Histological and Molecular Developmental Mechanism of Striga Weed (*Striga hermonthica*) Parasitizing Maize (*Zea mays*)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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

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DEDICATION

This thesis is dedicated to my late son Osteen Mutugi and my parents William and Asenath.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDICES

ABBREVIATIONS AND ACRONYMS

bp	base pairs
cDNA	Complementary deoxyribonucleic acid
CYS	Cysteine protease gene
CIMMYT	International maize and wheat improvement centre
ddH2O	Double distilled water
DMBQ	2, 6-dimethoxy-p-benzoquinone
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
EXP	*Expansin* gene
FAA	Formalin acetic alcohol
FAO	Food and agriculture organization
IR	Imidazolinone resistance
Kb	kilo base
Kg	kilogram
KSTP'94	Kakamega *S. hermonthica* tolerant population 94
LB	Luria bertani
LGB	larger grain borer
mRNA	Messenger ribonucleic acid
MiRNA	Micro interfereing ribonucleic acid
M6PR	Mannose 6-phosphate reductase genes
NCBI	National centre for biotechnology information
Nt	Neucleotide
OD	Optical density
OPV	Open pollinated varieties
ORF	Open reading frame
PCR	Polymerase chain reaction

PDS	Phytoene desaturase gene
PME	Pectin methyl esterase
QOR	Quinone oxidoreductase genes
QRT-PCR	Quantitative real time polymerase chain reaction
RISC	RNA-induced silencing complex
RNAi	Ribonucleic acid interference
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNAs	Small interfering ribonucleic acids
SSA	Sub Saharan Africa
TVP	Tvpirin genes
VIGS	Viral induced gene silencing

ABSTRACT

Smallholder farmers in sub-Saharan Africa (SSA) have for decades battled with *Striga hermonthica,* a root parasitic angiosperm that constrains maize production leading to yield losses of up to 100%. The most sustainable strategies in control of *S. hermonthica* can only arise from a clear understaning of the parasite molecular mechanism during parasitism. Currently there is no clear understanding of mechanisms involved in haustoria development and the resistance mechanisms in some existing maize genotypes. Such an understanding could lead to several management approaches via RNA interference and gene overexpression techniques. This potential for developing resistance against *S. hermonthica* is also further constrained by the paucity of candidate genes to target and lack of efficient high throughput gene screening protocols. Viral induced gene silencing (VIGS) provides an easy and effective strategy in screening for putative candidate genes for targeting through knockdown techniques but has not been optimised in *S. hermonthica*. This studys' specific objectives were: (i) To Advance our understanding of the resistance mechanisms and biology of *S. hermonthica* parasitism on a susceptible (*Namba nane*) and tolerant (*KSTP'94*) open pollinated varieties of maize in Kenya, (ii) To identify some of the genes involved in *S. hermonthica* haustoriagenesis, (iii) To develop a VIGS protocol for functional genomics in the parasitic plant *S. hermonthica*. The two maize varieties were planted in *S. hermonthica* infested soil collected from Alupe followed by data collection on the number of germinated *S. hermonthica* plants and haustoria, in the different varieties. Further histological analysis were done by cross-sectioning formalin acetic alcohol (FAA) fixed *S. hermonthica* haustoria attached to maize root of the different varieties. Using a predictive literature search on other well studied parasitic plants like *Orobanchea* and *triphysaria*, gene specific primers were generated and used to clone followed by sanger sequencing of the fragments. For the VIGS protocol development, the tobacco rattle virus (TRV2 and TRV1) in agrobacterium GV3101 designed to silence *phytoene desaturase (PDS)* gene were introduced into S. *hermonthica* using agro-drench and agro-infiltration methods. The results show that the maize varieties response to *S. hermonthica* was different in the sense that there was delayed parasite emergence on the *KSTP'94* (90 days after planting) compared to *Namba nane* where the parasite started emerging at 42 days after planting. Additionally, there was an average of 5±0.5 Striga plants with only

