CACUAACUCCAGUCCCUACUAA	0
24	stcJ (735 - 756)	CCCGCGCUGCACUGCCUAUAGU	0
25	stcJ (736 - 757)	CCGCGCUGCACUGCCUAUAGUA	0
26	stcJ (737 - 758)	CGCGCUGCACUGCCUAUAGUAU	0
27	stcJ (739 - 760)	CGCUGCACUGCCUAUAGUAUGA	0
28	stcJ (768 - 789)	CGCGCGAGUGCACUCUUCGAAU	0
29	stcJ (771 - 792)	GCGAGUGCACUCUUCGAAUCUU	0
30	stcJ (772 - 793)	CGAGUGCACUCUUCGAAUCUUA	0
31	stcJ (805 - 826)	CACGACCGUGGCUCCUUGGUUA	0
32	stcJ (808 - 829)	GACCGUGGCUCCUUGGUUAGAA	0
33	stcJ (810 - 831)	CCGUGGCUCCUUGGUUAGAACA	0
34	stcJ (848 - 869)	CGACCGUUACUGGCGUAUCUAU	0
35	stcJ (852 - 873)	CGUUACUGGCGUAUCUAUCCUU	0

Figure 14. Effective siRNA sequences from stcJ fragment cloned into pStargate and expressed in maize. The siRNAs were querried against a maize genome database and revealed no potential off targets in this index.

Target dataset = Zea mays (maize) DFCI Gene Index (ZMGI) version 19 release on 8/12/2010

Query sequence fragments do not have hits in target dataset

Predicted efficient siRNAs

Number	Fragment	siRNA AS (3' - 5')	Hits
1	stcA (19 - 40)	CGUGGCAGGCGAGCCUGUUACA	0
2	stcA (22 - 43)	GGCAGGCGAGCCUGUUACAGUU	0
3	stcA (23 - 44)	GCAGGCGAGCCUGUUACAGUUU	0
4	stcA (46 - 67)	CUCCGGGGCCCGUACUAGCAUA	0
5	stcA (82 - 103)	GGUUUAAGGCAGGCGAAGACUU	0
6	stcA (83 - 104)	GUUUAAGGCAGGCGAAGACUUA	0
7	stcA (89 - 110)	GGCAGGCGAAGACUUAGUACUU	0
8	stcA (90 - 111)	GCAGGCGAAGACUUAGUACUUU	0
9	stcA (91 - 112)	CAGGCGAAGACUUAGUACUUUA	0
10	stcA (94 - 115)	GCGAAGACUUAGUACUUUACGU	0
11	stcA (95 - 116)	CGAAGACUUAGUACUUUACGUA	0
12	stcA (96 - 117)	GAAGACUUAGUACUUUACGUAA	0
13	stcA (101 - 122)	CUUAGUACUUUACGUAAGGAAA	0
14	stcA (241 - 262)	CGCCAUUUCACACCCUAGUUCA	0
15	stcA (242 - 263)	GCCAUUUCACACCCUAGUUCAU	0
16	stcA (297 - 318)	GGGGCCGACCCAUCGCUUGUUA	0
17	stcA (301 - 322)	CCGACCCAUCGCUUGUUAGGUU	0
18	stcA (302 - 323)	CGACCCAUCGCUUGUUAGGUUA	0
19	stcA (306 - 327)	CCAUCGCUUGUUAGGUUACGAU	0
20	stcA (307 - 328)	CAUCGCUUGUUAGGUUACGAUA	0
21	stcA (311 - 332)	GCUUGUUAGGUUACGAUAAUGU	0
22	stcA (368 - 389)	GAACACCUUAUCCGCGUAGUUA	0
23	stcA (371 - 392)	CACCUUAUCCGCGUAGUUAUUA	0
24	stcA (373 - 394)	CCUUAUCCGCGUAGUUAUUACU	0
25	stcA (374 - 395)	CUUAUCCGCGUAGUUAUUACUA	0
26	stcA (380 - 401)	CGCGUAGUUAUUACUAAUUGCU	0
27	stcA (381 - 402)	GCGUAGUUAUUACUAAUUGCUU	0
28	stcA (405 - 426)	GUGGAGGAGCGGAACCAAUUGU	0
29	stcA (408 - 429)	GAGGAGCGGAACCAAUUGUUCA	0
30	stcA (462 - 483)	CCUGCUGGUCGGCGGGUUUACU	0
31	stcA (463 - 484)	CUGCUGGUCGGCGGGUUUACUA	0
32	stcA (496 - 517)	CCGACAGCGGCGGCCUAGAGUA	0
33	stcA (497 - 518)	CGACAGCGGCGGCCUAGAGUAA	0
34	stcA (502 - 523)	GCGGCGGCCUAGAGUAACGUUU	0
35	stcA (503 - 524)	CGGCGGCCUAGAGUAACGUUUU	0
36	stcA (506 - 527)	CGGCCUAGAGUAACGUUUUCGA	0
37	stcA (515 - 536)	GUAACGUUUUCGAGAGUUAGUA	0
38	stcA (538 - 559)	CGAGGUACACACGUCAAGUACA	0
39	stcA (539 - 560)	GAGGUACACACGUCAAGUACAA	0
40	stcA (541 - 562)	GGUACACACGUCAAGUACAAGA	0
41	stcA (558 - 579)	CAAGAGCCCUAGGGCUCGUAUU	0

Figure 15. Effective siRNA sequences from stcA fragment cloned into pStargate and expressed in maize. The siRNAs were querried against a maize genome database and revealed no potential off targets in this index.

5.4 Discussion

5.4.1 Maize transformation

The use of immature embryos in maize transformation and regeneration has been extensively reported (Ahmadabadi *et al.,* 2007; Abebe *et al.,* 2008; Muoma *et al.,* 2008; Ombori *et al.,* 2008). Success of any transformation procedure is however dependent on the quality and stage of development of the immature embryos as well as the conditions during transformation (Ishida *et al.,* 2007). To ensure this and avoid emerging challenges, embryos for use in transformation were only harvested from healthy plants and at the right time (10-14 days post pollination). Uptake of the hygromycin phosphotransferase (*hpt)* gene by developing calli was vital for their survival on selection media supplemented with hygromycin sulfate.

The *hpt* gene codes for a kinase, which inhibits protein synthesis by interfering with translocation, hence causing mistranslation (Rafiq *et al.,* 2004). Untransformed cells exposed to hygromycin died off due to the effect of the antibiotic but those with the gene survived. Hygromycin was therefore instrumental and effective in identifying transformed calli with relatively high frequencies of transformation achieved in maize inbred line CML 144. Success of a maize genetic improvement program requires not only the ability to deliver transgenes into the cell, but also to produce several transgenic events that stably inherit and express the transgene in the subsequent generations (Ombori *et al*., 2013). While PCR amplification of the transgene often indicates transfer of this transgene into transformed plants, other molecular biology techniques such as southern blot hybridization analysis are needed to verify integration of the transgene into the host genome and also assess the number of copies of the transgenes inserted (Ishida *et al*., 1996).