10.7±0.7% infestation rate while *Namba nane* had an average of 26±0.7 *S. hermonthica* plants per variety with 50.3±0.9% infestation rate. The average number of haustoria in the *KSTP'94* was low (18±2.6) compared to 43±2.3 in *Namba nane* variety at 112 days after planting. Histologically, *KSTP'94* variety exhibited resistance to haustoria penetration at the host cortex and endodermis and only in few cases did the parasite haustoria make connections to the vasculature. In *Namba nane* variety, the haustoria penetrated easily through the cortex and endodermis and made connections to the vasculature. Five key haustoria formation genes were found to be expressed in *S. hermonthica* haustoria through RT-PCR; *Expansin*, *Cysteine protease*, *Mannose 6-phosphate reductase*, *Tvpirin* and *Quinone oxidoreductase*. The effectiveness of agrodrench and agroinfiltration guided VIGS was determined in Striga via photobleaching phenotypes on leaves at 14 and 7 days post infection, respectively. The photobleaching however cleared at 28 days after planting. The transformation efficiency for VIGS protocol was 60±2.9% in agro-infiltration and 10.3±1.5% in agrodrench. Summarily, the results provide baseline information on the possible responses to local maize germplasm to *S. hermonthica* and the candidate genes involved in haustoria formation. The study developed a VIGS protocol that could be used for genomic studies in *S. hermonthica*. Further the genes identified in *S. hermonthica* could be used in parasite management via RNAi constructs.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Maize (*Zea mays*) is the third most important cereal crop in the world after wheat and rice, contributing substantially to the total cereal grain production in the world economy as a trade, food, feed, and industrial grain crop (FAO, 2010; IITA, 2011). The cereal crop constitutes the staple diet of many people in sub-Saharan Africa (SSA) and in Africa at large (FAO, 2010; CIMMIT, 2011). There is a high annual consumption level of 79 kg per capita in Africa and 125 kg per capita in Kenya (De Groote *et al*., 2002). Biotic and abiotic constraints affect maize production translating to US$7 billion worth of cereal grain loss annually consequently leading to food insecurity in SSA region (AATF, 2006; Ejeta, 2007; M'mboyi *et al*., 2010). *Striga hermonthica* a root obligate hemiparasitic weed is one of the biotic factors, that limits maize production and results in up to between 40% and 100% annual yield loss (AATF, 2006; Rich and Ejeta, 2008). Yield losses associated with *Striga spp* infestation depend on the crop cultivar, weather conditions and the degree of infestation (Berner *et al*., 1995). Interestingly, by the time the parasite emerges from the soil, damage is already done to the host plant. Although the parasite is capable of photosynthesis, some experimental evidence in cereals has indicated that approximately 38% to 85% of the parasite carbon is obtained from the host (Graves *et al*., 1989; Graves *et al*., 1990).

Conventional approaches used to control *S. hermonthica* are generally the cultural practices used by the smallscale farmers. These include practices such as use of maize seeds that are dressed with certain herbicides, crop rotation, intercropping, fallowing, handweeding, management of soil fertility, use of herbicides, development of *S. hermonthica* resistant germplasm and bio-control using *Fusarium* pathogens (AATF, 2006; Khan *et al*., 2006; De Groote *et al*., 2007; M'mboyi *et al*., 2010). Despite all these strategies in place to control *Striga spp*, their practicability remains remote and some are expensive to the small-scale farmers in SSA (M'mboyi *et al*., 2010). Genetic improvement of maize either through genetic modification (GM) or non-GM approach may offer a

solution against the effects of *Striga spp*. However, these approaches are constrained by limited information on the biological processes leading to parasitism and the resistance mechanisms related to parasite-host interactions, as well as scarcity of sources for resistance (Amusan *et al*., 2008; Runo *et al*., 2012). Additionally, there is lack of information regarding the genes responsible for haustoria formation in *Striga spp* which could be targeted for silencing.

Majority of the smallscale farmers opt for Open pollinated varieties (OPVs) such as *KSTP´94* and *Namba nane*. This is because the OPVs are early maturing, requires less inputs, and better culinary qualities (macharia *et al*., 2010; Omondi *et al*., 2014). In this study, the differential host responses to *S. hermonthica* infection in susceptible (*Namba nane*) and tolerant (*KSTP'94*) open pollinated varieties of maize in Kenya were examined. The *KSTP'94* maize variety was released by Kenya Agricultural Research Institute (KARI) in the year 2000 with special attributes of tolerance to *S. hermonthica* infection, but the resistance mechanism has not been determined. *Namba nane* variety has been known to be highly susceptible to *S. hermonthica* infestation and is the popular maize variety in western Kenya, a region greatly affected by the parasite. The expression of putative genes key in *S. hermonthica* haustoriagenesis; *Expansin*, *Cysteine proteinase*, *Mannose 6-phosphate reductase*, *Tvpirin*, and *Quinone-oxidoreductase* genes in *S. hermonthica* tissues were also evaluated. The genes have been previously reported to be responsible for haustoria formation in various parasitic weeds (O'malley and Lynn, 2000; Aly *et al.*, 2009; Bleischwitz *et al.*, 2010; Bandaranayake, 2010).

RNA interference (RNAi) against genes for haustorium formation in parasitic plants has been proposed as an effective and sustainable strategy in management of the parasitic weeds (Alakonya *et al*., 2012). RNAi has potential for developing resistance against *S. hermonthica* by engineering host-derived resistance. However, the strategy has not been extensively tested due to lack of candidate genes. Additionaly, RNAi application, is constrained by lack of efficient high throughput screening protocols for such candidate genes. Recently, strategies based on developing resistance against parasites using RNAi have been widely explored in resistance against viruses, root knot nematodes, and insect pests and hold promise in parasitic plants control, (reviewed in Runo *et al*., 2011). Viral induced gene silencing (VIGS) is a powerful tool for plant functional genomics and provides an easy and effective strategy in

screening for putative candidate genes to target through RNAi. Therefore the efficacy of agrodrench and agroinfiltration using VIGS constructs tageting *phytoene desaturase* (PDS) gene in *S. hermonthica* was assesed.

1.2 Problem statement

Maize is the staple food in the SSA (FAO, 2010; CIMMIT, 2011). However, due to biotic and abiotic constrains, its production is below the demand hence results in food insecurity (AATF, 2006; Ejeta, 2007; M'mboyi *et al*., 2010). *Striga hermonthica* is one of the major biotic constrains affecting maize production (AATF, 2006; Rich and Ejeta, 2008). The habitats of this parasitic weed are predominantly semi-arid areas of Africa and may spread epidemically on cultivated land, destroying cereal crops and causing severe economic losses of up to 100% (Diallo *et al*., 2005). Moreover, the seeds can remain dormant and viable in the soil for more than 20 years, endangering the future crops during this period (Berner *et al*., 1995; De Groote *et al*., 2007). Increasing food demand due to increasing population density in Africa, causes more frequent land use, resulting in an expanding *S. hermonthica* infestation and hence more food insecurity threats. Several methods of *S. hermonthica* control have been developed, but they are of limited practical use to small scale farmers hence allowing the epidemic potential of *S. hermonthica* to increase. This calls for development of new, sustainable and affordable approaches to manage *S. hermonthica*.

1.3 Justification

African maize smallholder farmers struggle to overcome the *S. hermonthica* weed menace, whose spread has been going on uncontrollably, leading to continued food insecurity and poverty. This situation calls for a sustainable method to overcome this parasitic weed menace in Africa in order to enhance food security. The most attractive strategy would involve exploiting germplasm resistance. However, this is constrained by lack of clear understanding of mechanisms involved in haustoria development and the resistance mechanisms in some existing tolerant maize genotypes. Genetic engineering offers promise especially through RNAi which faces the problem of lack of information on candidate genes to target. Although RNAi has been used against *S. hermonthica,* only a handful of genes have been screened (Ejeta, 2007). This is because the previous researchers opted for screening via the stable transformation strategy that is laborious and

time consuming. Viral induced gene silencing is a transient transformation mechanism that circumvents stable transformation limitations and provides an easy and effective strategy for screening candidate genes for target against *S. hermonthica*. This work aimed at identifying the resistance mechanisms in tolerant varieties so as to expand the understanding of *S. hermonthica* resistance mechanisms. Furthermore, the study also identified putative genes that could be key in *S. hermonthica* haustoria formation. Finally, an Agrodrench and Agroinfiltration VIGS based protocol for use in screening such genes in *S. hermonthica* was developed.

1.4 General objective

To investigate histological and molecular developmental mechanism of *S. hermonthica* parasitizing maize (*Zea mays*).

1.4.1 Specific objectives

i. To investigate haustorium development process in *S. hermonthica* tolerant maize variety (*KSTP'94*) and susceptible variety (*Namba nane*)
ii. To clone and sequence the genes expressed in *S. hermonthica* haustoria
iii. To validate Viral Induced Gene Silencing (VIGS) as a tool for functional genomic in *S. hermonthica*

1.5 Null hypothesis

i. The haustoria development process in *S. hermonthica* tolerant variety (*KSTP´94*) is not different to that in susceptible variety (*Namba nane*)

ii. Genes key in *S. hermonthica* haustoria formation can not be cloned and sequenced

iii. Viral induced gene silencing cannot be used for functional genomic studies in *S. hermonthica*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Maize and its economic importance

Maize (*Zea mays*) constitutes the major staple diet in Sub-saharani Africa and it's grown widely throughout the world in a range of agroecological environments (FAO, 2010; IITA, 2011). It has extensive geographical reach growing from sea level to elevations exceeding 3,000 meters, in the tropics and subtropics, and in cold, hot, rainy, and dry areas (Dowswell *et al*., 1996; IITA, 2011). Worldwide consumption of maize is more than 116 million tons, with Africa consuming 30% and SSA 21%. The cereal crop constitutes the staple diet of many people in sub-Saharan Africa (SSA) and in Africa at large (FAO, 2010; CIMMIT, 2011). Over 300 million Africans depend on maize as their main source of food (IITA, 2011). A high annual consumption level of 79 kg per capita in Africa and 125 kg per capita in Kenya as been reported (De Groote *et al*., 2002).

Maize in SSA is mainly grown by smallscale farmers, where most of them preffer OPVs as opposed to the hybrid´s. This is because the OPVs are early maturing, requires less inputs, and better culinary qualities (Macharia *et al*., 2010; Omondi *et al*., 2014). Maize has the highest numbers of ways it can be utilized, with all parts of the plant finding economic value. The grain, cob, stalk, leaves and tassel can all be used to produce a large variety of food and non-food products. The grain is used as food or fermented to produce a wide range of foods and beverages, livestock feed, industrial inputs of starch, oil, sugar, protein, cellulose, and ethyl alcohol. Roots are used for mulching, manure, or burned as fuel. Maize is rich in essential minerals, vitamins A, B, C and E, carbohydrate, protein and iron, as well as dietary fiber (M'mboyi *et al*., 2010; IITA, 2011). Africa as a whole uses 95%, of its production as food compared to other world regions that use most of its maize as animal feed (IITA, 2011).