5.4.2 Evaluation of *A. flavus* infection procedure and the effect of transgenic maize on *A. flavus* aflatoxigenecity

Studies have classified techniques for inoculating maize with *A. flavus* for field and laboratory studies into two main categories; wounding and non-wounding (Windham & Williams, 2007). While the wounding techniques involve causing physical injury to the kernels, the non-wounding method entails spraying a small volume of *A. flavus* conidial suspension onto the ears being infected. Spray-inoculations are the most commonly used assay techniques and have previously been successful in field and laboratory studies in Africa and further, they do not predispose the kernels to other factors that could influence such studies (Cardwell *et al*., 2000). Since aflatoxin contamination is sporadic from growing season to season, the technique used for infection needs to uniformly infect ears with *A. flavus* for effective assaying (King *et al.,* 1982). Silk is the main maize infection court by *A. flavus*, therefore the infection process employed in this study mimicked what happens in nature.

On the other hand, covering the infected silks using a plastic bag enhanced infection beyond what happens in nature, due to the moist environment, and ensured that the resident culture would easily out-compete later infection by different *A. flavus* strains and/or other fungal and species. This is in line with other studies that have reported that environmental conditions have a significant impact on *A. flavus* infection and aflatoxin accumulation (Widstrom *et al.,* 1990). After 3 days, massive *A. flavus* growth on the inoculated silks was visible and therefore indicated successful infection. The successful fungal colonization of infected ears was expected since the genes targeted *StcA* and *stcJ* for silencing are key in aflatoxin biosynthetic pathway and not responsible for fungal growth (Yu, 2012). After inoculations and recovery of the infecting fungi, steps are required to ensure an accurate and precise measurement of aflatoxigenecity of re-isolated *A. flavus* isolate as well as in plant aflatoxin accumulation (Windham & Williams, 2007; Atanda *et al.,* 2011).

Successful re-isolation of the fungi was achieved using culture media. *Aspergillus flavus* is not fastidious in its nutritional requirements and as such, it has been found to grow on nearly all fungal media (Shier *et al.,* 2005). Its isolation therefore relies on sensitivity to certain culture conditions, key among them being certain antibiotics (dichloran and Rose Bengal), ability to grow at relatively high temperatures (37º) and its tolerance to low moisture content (Shier *et al.,* 2005). In this study, the use of chloramphenicol (to eliminate bacteria), media supplemented with Rose Bengal and incubation at (37º) were instrumental in successful re-isolation of *A flavus* from maize, in a similar fashion to other previously reported studies (Pitt 1992; Cotty 1994).

Various cultural methods for identification of aflatoxigenicity in fungal isolates have also been reported based on a variety of qualitative and quantitative attributes (Gilbert and Vargas 2003; Maragos 2004). Such methods rely on the ability to detect fluorescence of aflatoxins produced by a colony growing on agar media (Atanda *et al.,* 2006). In this study, *in vitro* aflatoxigenecity of the isolates was determined using the NRDCA medium (Atanda *et al.,* 2011). According to Shier *et al.* (2005), aflatoxin B1 and B2 produce an intense blue fluorescence under U.V long wave-length light. The diminished florescence level in cultures recovered from transgenic events indicated that there were less aflatoxins produced by those isolates as compared to those from wild type maize kernels. This indicated several things: that the silencing constructs had downregulated the aflatoxin biosynthesis of the toxigenic isolates upon colonization of transgenic maize. Further this also showed that there existed a pathway or mechanism of siRNA trafficking between *A. flavus* and maize.

Finally this result indicated that the siRNAs that trafficked could act at longer distances and across species. These results are similar to those reported by (Niu *et al.,* 2006; Huvenne and Smagghe 2009; Nowara et al., 2010; Alakonya *et al*., 2012; Duan *et al*., 2012; Nunes and Dean 2012) where a host-fungal junction has been proposed. This method for aflatoxin detection while not confirmatory, acted as a pre-screen before actual quantification using quantitative chromatographic and immune based procedures.

Numerous studies have reported the use of ELISA in determining the levels of aflatoxins in grain based foodstuffs. (Chun *et al.,* 2007; Gunterus *et al.,* 2007; Nuryono *et al.,* 2007 and Ayejuyo *et al.,* 2011). This method has been proven to be simple, sensitive, less costly and safe due to the reagents involved as compared to the other techniques therefore validating its use in this study (Ayejuyo *et al.*, 2011).

5.4.3 Effect of RNAi cassettes on aflatoxin biosynthesis and accumulation in maize

The currently acceptable limits for aflatoxins in food and feed Kenya are 10 and 5ppb for total and B1 aflatoxins respectively (Lewis *et al.,* 2005; KEBS 2007; Ndung'u *et al.,* 2013). Results from this study showed a significant reduction in the levels of aflatoxins accumulating in transgenic maize which fell below the maximum limits permitted. Moreover, *in vitro* quantification of the aflatoxins indicated that, to some level, culturing of *A. flavus* on media temporarily reduces its aflatoxigenecity, but when the fungi colonizes maize it enhances its aflatoxigenecity. This could be because the components in culture media are not able to mimic what happens in nature in totality and therefore there are other components in maize that could be missing. A further check on the expression level of the target gene expression revealed a weaker signal in isolates from transgenic as compared to those from non-transformed plants. More importantly, these results confirmed that HIGS was effective in significantly limiting/reducing aflatoxin production by the fungus.

Initial investigations for genetic engineering strategies against aflatoxin biosynthesis in fungi focused majorly on *in vitro* knockdown/knockout (Brown *et al*., 1995; Keller *et al*., 2005). This study successfully applied knowledge on this basic research approach with HIGS to generate transgenic maize accumulating low levels of aflatoxins. Based on the data presented, it is postulated that transgenic maize transformed with the silencing constructs synthesized dsRNAs that were later converted into complementary siRNAs against the target *stcA* and *stcJ* mRNA sequences. Transfer of these molecules from host plants into colonizing *A. flavus* induced silencing of the fungal genes homologous to the expressed precursors required for aflatoxin biosynthesis. This occurred through

targeted degradation where Dicer guided the 21-28bp silencing molecules onto target mRNAs and led to chopping of the respective homologous sequences resulting in downregulation of respective gene expression. These results further emphasize the importance of the targeted genes in relation to the role they play in sterigmatocystin and aflatoxin biosynthesis. It has been reported that *stcJ* together with another gene (*stcK)* encode α and β sub-units respectively of a fatty acid synthase which are required for the formation of norsolorinic acid, NOR, from a hexanoyl starter unit of the aflatoxin and sterigmatocystin biosynthesis pathway (Yu, 2012). Disrupting the *stcJ* gene in this study led to synthesis of less or no sterigmatocystin and subsequently resulted in low aflatoxin production in colonizing fungi. Similar results have been reported by Brown *et al.* (1996) who showed that mutants (engineered *in vitro*) that had *stcJ* or *stcK* disrupted produced no sterigmatocystin and subsequently could not synthesize aflatoxins.

On the other hand, *stcA* encodes a polyketide synthase and is responsible for the subsequent extension steps of the hexanoate starter, NOR, to form noranthrone that is required for the subsequent steps of aflatoxin biosynthesis. Mutants with disrupted *stcA* have been shown to be unable to produce noranthrone and eventually failed to synthesize aflatoxins (Chang *et al*., 1995). In the current study, it is postulated that downregulation of *stcA* resulted in disruption of mutants ability to extend the synthesis of NOR and hence formation of low levels of aflatoxins. The level of inhibition, though varied, indicates that each of the genes targeted have different levels of contribution to the aflatoxin biosynthesis pathway. Furthermore, the low levels of aflatoxins recorded during screening assays in this study are attributed to the controlled environment (temperature and relative humidity) under which infection was done. Higher levels of the toxin (>1000ppb) have previously been reported in maize although the conditions under which infection occurred were not provided (Lewis *et al.,* 2005). A free online and publicly accessible tool for designing effective siRNAs and prediction of off target sequences was used in this study. This is vital during design and cloning of silencing constructs to ensure that the generated dsRNAs and their corresponding siRNAs do not exert off target effects that negatively impact on the host plant (Koch & Kogel 2014). In

this study, no potential off target sequences were identified in maize indicating the suitability of the designed constructs and the generated siRNAs in silencing of only target sequences in *A. flavus.* This is further supported by the lack of significant differences in plant height and dry biomass between transgenic maize and their non-transformed counterparts. This further confirms the thought that the targeting of *aflR* could have been mis-targeted and disrupted an important process in maize.

5.5 Conclusion

Downregulation of aflatoxin biosynthesis in *A. flavus* and its subsequent accumulation in transgenic maize was achieved through targeting of the rate limiting steps controlled by *stcA* and *stcJ* indicating that the genes are key in aflatoxin biosynthesis. The variation in toxin reduction between *stcA* and *stcJ* cassettes while indicating that each of these genes has a significant contribution to the aflatoxin and sterigmatocystin biosynthesis pathway, also showed that *stcJ* has a bigger role than *stcA.* Disrupting these genes through *in planta* expression resulted in reduced aflatoxin production indicating existence of a maize-fungal conduit for siRNA trafficking and an existence of siRNA processing/amplification machinery in both maize and fungi. Finally the genes reported in this chapter are better options than transcription factor *aflR* (chapter four above) as no negative effects were observed on the maize plant.

5.6 Recommendation

Further screening needs to be carried out on the transgenic maize using available molecular techniques to ascertain stability of the siRNAs in stored kernels. Since stability of the generated siRNAs is unknown in stored grains, assessment is required to verify whether the silencing molecules will remain in the maize for long given that fungal infection could still occur during storage. It is also necessary to test transgenic maize in the field to assess the effect of natural infection. Assays carried out in this study were under controlled conditions with infection using *A flavus* done manually. On field trials could offer a better alternative for assaying transgenics through involvement of

natural infection by both aflatoxigenic and non aflatoxigenic fungi. The possibility of stacking the genes through crossing the lines could also prove more beneficial than single genes.

CHAPTER SIX

GENERAL DISCUSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Based on the current knowledge on management of aflatoxins in foodstuffs, no single method exists that is totally effective against aflatoxins (Hell and Mutegi 2011; Kimatu *et al.,* 2012; Walker 2013). Transgenic approaches, which have several advantages over other aflatoxin control methods including conventional breeding and biological control are also faced with regulator drawbacks as well as consumer skeptism. The transgenic approach mainly focuses on expression of foreign transgenes such as insecticidal proteins (due to a correlation between insect damage and aflatoxin contamination), antifungal peptides and proteins in host plants (Munkvold 2003; Barros *et al*., 2009). The current knowledge is backed by *in vitro* studies that have shown downregulation of aflatoxin biosynthetic pathway genes with subsequent toxin suppression and the postulation of host induced gene silencing technology as having potential to eliminate aflatoxins in foodstuffs (Alakonya and Monda 2013).

The results presented by this study therefore focused on generating proof of concept that HIGS of key aflatoxin and sterigmatocystin biosynthesis pathway genes could be effective against aflatoxin accumulation in maize and groundnut kernels. Successful optimization of a regeneration protocol for ICGV-CG2 and CG2 groundnut genotypes, as reported in this study, was imperative for ease of transfer of the silencing constructs in these germplasm. This is in line with studies indicating that an efficient and reproducible protocol for generation of transgenics is a pre-requisite to any genetic engineering program (Anuradha *et al.,* 2006; Iqbal *et al*., 2011). Generation of transgenic maize expressing the silencing molecules was also achieved in this study. Since the success of a maize genetic improvement program requires not only the ability

to deliver transgenes into the cell, but also to produce several transgenic events that stably inherit and express the transgene in the subsequent generations (Ishida *et al*., 1996), expression of the construct up to T_2 generation confirmed stability of the transformants.

On the other hand, the method for maize infection used in this study is the most commonly used assay technique and has previously been successful in field and laboratory studies in Africa (Cardwell *et al*., 2000). Furthermore, successful fungal colonization of infected ears was not a failure of strategy since the genes targeted (*aflR*, *stcA* and *stcJ)* are key in aflatoxin biosynthetic pathway and not responsible for fungal growth (Yu, 2012). There was a marked reduction in aflatoxin accumulation below the maximum levels permitted in food stuffs in Kenya (KEBS 2007). The *stcJ* construc*t* produced the highest level of reduction followed by *aflR* and *stcA*, an important indication of the roles played by these genes in the biosynthetic pathway (Yu 2012). Targeting of *aflR* however produced detrimental effects on plant growth characteristics and yield. This is postulated to be due to a cross reaction between *aflR* siRNAs and other plant growth factors (Jackson *et al*., 2003; Xu *et al.,* 2006). Transcription factors have many binding sites with *aflR* being shown to bind to deviated sequences rather than its typical motifs (Yu, 2012).

Overall, these results indicate that transgenic maize synthesized primary siRNAs that were complimentary to the cloned sequences in *A. flavus* from the double stranded RNA constructs transformed in maize. Upon colonization, the primary siRNAs then trafficked into infecting fungi, possibly through a plant-fungal cellular interface (Micali *et al*., 2011; Voegele and Mendgen, 2003). On reaching the fungi, the siRNA signals were amplified through Dicer-dependent RNA silencing mechanism and chopped the complementary mRNA transcripts in a homology-dependent manner (Hamilton and Baulcombe 1999) resulting in gene expression downregulation.

6.2 Conclusions

From the findings of this study the following conclusions can be inferred:-

1. Groundnut genotypes ICGV-CG2 and CG2 are regenerable using cotyledons and optimum combination levels of BAP and 2,4-D.