2.2 Constraints to maize production in SSA

A combination of factors such as drought, pests, poor soils, parasitic weeds, fungal and bacterial diseases, poorly developed markets and rural infrastructure has delayed efforts to improve the maize productivity in SSA (IITA, 2011). Bio-physical constraints to maize

production can be categorized into biotic and abiotic. The major biotic constraints to maize production in SSA include Striga weeds, diseases such as maize streak virus and ear rots and pests such as the larger grain borer (LGB) (*Prostephanus truncatus*), stem borers and maize weevil (*Sitophilus spp*) (Diallo *et al*., 2005). Among the biotic factors, *Striga hermonthica*, a root hemi-parasitic weed is the most devastating and dorminant constraint affecting maize production and causes up to 100 % yield losses leading to US$7 billion losses annually (Diallo *et al*., 2005). Drought stress is the dominant abiotic constraint to maize production in SSA, causing up to 17% losses. Low and declining soil fertility due to lack of affordable fertilizers by the small scale farmers is also one of the major abiotic factors (Diallo *et al*., 2005; IITA, 2011). Low soil nitrogen is ranked among the most important constraints to maize productivity in the east and central African region, contributing to production losses of about 30%, equivalent to US$500 million (Diallo *et al*., 2005).

2.3 *Striga hermonthica* biology and its impact on the host

Striga hermonthica is an annual chlorophyllous plant that grows up to 80 cm in height. The weed has hairy, hard quadrangle-shaped and fibrous stem with narrow leaves. The inflorescence has spike-shaped raceme bearing up to 60 flowers for the terminal and 10 -20 for the lateral inflorescence with bright pink, rose-red, white, or yellow color (Muscleman, 1980). The root system in *S. hermonthica* is vestigial, in which the germinated seed radical produces a host attachment organ called haustorium. The haustoria function is in host attachment, invasion and in the physiological redirection of host resources into the parasite (Hood *et al*., 1998; Yoder, 1999; Westwood *et al.*, 2010). The *S. hermonthica* haustoria are classfied as either primary or secondary, depending on whether they are terminally or laterally localized on the roots, respectively (Yoder, 1999).

The seeds are very tiny with a diameter ranging between 0.15-0.3 mm and the weed is estimated to produce as high as 40,000-500000 seeds per plant. *Striga hermonthica* seeds are triggered to germinate by host and non-host derived chemical signals called strigolactones (Amusan *et al*., 2008). *Striga hermonthica* normally emerges about 4-7 weeks after planting maize, and the germinated seedlings attach to host's roots within 3-7 days. If not stimulated to germinate, seeds may stay dormant in the soil for over 20 years (Berner *et al.*, 1995; De Groote *et al.*, 2007). The Striga genus is composed of 30–35

species, over 80% of which are found in Africa, while the rest occur in Asia and the United States. The life cycle of *S. hermonthica* is highly synchronized with that of its host and generally involves the stages of germination, attachment to host, haustoria formation, penetration and establishment of vascular connections, accumulation of nutrients, flowering and seed production (Parker and Riches, 1993).

Striga hermonthica and *S. asiatica* species are the most important cereal weeds, whereas *S. gesnerioide* parasitizes legumes and is a serious constraint to cowpea production (De Groote *et al*., 2007). *Striga hermonthica* affects its host by slowing down the growth as a result of impeding its photosynthesis process, using its nutrients and water hence causing a deficit (Khan *et al*., 2006). Once the parasitic weed spreads to a field the damage it causes increases every season if nothing is done to combat it (Khan *et al*., 2006). Although *S. hermonthica* is capable of photosynthesis once it emerges, it relies on host plants for a significant portion of its carbon supply (Rich and Ejeta, 2007). Its infection results in chlorosis, wilting, stunting, and death to the host, resulting to losses of up to 100% (Hood *et al*., 1998; Rich and Ejeta, 2007). The cereal yield decreases by 1.2 million tons annually in East Africa alone (CIMMYT, 2011).

2.3.1 *Striga hermonthica* maize interaction

The signal exchange between *S. hermonthica* and its hosts leads to successful parasitism. Germination of *S. hermonthica* seeds only takes place in response to chemical signals, most commonly strigolactones, produced by the host and in some cases non-host species (Parker and Riches, 1993; Yoder, 1999). The haustoria formation and subsequent attachment to the host is further guided by the host-derived chemical signals. After penetration to the cortex, haustoria cells undergo a differentiation process and form vessels that form a continuous bridge with the host xylem that serve as a conduit for host derived nutrients and water (Dorr, 1997). After a connection being established between host and parasite, the parasite exhibits a holoparasitic subterranean stage of development at which time damage is inflicted. The parasite then emerges from the soil, develops chlorophyllous shoots (hemi-parasitic stage) and produces flowers and seeds (Bagonneaud-Berthorne *et al*., 1995). *Striga hermonthica* causes most damage to its host before it emerges and therefore, the use of post-emergent herbicides is not of alue in controlling the weed (De-Groote *et al*., 2007).