2. The targeting of the transcription factor *aflR* and rate limiting enzymes *stcA* and *stcJ* was successful in downregulation of aflatoxin biosynthesis and reduced accumulation of the toxin in maize to levels permitted in food by KEBS and way below the 20ppb set by the American Food and Drug administration.

3. The varying reduction in toxin accumulation in transgenic maize by targeting *stcA* and *stcJ* cassettes showed that *stcJ* has a bigger role than *stcA* in the targeted step of the aflatoxin biosytnthetic pathway.

4. Targeted downregulation of *stcJ* and *stcA* is a better option than transcription factor *aflR* (which resulted in other non-intended effects) as no negative effects were observed on the host plant.

5. *In planta* expression and subsequent downregulation of gene expression in colonizing fungi indicated existence of a maize-fungal interphase for siRNA trafficking as well as siRNA processing/amplification machinery in both maize and fungi.

6.3 Recommendations

1. The optimized regeneration protocol sets up a platform for genetic manipulation of the two groundnut genotypes against different biotic and abiotic challenges and may therefore be adopted during improvement programs.
2. The detrimental/off-target effects on plants transformed with the RNAi construct against the transcription factor *aflR'*s indicate that targeting of transcription factors in HIGS experiments should be avoided due to their influence on many targets in the plant genomes.
3. Further molecular screening needs to be carried out on the transgenic maize to ascertain stability of the siRNAs in stored kernels which is an important stage in falatoxin accummulation.
4. It may be important to test the developed transgenic maize in the field to assess the effect of natural infection. However, the current legislations governing genetically modified organisms could be a bottleneck to achieving this in the near future.
5. It will also be important to test the efficacy of a stacked construct simultaneously targeting the rate limiting steps *stcA, stcJ* and *stcK* so as to benefit from the complementarity of the step.

REFERENCES

Abdel-Hadi A. M., Caley D. P., Carter D. R. F and Magan N. (2011). Control of aflatoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* using RNA silencing technology by targeting *aflD* (nor-1) Gene. *Toxins*, 3:647-659.

Abebe D. Z., Teffera W and Machuka J. S. (2008). Regeneration of tropical maize lines (*Zea mays*) from mature zygotic embryo through callus initiation. *African Journal of Biotechnology*, 7 (13), 2181-2186.

Afla-guard (2005). Aflatoxin in Africa. www.circleoneglobal.com/aflatoxin_africa.htm.

Alakonya A., Kumar R., Koenig D., Kimura S., Townsley B., Runo S., Garces H. M., Kang J., Yanez A., David-Schwartz R., Machuka J and Sinha N. (2012). Interspecific RNA interference of SHOOT MERISTEMLESS-Like disrupts *Cuscuta pentagona* plant parasitism. *Plant Cell*, 24:3153–3166.

Alakonya A. E and Monda E. O. (2013). A New Approach in Aflatoxin Management in Africa: Targeting Aflatoxin/Sterigmatocystin Biosynthesis in *Aspergillus* Species by RNA Silencing Technique http://dx.doi.org/10.5772/51440.

Anami E., Mgutu J., Rasha A., Nelissen H., Inzé D., Lijsebettens V and Machuka J. (2008). Progress in transformation and regeneration of tropical inbred maize lines in Kenya. *Maize Gen. Coop. Newsletter* 82: 23.

Anuradha T. S., Jami S. K., Datla R. S and Kirti P. B. (2006). Genetic transformation of peanut (*Arachis hypogaea* L.) using cotyledonary node as explant and a promoterless gus::nptII fusion gene based vector. *J. of biosci.* 31: 235-246.

Atanda O. O., Akpan I and Enikuomehin O. A. (2006). Palm kernel, an alternative culture medium for rapid detection of aflatoxins in agricultural commodities. *Afri. J. of Biotech.* 5: 1029–1033.

Atanda O. O., Ogunrinu M. C and Olurunfemi F. M. (2011). A neutral red desiccated coconut agar for rapid detection of aflatoxigenic fungi and visual determination of aflatoxins. *World myco. J.* 4(2), 147–155.

Ayejuyo O. O., Olowu R. A., Agbaje T. O., Atamenwan M and Osundiya M. O. (2011). Enzyme - Linked Immunosorbent Assay (ELISA) of Aflatoxin B1 inGroundnut and Cereal Grains in Lagos, Nigeria. *Res. J. Chem.Sci*. 1(8), 1-5.

Bandyopadhyay R and Cotty P. (2011). Presentation at Annual Meeting of APS/IAPPS. 6-10 August 2011, Honolulu, Hawaii.

Bankole S. A., Schollenberger M and Drochner W. (2006). Mycotoxin contamination in food systems insub-Saharan Africa: A review. *Myco. Resea.* 22:163–169.

Barros G, Magnoli C, Reynoso M. M, Ramirez M. L, Farnochi M. C, Torres A, Dalcero M, Sequeira J, Rubinstein C and Chulze S. (2009). Fungal and mycotoxin contamination in *Bt* maize and non- Bt maize grown in Argentina. *World MycotoxinJ*. 2: 53-60.

Baulcombe D. (2004). RNA silencing in plants. *Nature,* 431:356–363.

Bennett J. W and Klich M. (2003). Mycotoxins. *Clin. Microbiol. Rev.* 16:497–516.

Bhatnagar D., Cotty P. J and Cleveland T. E. (2000). Genetic and biological control of aflatoxigenic fungi: *Micro. food conta*. 208–240.

Birthal P. S., Rao P. P., Nigam S. N., Bantilan C. S and Bhagavatulu S. (2010). Groundnut and Soybean Economies in Asia: Facts, Trends and Outlook. Patancheru: *Intl. Crops Resea. Inst. for the Semi-Arid Tropics.*

Brodersen P and Voinnet O. (2006). The diversity of RNA-silencing pathways in plants. *Trends Genet.* 22:268–280.

Brown D. W., Yu J. H., Kelkar H. S., Fernandes M., Nesbitt T. C., Keller N. P., Adams T. H and Leonard T. J. (1996). Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci*. 93:1418-1422.

Cardwell K. F., Kling J. G., Maziya-Dixon B and Bosque-Perez N. A. (2000). Interaction between *Fusarium verticilliodes*, *Aspergillus flavus*, and insect infestation in four maize genotypes in lowland Africa. *Phytopathology*. 90(3), 276–284.

Cary J. W., Ehrlich K. C., Wright M., Chang P. K and Bhatnagar D. (2000)a. Generation of *aflR* disruption mutants of *Aspergillus parasiticus*. *Appl. Microbiol. Biotechnol.* 53. 680–684.

Cary J. W., Linz J. E and Bhatnagar D. (2000)b. Aflatoxins: biological significance and regulation of biosynthesis. *Technomic.* 317–361b.

Cary J. W., Rajasekaran K, Brown R. L., Luo M, Chen Z and Bhatnagar D. (2011)a. Developing Resistance to Aflatoxin in Maize and Cottonseed *Toxins 3*, 678-696

Castel S. E and Martienssen R. A. (2013). RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nat. Rev. Genet.* 14:100–112.

CDC (Center for Disease Control). (2004). *Outbreak of aflatoxin poisoning- Eastern and Central provinces, Kenya, January-July.Morbility and Mortality Weekly Report*. 53. 790-792.

Chandra A and Pental D. (2003). Regeneration and genetic transformation of grain legumes : An overview. *Current Science,* 84(3), 381–387.

Chang P. K., Cary J. W., Yu J., Bhatnagar D and Cleveland T. E. (1995). *Aspergillus parasiticus* polyketide synthase gene, *pksA*, is required for aflatoxin Bbiosynthesis. *Mol.Gen. Genet.* 248:270-277.

Chulze S. N. (2010). Strategies to reduce mycotoxin levels in maize during storage: areview. *Food Add. and Conta.* 27:651-657.

Chun H. S., Kim H. J., Hwang J and Chung D. (2007). Determination of aflatoxin levels in nuts and their products consumed in South Korea. *Food Chemistry.* 102:385-391.

Cleveland T. E., Dowd P. F., Desjardins A. E., Bhatnagar D and Cotty P. J. (2003). United States department of agriculture-agricultural research service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops. *Pest Manag. Sci. 59*: 629–642.

Clough S. J and Bent A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated Transformation of *Arabidopsis thaliana*. *The Pla. Jour.* 16:735–743.

Cotty P. J. (1994). Comparison of four media for the isolation of *Aspergillus flavus* group fungi. *Mycopathologia* 125:157–162.

Craufurd P. Q., VaraPrasad P. V and Summerfield R. J. (2002). Dry matter production and rate of change of harvest index at high temperature in peanut. *Crop Sci.* 42:146–151.

Craufurd P. Q., Prasad P. V., Kakani V. G, Wheeler T. R and Nigam S. N. (2003). Heat tolerance in groundnut. *Fiel. Crps Res.* 80:63–77.

Davuluri G. R., van Tuinen A., Fraser P. D., Manfredonia A., Newman R., Burgess D., Brummell D. A., King S. R., Palys J., Uhlig J., Bramley P. M., Pennings H. M and Bowler C. (2005). Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol*. 23:890–895.

Dehghan P., Zaini F., Rezaei S., Jebali A., Kordbacheh P and Mahmoudi M. (2008). Detection of *Aflr* Gene and Toxigenicity of *Aspergillus flavus* Group Isolated from Patients with Fungal Sinusitis. *Iranian. J. Publ Health*. 37(3), 134-141.

Dorner J. W. (2002). Simultaneous quantitation of *Aspergillus flavus /A. parasiticus* and aflatoxin in peanuts. *Journal of AOAC Internl*. 85:911–6.

Dorner J. W, Cole R. J and Wicklow D. T. (1999). Aflatoxin reduction in corn through field application of competitive fungi. *J. of Food Protect*. 62: 650-656.

Duan C. G., Chun-Han W and Hui-Shan G. (2012). Application of RNA silencing to plant disease resistance. *Sience.* 3:5.

Dunoyer P., Himber C and Voinnet O. (2006). Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. *Nat. Genet.* 38:258–263.

FAOSTAT (2010). Available at: http:// faostat. fao.org.

FAOSTAT (2011). Available at: http:// faostat. fao.org.

Fellinger A. (2006). Worldwide mycotoxin regulations and analytical challenges.World Grain Summit: Foods and Beverages, September 17–20, 2006, San Francisco, California, USA.

Flaherty J. E and Payne G. A. (1997). Over-expression of *aflR* Leads to Up-regulation of Pathway Gene Transcription and Increased Aflatoxin Production in *Aspergillus flavus. App. and Environ. Microbio.* 63(10), 3995–4000.

Geetha N, Venkatachalam P and LakshmiSita G. (1999). *Agrobacterium*-mediated genetic transformation of Pigeonpea (*Cajanus cajan* L) and development of transgenic plants via Direct Organogenesis; *Plant Biotechnol.* 16:213–218.

Gelvin S. (2003). Improving plant genetic engineering by manipulating the host. *Trends Biotechnol*. 21:95-98.

Ghildiyal M and Zamore P. D. (2009). Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* 10:94–108.

Gilbert J and Vargas EA (2003). Advances in sampling and analysis for aflatoxins. *Jour.of Toxi. - Toxins Reviews*. 22(2 and 3), 385-426.

Gong YY., Hounsal A., Egal S., Turner P. C., Sutcliffe A. E., Hall A. J., Cardwell K. F and Wild C. P. (2004). Post-weaning exposure to aflatoxin results in impaired child growth. A longitudinal study in Benin, West Africa. *Env. Heal. Pers.*112:1334-1338.

Gunterus A., Roze L. L., Beaudry R and Linz J. E. (2007). Ethylene inhibits aflatoxin biosynthesis in *Aspergillus parasiticus* grown on peanuts. *Food Microbio.* 24: 658-663.

Haidukowski M., Pascale M., Perrone G., Pancaldi D., Campagna C and Visconti A. (2004). Effect of fungicides on the development of Fusarium head blight, yield and deoxynivalenol accumulation in wheat inoculated under field conditions with *Fusarium graminearum* and *Fusarium culmorum*. *Jour. Sci. of Food and Agric.* 85:191–198.

Hamilton A. J and Baulcombe D. C. (1999). A species of small antisense RNA in post transcriptional gene silencing in plants. *Science*. 286:950–952.

Hell K and Mutegi C. (2011). Aflatoxin control and prevention strategies in key crops in sub-Sahara Africa. *Afric. J. ofMicr. Res*.5: 459-466.

Hell K., Fandohan P., Bandyopadhyay R., Cardwell K., Kiewnick S., Sikora R and Cotty P. (2008). Pre- and post-harvest management of aflatoxin in maize. *In Leslie, J.F.*

Helliwell C and Waterhouse P. (2003). Constructs and methods for high-throughput gene silencing in plants *Methods*. 30(4), 289–295.

Horn B. W, Olarte R. A, Peterson S. W and Carbone I. (2013). Sexual reproduction in *Aspergillus tubingensis* from section *Nigri*. Mycol. 105: 1153-1163.

Huvenne H and Smagghe G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J. Insect Physiol*, 56:227–235.

IITA (International Institute for Tropical Agriculture). (2003). Blocking growth-stunting toxins in food. *Science in Africa*. www.scienceinafrica.co.za.

Iqbal M. M., Nazir F., Iqbal J., Tehrim S and Zafar Y. (2011). *In vitro* micropropagation of peanut (*Arachis hypogaea*) through direct somatic embryogenesis and callus culture. *Int J. Agric. Biol.* 13:811–814.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T and Kumashiro T. (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat Biotechnol* 14:745–750.

Ishida Y., Hiei Y and Komari T. (2007). *Agrobacterium*-mediated transformation of maize. *Nat. protocols* 2:1614–1621.

Jackson A. L and Linsley P. S. (2004). Noise amidst the silence: off-target effects of siRNAs? *Trends Genet.* 20(11), 521-524.