2.3.2 Management of *Striga hermonthica*

Different control measures have been recommended in tackling the negative effects of *S. hermonthica* (Teka, 2014). The weed can be managed using one or more methods: use of cultural and mechanical control practices, nitrogen fertilizers, push pull technology, biological control practices, resistant host crops, use of herbicides and integrated Striga control methods (Teka, 2014; Avedi *et al*., 2014). One way to control *S. hermonthica* is to use maize seeds that are coat dressed with certain systemic herbicides such as imidazolinone herbicides (e.g imazapyr). Breeders have developed the Imazapyr resistant (IR) maize varieties that cause any geminating *S. hermonthica* attempting to get in contact to be destoyed (Berner *et al.*,1997; AATF, 2006; De Groote *et al*., 2007). This protects the host seed from *S. hermonthica* infestation and improves germination. However, the seeds can only be from imazapyr resistance (IR) maize cultivars. Additionaly, these dressed seeds are expensive and cannot be recycled (Khan *et al*., 2006). Imazapyr is commonly marketed under the tradename STRIGAWAY® (AATF, 2006).

Intercropping is another cultural practice used by small scale farmers to control *Striga spp*. This strategy is advantageous because the risk of harvest losses decreases as several crops are grown at the same time (Khan *et al*., 2009, Teka, 2014). Intercropping cereal crops and legumes also increases the soil fertility and provides shade that gives *S. hermonthica* a disadvantage (Khan *et al*., 2006; Midega *et al*., 2013). *Striga spp* germination is known to be suppressed by roots exudes of some legumes such as groundnuts and soyabean (AATF, 2006). Crop rotation, fallowing and mulching are other cultural practices used to control *Striga spp* (Midega *et al*., 2013). Fallowing can be included in the crop rotation in order to increase soil fertility which makes the conditions less favorable for *S. hermonthica*. Morever, the strategies decrease the parasites' seed bank in the soil (De Groote *et al*., 2007). Due to the ever-increasing human population, the use of fallow has become impractical with time because of intensified land demand (Berner *et al*., 1995).

Push and pull, also known as 'suicidal germination' strategy is another agroecological approach used to control *S. hermonthica*. This involves use of crops such as desmodium whose roots produces strigolactones that stimulate *S. hermonthica* to germinate (AATF, 2006; Khan *et al*., 2010; Midega *et al*., 2013; Maina and Mwangi, 2014, Teka, 2014). Desmodium stimulates the *S. hermonthica* seeds to germinate but cannot serve as a host as

the *S. hermonthica* seedlings are not able to attach to it (AATF, 2006). The germinated *S. hermonthica* seedlings stimulated to grow are then pulled out or sprayed with herbicides before flowering to minimize the seed bank (De Groote *et al*., 2007). Desmodium also provides high quality livestock feed, apart from suppressing Striga weed (AATF, 2006). The crop is also known to exude substances that are allelopathic to Striga plants.

Use of broadleaf herbicides, in both pre-emergent and post-emergent field application, is also a valuable control measure of *Striga spp*, but unfortunately this strategy is expensive to most small scale farmers in SSA. Herbicides such as 2-4 D, Oxyfluorfen and WitchAway have been successfully employed against *Striga* in southern USA (AATF, 2006, Teka, 2014). Many African farmers practice cereal-legume intercropping, a practice that is incompatible with field spraying (AATF, 2006). However, the use of herbicides as a means to control the weed is not effective because the challenge is that most damage to its host occurs before *S. hermonthica* emerges and therefore post- emergent herbicide field applications has little effect to the Striga (Berner *et al*., 1995). The strategy also remains expensive and unaffordable to the small-scale farmers in SSA (AATF, 2006).

Use of *Striga spp* tolerant varieties is another strategy used in controlling the parasite. Several Striga tolelant varieties are available as either open pollinated or hybrid varieties. While resistant varieties may yield better under moderate levels of *Striga* infestation, they do little to reduce *Striga.* Genetic improvement of maize either through genetic modification or non-genetic modification approach offers resilient germplasm against the parasitic weed constraints (Alakonya, 2011). Exploiting the resistance offered by the tolerant germplasm would be a sustainable solution to striga menace. The strategy is widely considered to be the most cost-effective and sustainable management strategy for parasitic weeds and pest control. However, these approaches are constrained by limited information on the biological processes and resistance mechanisms related to parasite-host interactions, as well as scarcity of sources for resistance (Amusan *et al*., 2008; Runo *et al*., 2012). Additionaly, the conventional breeding of resistant crops is time consuming and limited by the deficiency of natural resistance genes (Johnson, 2000; Stuthman *et al*., 2007). Furthermore, it's unfortunate that most pathogens, pests and weeds have the ability of persistent genetic variation and adaptive evolution. The virulent biotypes emerge rapidly

under the selection pressure of resistance gene generations to overcome the cultivar resistance (McDonald and Linde, 2002). Therefore, alternative control strategies are urgently needed for the development of more durable resistant cultivars.