Jackson A. L., Bartz S. R., Schelter J., Kobayashi S. V., Burchard J., Mao M, Li B., Cavet G and Linsley P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol*, 21:635−637.

Janila P., Nigam S. N., Manish K. P., Nagesh P and Varshney K. (2013). Groundnut improvement, use of genetic and genomic tools. *Frontiers in pla. Sci*.4: 23.

Jeurissen S. M., Seyhan F., Kandhai M. C., Dekkers S., Booij C. J., Bos P. M and vander-Klerx-Fels H. J. (2011). An indicator based 'traffic light' model to pro-actively assess the occurrence of mycotoxin in tree nuts.

Kakani A., Li G and Peng Z. (2009). Role of AUX1 in the control of organ identity during *in vitro* organogenesis and in mediating tissue specific auxin and cytokinin interaction in *Arabi*. *Planta.* 229: 645-657.

Karimi M, Inzé D and Depicker A. (2002). Gateway vectors for *Agrobacterium*-mediated plant transformation. *Tre. in Pla. Sci*. 7(5), 1360-1385.

KEBS. (2007). Kenya Standard KS 694-1: 2007. Shelled groundnut (*Arachis hypogaea* Linn.) Specification. Part 1: Raw Groundnut for Table Use. Kenya Bureau of Standards Documentation Centre, Nairobi, Kenya.

Keller N. P., Watanabe M. C., Kelkar H. S., Adams T. H and Townsend C. A. (2000). Requirement of monooxygenase-mediated steps for sterigmatocystin biosynthesis by *Aspergillus nidulans*. *Appl. Environ. Microbiol.* 66:359–366.

Ketting R. F. (2011). The many faces of RNAi. *Dev. Cell*. 20:148–161.

Khayoon W. S., Saad B., Salleh B., Ismail N. A., Manaf N. H and Latiff A. A. (2010). A reversed phase high performance liquid chromatography method for the determination of fumonisins B1 and B2 in food and feed using monolithic column and positive confirmation by liquid chromatography/tandem mass spectrometry. *Analytica Chimica Acta*. 679(1–2), 91–97.

Kim H. J., Ono E., Morimoto K., Yamagaki T., Okazawa A., Kobayashi A and Satake H. (2009). Metabolic engineering of lignan biosynthesis in Forsythia cell culture. *Pla. Cell Physiol*. 50:2200–2229.

Kim S. W., Kim T. J and Liu J. R. (2003). High frequency somatic embryogenesis and plant regeneration in petiole and leaf explants cultures and petiole-derived embryogenic cell suspension cultures of *Hylomecon vernalis*. *Pla. Cell. Tiss. Org. Cult.* 74:163–167.

Kimatu J. N, McConchie R, Xie X and Nguluu S. N. (2012). The significant role of post-harvest management in farm management, aflatoxin mitigation and food security in sub-Sahara *Africa. Gren. J. of Agri. Sc.*2:279-288.

King S. B and Scott G. E. (1982). Field inoculation techniques to evaluate maize for reaction to kernel infection by *Aspergillus flavus*. *Phytopathology* 72(7), 782–785.

Klich M. A and Pitt J. (1988). Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Trans. Be. Mycol. Soc*. 91: 99-108.

Koch A and Kogel K. H. (2014). New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. *Plant Biotech J.* 12:821–831.

Koch A., Kumar N., Weber L., Keller H., Imani J and Kogel K. H. (2013). Host-induced gene silencing of cytochrome P450 lanosterol C14ademethylase-encoding genes confers strong resistance to *Fusarium* species. *Proc. Natl Acad. Sci. USA*, 110:19324–19329.

Lanyasunya T. P., Wamae L. W., Musa H. H., Olowofeso O and Lokwaleput I. K. (2005). The risk of mycotoxins contamination of dairy feed and milk on smallholder dairy farms in Kenya. *Pakistan Jou. of Nutri.* 4:162–169.

Lerda D. (2010). Mycotoxin fact sheet. 3rd edition. JRC technical notes. IRRM, Geel

Lewis L., Onsongo M., Njapau H., Schurz-Rogers H., Luber G., Kieszak S., Nyamongo J., Backer L., Dahiye AM and Misore A. (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environ. HealthPerspect.* 113:1763–1767.

Lilley C. J., Davies L. J and Urwin P. E. (2012). RNA interference in plant parasitic nematodes: a summary of the current status. *Parasitology*. 139:630–640.

Lippman Z and Martienssen R. (2004). The role of RNA interference in heterochromatic silencing. *Nature* 431:364–370.

Machuka J. (2005). Plant transformation: Goals, strategies and relevance for African crop improvement and production of high value biologics. *Afri. Cro. Sci. Co. Proc.* 7:1297-1303.

Mahanti N., Bhatnagar D., Cary J. W., Joubran J and Linz J. E. (1996) Structure and function of *fas-1A*, a gene encoding a putative fatty acid synthetase directly involved in aflatoxin biosynthesis in *Aspergillus parasiticus*. *Appl. Environ. Microbiol*. 62:191-195.

Maina S. M., Emongor Q., Sharma K. K., Gichuki S. T., Gathaara M and De Villiers S. M. (2010). Surface sterilant effect on the regeneration efficiency of cotyledon explants of groundnut (*Arachis hypogeal* L.) varieties adapted to eastern and Southern Africa. *Afri. J. of Biotech* .9:2866–2871.

Mao Y. B., Cai W., Wang J., Hong G., Tao X and Wang L. (2007). Silencing a cotton bollworm *P450* monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat. Biotechnol* 25:1307-13.

Maragos C. M. (2004). Emerging technologies for mycotoxin detection. *J. Toxicol. Toxin Rev*. 23: 317–344.

McDonald T., Brown D., Keller N. P and Hammond T. M. (2005). RNA silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. *Mol. Plant-Microbe Interact,* 18:539−545.

MERCK (Medical Research Council) (2006). Aflatoxin in peanut butter. Science in Africa.

Micali C. O., Neumann U., Grunewald D., Panstruga R and O'Connell R. (2011). Biogenesis of a specialized plant-fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. *Cell. Microbiol*. 13:210–226.

Misra J. B., Ghosh P. K., Dayal D and Mathur R. S. (2000). Agronomic, nutritional and physical characteristics of some Indian groundnut cultivars. *Indian J. Agric. Sci.* 70:741–746.

Murashige T and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.*15:473–497.

Mutegi C. K., Ngugi H. K., Hendriks S. L and Jones R. B. (2009). Prevalence and factors associated withaflatoxin contamination of peanuts from Kenya. *Intern. J. of Food Microbio.*130:27–34.

Mutegi C. K., Ngugi H. K., Hendriks S. L and Jones R. B. (2012). Factors associated with the incidenceof *Aspergillus* section *Flavi* and aflatoxin contamination of peanuts in the Busia and Homa bay districts of western Kenya. *Plant. Pathol.* 61: 1143–1153.