2.3.2.1 RNA interference in parasitic plants management

RNA interference occurs in eukaryotes and functions to regulate gene expression through RNA silencing. It is a mechanism involving the cleavage of a double stranded (dsRNA) into short sequences of 21-25 nucleotides in length called short interfering RNAs (siRNAs). The cleavage of the dsRNA is by an enzyme called DICER that has Rnase III domains. These RNAs, known as siRNAs and microRNAs (miRNAs), are then incorporated into 'RNA-induced silencing complex' (RISC). The RISC then directs the degradation of endogenous mRNAs that are homologous to the small interfearing RNAs causing post transcriptional gene silencing (PTGS) (Hannon, 2002; Baulcombe, 2004; Meister and Tuschl, 2004). The RNA silencing has been reported to occur due to either of the three RNAi mechanism pathways; the first pathway is cytoplasmic siRNA silencing. This pathway has been reported to have evolved as a defense mechanism against plant viruses, where the dsRNA could result from viral replication intermediate or a secondary-structure feature of single-stranded viral RNA. In plant DNA viruses, dsRNA may be formed by the annealing of overlapping complementary transcripts (Hamilton and Baulcombe, 1999).

The second pathway involves silencing of endogenous messenger RNAs by miRNAs. These miRNAs are involved in negative regulation of gene expression, and they do so by base pairing to specific mRNAs, resulting in either RNA cleavage or termination of protein translation. Like siRNAs, the miRNAs are short 21-25 nucleotide RNAs derived by DICER cleavage of a precursor (Bartel, 2004). The third pathway is DNA methylation which acts to suppress transcription. This mechanism is thought to be important in protecting the genome against damage caused by transposons (Lippman and Martienssen, 2004).

Using RNAi as a tool for crop improvement is advantageous over other methods. The RNAi signal can be both local (cell – cell) and systemic (spread through the vascular system). This indicates that a signal introduced in one part of the plant can easily spread to

other distant parts and there is more flexibility in regulating gene expression as compared with other methods such as mutation or loss of function mutations (Bauchera *et al*., 1998; Voinnet and Baulcombe, 1997). Tomilov *et al*. (2006) demonstrated that interfering hairpin constructs transformed into host plants could silence expression of the targeted genes in the parasite. Previous researchers reported reduction in Orobanche viability by RNAi-mediated silencing (Aly *et al*., 2009). However, the practicability of RNAi in *S. hermonthica* management has been constrained by lack of methods to deliver the silencing molecules and lack of candidate genes to target.

2.4 Viral induced gene silencing (VIGS)

Virus-induced gene silencing (VIGS) is an RNA-mediated post-transcriptional gene-silencing mechanism that works to protect plants against invasion by foreign genes (Baulcombe, 1999). The VIGS mechanism has allowed a better understanding of how plants defend themselves against plant viruses, and has emerged as a functional genomics tool for knocking out gene expression of target plant genes in some plants (Liu *et al*., 2002a; Sharma *et al*., 2003; Yoshioka *et al*., 2003). The mechanism is also used as a forward genetics tool to identify a desired phenotype (Lu *et al*., 2003b). Plant based virus vectors carrying plant sequences homologous to endogenous plant genes trigger gene silencing through a homology-dependent RNA degradation mechanism also known as RNA silencing. The small interfering RNA (siRNA), derived from targeted dsRNA of virus or host and expressed from the viral genome, directs the degradation RISC complex to the corresponding host mRNA resulting in a loss-of function phenotype in the host due to RNA degradation (Baulcombe, 2002; Lu *et al*., 2003a; Robertson, 2004; Voinnet, 2001).

Viral induced gene silencing is not a stable transformation strategy and transiently suppresses the expression of the targeted gene by degrading mRNA transcripts hence does not alter the gene itself unlike conventional mutagenesis. Thus VIGS allows, unlike stable transformation procedures, the study of genes that would normally lead to a lethal phenotype when disrupted (Kim *et al*., 2003b; Lu *et al*., 2003a). Therefore, VIGS is a powerful technique with the potential to silence specific genes and study rapid loss of gene function, and it has already been employed successfully in a wide range of species. Furthermore reproducible protocols for stable genetic transformation are extremely challenging to establish for the majority of plant species (Becker and Lange, 2009).

2.4.1 Molecular mechanism of VIGS

Viral induced gene silencing is based on post-transcriptional gene silencing (PTGS) (Baulcombe, 1999). The commonly used viral vectors for VIGS are manipulated to incorporate a gene fragment from the targeted host plant. Plants employ PTGS as their antiviral defense mechanism to counteract viral proliferation, hence employing an RNA degradation mechanism very similar to the pathways of RNAi (Becker and Lange, 2009). The VIGS vectors are standard binary Ti-plasmid derived vectors used for *Agrobacterium tumefaciens*-mediated plant transformation in which part of a viral genome is inserted. This viral genome has a multiple cloning site to insert the endogenous target gene of the host plant. The host gene fragments of 200 to 1300 bp's length targeting the middle regions of mRNAs and without the homopolymeric regions, such as poly-A tails suffice are used (Liu and Page, 2008).

Plant inoculation with viral vectors is most commonly achieved through *A. tumefaciens* infection. The T-DNA containing the viral genome is integrated into the host genome of at least one cell, transcribed, and translated. This leads to the production of double-stranded RNAs (dsRNAs) from the viral ssRNA template by self-assembly of viral ssRNA into secondary structures or complementary sequences derived from longer positive and negative viral ssRNA strands (Donaire *et al*., 2008). The Dicer-like proteins then cleave these viral dsRNAs into short interfering RNAs (siRNAs) duplexes that are approximately 21–24 nucleotides (nt) in length. These siRNA in turn are incorporated as single strand RNA molecules into RISC (RNA-induced Silencing Complex) that screens for and destroys RNAs complementary to the siRNA (Waterhouse and Fusaro, 2006; Ding and Voinnet, 2007).

The virus-derived silencing signal is further amplified and spread systemically throughout the plant. Small interfering RNAs (siRNAs) about 21 nt in length are assumed to mediate the short range transport and the RNA-dependent RNA Polymeras e6 (RDR6) is required for long-range transport, possibly by amplifying the silencing signal (Kalantidis *et al*., 2008). This systemic spread of the silencing signal occurs regardless of the successful movement of the virus particles in the plant. When VIGS is applied to a susceptible plant, the host targeted mRNA is degraded in large portions (Becker and Lange, 2009).

2.4.2 Application of viral induced gene silencing

Gene silencing in response to genetically manipulated RNA viral vectors was originally observed in *Nicotiana tabacum*. This was through silencing of *Phytoene desaturase* (PDS) gene which is involved in the carotenoid biosynthesis pathway. Incorporating parts of the PDS cDNA into a hybrid viral vector composed of sequences from the tobacco mosaic virus and the tomato mosaic virus led to success of VIGS in tobacco (Becker and Lange, 2009). Kumagai *et al*. (1995) reported that virally delivered PDS antisense RNA resulted in inhibition of the carotenoid synthesis and the inhibition spread systemically throughout the entire plant. Reduced levels of photo-protective carotenoids lead to the rapid destruction of chlorophyll by photo-oxidation that subsequently resulted in a white leaf phenotype which can be easily followed visually (Kumagai *et al*., 1995). To monitor the success of VIGS, *Phytoene desaturase* gene homologs have been used in proof-of-concept experiments in a large variety of species. Most and nearly all VIGS vectors are derived from viruses that were originally hosted in the Solanaceae. However, the host range of some of them was successfully extended to other Solanaceae specifically tomato (*Solanum lycopersicum*), bell pepper, and petunia (*Petunia hybrida*) (Chung *et al*., 2004; Brigneti *et al*., 2004; Liu *et al*., 2002; Chen *et al*., 2004).

The Tobacco rattle virus (TRV) is used preferentially as the VIGS vector in dicots due to its high susceptibility in a wide host range and because of its mild disease symptoms after infection. Viral induced gene silencing has also been shown to be effective in *Rosaceae* species like Arabidopsis, pea (*Pisum sativum*), and cassava (*Manihot esculenta*) (Pflieger *et al*., 2008; Constantin *et al*., 2004; Fofana *et al*., 2004). Recently, an additional VIGS vector system was developed from the apple latent spherical virus (ALSV) that targets a diverse range of higher eudicots including Solanaceae, Arabidopsis, curcubit, and legume species (Igarashi *et al*., 2009). Monocot crop species like barley, rice, wheat, and maize have also been reported to be susceptible to VIGS (Scofield and Nelson, 2009).

Viral induced gene silencing is also reverse genetic tool to study the gene function in species recalcitrant to stable genetic transformation. The VIGS mechanism is additionaly used in analysis of genes involved in plant-pathogen/parasite interaction. The mechanism has been used to study stem rust resistance in barley, the genes involved in interactions with phytophtora, or methyl jasmonate signaling during herbivore attack in tobacco

(Brueggeman *et al*., 2008; Kanzaki *et al*., 2008; Wu *et al*., 2008). Viral induced gene silencing also serves as a major new tool in the field of evolutionary developmental genetics. Recent work includes the characterization of a gene important for the symbiotic root nodulation of pea, the contribution of floral homeotic B class genes to the unusual columbine staminodia floral organs, and the lineage-specific gene function acquisitions and losses of the Crabsclaw-like carpel developmental regulators during angiosperm evolution (Kramer *et al*., 2007; Constantin *et al*., 2008; Orashakova *et al*., 2009).

The silencing effects of VIGS remain transient in the majority of all cases and the timing of its appearance as well as its duration is species-specific. One example includes the barley stripe mosaic virus (BSMV)-induced VIGS whose effects in barley last one to two weeks and the TRV mediated silencing in California poppy (*Eschscholzia californica*) lasts after 16 weeks. Morever, the apple latent spherical virus (ALSV)-mediated silencing is maintained in soybean throughout the plant's life and is even transmitted to the next generation (Holzberg *et al*., 2002; Wege *et al*., 2007; Yamagishi and Yoshikawa, 2009). Varying penetrance of the phenotype in vegetative and reproductive tissue that requires a larger number of plants to be screened for phenotypes is another challenge of this technique. Silencing effects are often found in sectors dividing the whole plant or are restricted to plant organs formed from few consecutive nodes (Wege *et al*., 2007).