Ndung'u J. W., Makokha A. O., Onyango C. A., Mutegi C. K., Wagacha J. M., Christie M. E and Wanjoya A. K. (2013). Prevalence and potential for aflatoxin contaminationin groundnuts and peanut butter from farmers and traders in Nairobi and Nyanza provinces of Kenya. *Journal of Appl. Biosci.* 65:4922 – 4934.

Nigam S. N., Manish K. P., Monyo E., Ozias-Akins P., Liang X., Guimarães P., Upadhyaya H. D., Janila P., Zhang X., Guo B., Cook D. R., Bertioli D. J., Michelmore R and Varshney R. (2012). Advances in *Arachis* genomics for peanut improvement: *Biotechnol. Adva*. 30:639–651.

Niu Q. W., Lin S. S., Reyes J. L., Chen K. C., Wu H. W and Yeh S. D. (2006). Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat Biotech.* 24:1420-8.

Nowara D., Gay A., Lacomme C., Shaw J., Ridout C., Douchkov D., Hensel G., Kumlehn J and Schweizer P. (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cel.* 22:3130–3141.

Nunes C. C and Dean R. A. (2012). Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies. *Mol. Plant Pathol*, 13:519–529.

Nuryono N., Agus A., Wedhastri S., Maryudani Y. B., Sigit S. F. M., Bohm J and Razzazi-Fazeli E. (2009). A limited survey of aflatoxin M1 in milk from Indonesia by ELISA. *Food Contro.* 20:721-724.

Odour R. O, Njagi E. N. M, Ndung'u S and Machuka J. S. (2006). *In vitro* regeneration of dryland Kenyan maize Genotypes through somatic embryogenesis. *Int. J. Bot.* 2(2), 146-151.

Olhoft P. M., Flagel L. E., Donovan C. M and Somers D. A. (2003). Efficient soybean transformation using hygromycin B selectionin the cotyledonary-node method. *Planta* 216:723–735.

Ombori O., Gitonga N and Machuka J. (2008). Somatic embryogenesis and plant regeneration form immature embryos of tropical maize (*Zea mays* L.) inbred lines. *Biotechnol.* 7(2), 224-232.

Ombori O., Muoma V. O. J and Machuka J. (2013). *Agrobacterium*-mediated genetic transformation of selected tropical inbred and hybrid maize (*Zea mays* L.) lines. *Plant Cell Tiss Organ Cult* 113:11–23.

Omer A. R., Matheka J. M., Ali A. M and Machuka J. (2013). Transformation of Tropical Maize with the *NPK1* Gene for Drought Tolerance. *International J. of Gen. Eng.* 3(2), 7-14.

Omer R., Ali A., Matheka J and Machuka J. (2008). Regeneration of Sudanese maize inbred lines and open pollinated varieties. *African J. of Biotech.* 7(11), 1759-1764.

Panstruga R. (2003). Establishing compatibility between plants and obligate biotrophic pathogens. *Curr. Opin. Plant Biol*. 6:320–326.

Payne G. A., Nystrom G. J., Bhatnagar D., Cleveland T. E and Woloshuk C. P. (1993). Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. *Appl. Environ. Microbiol*, 59:156–162.

Pitt J. I. (1992). Collaborative study on media for detection and differentiation of *Aspergillus flavus* and *A. parasiticus* and the detection of aflatoxin production. *Mod. Meth. in food Myco.. Elsevier.* 303-308.

Pitt J. I and Hocking A. D. (1997). Fungi and Food Spoilage, 2nd ed. Blackie Academic and Professional, London, UK.

Price D. R and Gatehouse J. A. (2008). RNAi-mediated crop protection against insects. *Trends Biotechnol*. 26:393–400.

Rachaputi N. R., Wright G. C and Kroschi S. (2002). Management practices to minimize pre-harvest aflatoxin contamination in Australian groundnuts. *Aust. Jour. of Exp. Agri.* 42: 595–605.

Robertson A. (2005). Risk of aflatoxin contamination increases with hot and dry growing conditions: *Intergrated Cro. Mangnt*. 185-186.

Rohini V. K and Rao K. S. (2001). Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable responses of transformants to leaf spot disease. *Plant Sci.* 16: 889–898.

Rossin C. B and Rey M. E. C. (2011). Effect of explant source and auxins on somatic embryogenesis of selected cassava (*Manihot esculenta* Crantz) cultivars. *South Afri J of Bot*. 77:59–65.

Runo S. (2011). Engineering host-derived resistance against plant parasites through RNA interference: challenges and opportunities. *Bioeng. Bugs*. 2:208–213.

Saxena S and Jonsson Z. O. (2003). Small RNAs with Imperfect Match to Endogenous mRNA Repress Translation: Implications for off-target activity of small inhibitory RNA in mammalian cells. *J. Biol. Chem*, 278 (45), 44312-4419.

Sharma A., Jani D., Raghunath C and Tyagi A. (2004). Transgenic plants as bioreactors. *Indian J. of Biotech.* 3:274-290.

Sharma K. K and Anjaiah V. (2000). An efficient method for the production of transgenic plants of peanut through *Agrobacterium tumefaciens*-mediated genetic transformation. *Plant Sci.* 159:7–19.

Shier W. T., Weaver M. A., Abbas H. K and Horn B. W. (2005). The Case for Monitoring *Aspergillus flavus* aflatoxigenicity for food safety assessment in developing countries. *Aflatoxin and Food Safety*. 291-312.

Singh A. K., Dwivedi S. L., Pande S., Moss J. P., Nigam S. N and Sastry D. C. (2003). Registration of rust and late leaf spot resistance peanut germplasm lines. *Crop Sci.* 43: 440–441.

Snove O and Holen T. (2004). Many commonly used siRNAs risk off-target activity. *Biochem. Biophys. Res. Commun,* 391(1), 256-263.

Sundar I. K and Sakthivel N. (2008). Advances in selectable marker genes for plant transformation. *Jour. of Pla. Physio.* 165, 1698—1716.

Tillman B. L and Stalker H. T. (2009). Peanut. Handbook of plant breeding. *Oil crops.* 4, 287–316.

Tinoco M. L., Dias B. B., Dall'Astta R. C., Pamphile J. A and Arag~ao F. J. (2010). *In vivo* trans-specific gene silencing in fungal cells by *in planta* expression of a double-stranded RNA. *BMC Biol*. 31, 27.

Tomilov A. A., Tomilova N. B and Yoder J. I. (2006). *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* transformed roots of the parasiticplant *Triphysaria versicolor* retain parasitic competence. *Planta.* 225, 1059–1071.

Trail F., Mahanti N., Rarick M., Mehigh R., Liang S. H., Zhou R and Linz J. E. (1995). Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. *Appl. Environ. Microbiol.* 61, 2665−2673.

Tu Z., He G., Li K., Chen M., Chang J., Cheng L., Yao Q., Liu D., Ye H., Shi J and Wu X. (2005). An improved system for competent cell preparation and high efficiency

plasmid transformation using different *Escherichia coli* strains. *Elec. Jour. of Biotech.* 8(1), 113-120.

Turner P., Sylla A., Gong Y., Diallo M., Sutcliffe A., Hall A and Wild C. (2005). Reduction of exposure to carcinogenic aflatoxins by postharvest intervention measures in West Africa; a community based intervention study. 365, 1950-1959.

Tzfira T and Citovsky V. (2006). *Agrobacterium*-mediated genetic transformation of plants, biology and biotechnology. *Curr. Opi. in Biotech.* 17, 147–154.

Ui-Tei K., Naito Y., Takahashi F., Haraguchi T., Ohki-Hamazaki H., Juni A., Ueda R and Saigo K. (2004). Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res.* 32, 936–948.

Upadhyaya H. D. (2005). Variability for drought resistance related traits in the mini core collection of peanut. *Crop Sci.* 45, 1432–1440.

Vaamonde G., Patriarca A., Pinto V. F., Comerio R and Degrossi C. (2003). Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. *Inter. J of Food Microbio.* 88, 79–84.

Van Egmond H. P., Schothorst R. C and Jonker M. A. (2007). Regulations relating to mycotoxins in food, perspectives in a global and European context. *Analytical and Bioanal. Che.* 389, 147–57.

Vaucheret H and Fagard M. (2001). Transcriptional gene silencing in plants, targets, inducers and regulators. *Trends Genet*. 17, 29–35.

Vaucheret H., Beclin C and Fagard M. (2001). Post-transcriptional gene silencing in plants. *J. Cell Sci.* 114, 3083–3091.

Vaucheret H., Vazquez F., Crete P and Bartel D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev*. 15, 1187–1197.

Venkatachalam P., Geetha N., Khandelwal A., Shaila M. S and LakshmiSita G. (2000). *Agrobacterium*-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis. *Curr. Sci*. 78, 1130–1136.

Voegele R. T and Mendgen K. (2003). Rust haustoria, nutrient uptake and beyond. *New Phytol*. 159, 93–100.

Wagacha J. M and Muthomi J. W. (2008). Mycotoxin problem in Africa, Current status, implications to food safety and health and possible management strategies, *Intern. J. of Food Microbio.* 124, 1-12.

Waliyar F., Reddy S. V and Kumar P. L. (2009). Review of Immunological methods for the quantification of aflatoxins in peanut and other foods, *Peanut Sci.* 36, 54–59.

Walker S. (2013). Aflatoxins, Finding solutions for improved food safety. International Food Policy Research Institute; *Focus 20*, Brief 7, November.

Wesley S. V., Helliwell C. A., Smith N. A., Wang M. B., Rouse D. T., Liu Q., Gooding P. S., Singh S. P., Abbott D., Stoutjesdijk P. A., Robinson S. P., Gleave A. P., Green A. G and Waterhouse P. M. (2001). Construct design for efficient, effective and highthroughput gene silencing in plants. *Plant. J*. 27, 581–590.

Widstrom N. W., McMillian W. W., Beaver R. W and Wilson D. M. (1990). Weather-associated changes in aflatoxin contamination of preharvest maize. *J. Prod. Agric.* 3(2), 196–199.

Windham G. L and Williams W P. (2007). A comparison of inoculation techniques for inducing aflatoxin contamination and *Aspergillus flavus* kernel infection on corn hybrids in the field. *Phytoparasitica* 35(3), 244–252.

Windham G. L., Williams W. P., Buckley P. M., Abbas H. K and Hawkins L. K. (2002). A comparison of inoculation techniques for *Aspergillus flavus* on corn. *Proc. Aflatoxin Elimination Workshop*. 139.

WHO (World Health Organization). (2006). Mycotoxins in African foods, implications to food safety and health. *AFRO Food Safety Newsletter*.

Xu P., Zhang Y., Kang L., Roossinck, M. J and Mysore K. S. (2006). Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physio*. 142, 429–440.

Yi J., Chang P. K., Cary J. W., Wright M., Bhatnagar D., Cleveland T. E., Payne G. A and Linz J. E. (1995). Comparative mapping of Aflatoxin pathway gene clusters in

Aspergillus parasiticus and *Aspergillus flavus*. *Appl. and Environ. Microbiology*. 61, 2365-2371.

Yin C., Jurgenson JE and Hulbert S. H. (2011). Development of a host-induced RNAi system in the wheat stripe rust fungus *Puccinia striiformis*. *Mol. Plant Microbe Interact*. 24, 554–561.

Yu J., Chang P. K., Cary J. W., Wright M., Bhatnagar D., Cleveland T. E., Payne G. A and Linz J. E. (1995). Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus Appl. Environ. Microbiol*. 61, 2365–2371.

Yu J., Chang P. K., Ehrlich K. C., Cary J. W., Bhatnagar D., Cleveland T. E., Payne G. A., Linz J. E., Woloshuk C. P and Bennett J. W. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.70*, 1253–1262.

Yu J. (2012). Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. *Toxins*. 4, 1024-1057.

Zhang M., Wang Q., Xu K., Meng Y., Quan J and Shan W. (2011). Production of dsRNA sequences in the host plant is not sufficient to initiate gene silencing in the colonizing oomycete pathogen *Phytophthora parasitica*. *PLoS One*. 6, 1–10.

Zhang Z., Chen H., Huang X., Xia R., Zhao Q., Lai J., Teng K., Li Y., Liang L., Du Q., Zhou X., Guo H and Xie Q. (2011). BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in Arabidopsis. *Plant Cel.* 23, 273–288.

Zhong S., Yueqiang L and Melvin D. B. (2012). Construction of Hairpin RNA-Expressing Vectors for RNA-Mediated Gene Silencing in Fungi *Plant Fung. Path. Meth. in Mol. Biol*. 835, 623-633.

Zidani S., Ferchichi A and Chaieb M. (2005). Genomic DNA extraction method from pearl millet (*Pennisetum glaucum*) leaves. *African J. of Biotech.* 4(8), 862-866.

APPENDICES

Publications accruing from this research work

2015: Joel Okoyo Masanga, Jonathan Matheka, Rasha Omer, Sheila Ommeh, Ethel Monda and Amos Alakonya. Development of maize germplasm with tolerance to aflatoxin accumulation through Host-induced gene silencing technology. ***In Press: Molecular Plant Pathology.***

2015: Joel Okoyo Masanga, Jonathan Matheka, Rasha Omer, Sheila Ommeh, Ethel Monda and Amos Alakonya. Down-regulation of transcription factor *aflR* in *Aspergillus flavus* confers reduction to aflatoxin accumulation in transgenic maize with alteration of host plant architecture *Plant cell Reports, Springer* 15,1794-1799.

2013: Joel Okoyo Masanga, Sheila Ommeh, Remy Kasili and Amos Alakonya. An optimized protocol for high frequency regeneration of selected groundnut (*Arachis hypogaea* L.) varieties from East Africa using cotyledons. *International Journal of Agriculture and Crop Sciences.* 6(20), 1421-1425.