2.5 Agro-innoculation and tobacco rattle virus VIGS constructs

Tobacco rattle virus (TRV) is among the several viral vector systems used to trigger VIGS. The VIGS vectors are widely used because they produce mild symptoms on the host and they have a wide host range (Ratcliff *et al*., 2001; Liu *et al*., 2002a; Dinesh-Kumar *et al*., 2003). Tobacco rattle virus belongs to the genus *Tobravirus* and is mostly transmitted by nematodes through the soil (Matthews, 1991; Visser and Bol, 1999). This virus contains bipartite positive-sense RNA genome (RNA1/RNA2) (Matthews, 1991). The RNAi encodes two viral replication proteins, a movement protein and a seed transmission factor, while RNA2 encodes the coat protein and a nematode transmission factor (Verchot-Lubicz, 2002; Visser and Bol, 1999). *Agrobacterium tumefaciens* is a soil-borne pathogen that causes neoplastic growth, referred to as 'crown gall,' in several dicotyledonous plants by entering mainly through wounds on roots and stem (Escobar and Dandekar, 2003). *Agrobacterium*-based binary vectors have been widely used for delivering viral vectors

into plants and this is a technique referred to as agroinoculation (Rochester *et al*., 1990; Evans and Jeske, 1993).

Agroinoculation is extensively used to deliver VIGS vectors into plants for RNA silencing (Liu *et al*., 2002b). Leaf infiltration is the most common agroinoculation method used in VIGS (Dinesh-Kumar *et al*., 2003; Lu *et al*., 2003a). However, the leaf infiltration method has limitations in that it is laborious for large-scale screening. On the other hand, certain plants, such as soybean and maize, are difficult to infiltrate. Leaf infiltration of TRV-based VIGS vectors do not induce efficient RNA silencing in many plants including varieties of tomato (Ekengren *et al*., 2003; Liu *et al*., 2002a). Leaf infiltration normally utilizes fully expanded leaves (Liu *et al*., 2002b; Ratcliff *et al*., 2001). These limitations prevent the efficient use of VIGS technology on some plant species and young seedlings.

A novel method of agroinoculation, called 'agrodrench' addresses some of the agroinfiltration problems normally associated with agroinoculation during VIGS (Choong *et al*., 2004). The agrodrench technique with a TRV-VIGS vector can be used for RNA silencing in diverse Solanaceae species and in young seedlings. Interestingly, agrodrench increases the efficacy of VIGS in roots compared with leaf infiltration. It has been proposed that agrodrench is a simple and effective agroinoculation technique that can be extensively used for infecting a wide range of plants to induce VIGS. Agrodrench involves drenching the plant rhizosphere with *A. tumefaciens* containing the viral vector within the T-DNA of a binary vector (Choong *et al*., 2004).

2.6 Gateway cloning

Engineering multiple expression vector constructs to accomplish goals for every target gene of interest using traditional ligase-mediated cloning is time-consuming and laborious, posing a technical barrier for high-throughput functional genomics or proteomics projects. The advent of Gateway cloning technology has considerably lowered such barriers (Hartley *et al*., 2000). Gateway cloning technique exploits the bacteriophage lambda recombination system, and passes the need for traditional ligase-mediated cloning. Once captured in a Gateway-compatible plasmid 'entry vector', an open reading frame (ORF) or gene flanked by recombination sites can be recombined into a variety of 'destination vectors' that possess compatible recombination sites. Destination vectors for protein

expression in *E. coli*, yeast, mammalian, and insect cells are commercially available and are marketed by Invitrogen (Carlsbad, CA, USA). These plant destination vectors have been designed for a variety of specific purposes including protein localization, promoter functional analysis, gene over expression**,** gene knockdown by RNA interference, production of epitope-tagged proteins for affinity purification or analysis of protein/protein interactions using fluorescence resonance energy transfer (FRET)**,** among other uses (Earley *et al*., 2006).

The Gateway cloning system exploits the accurate, site specific recombination system utilized by bacteriophage lambda in order to shuttle sequences between plasmids bearing compatible recombination sites. The preferred method for initially capturing sequences of interest is to use topoisomerase-mediated cloning, which eliminates the need for conventional DNA ligase-mediated molecular cloning (Shuman, 1994). In this approach, polymerase chain reaction (PCR) is used to amplify the target sequence using a forward primer that includes the sequence CACC at the 5' end. This sequence facilitates directional incorporation into Invitrogen's TOPO entry vector. The resulting recombinant plasmid has the target DNA sequences flanked by attL recombination sequences. Once flanked by attL recombination sites, the sequence can be recombined with attR sites using the LR clonase reaction mix (Invitrogen).

This reaction transfers the target sequence into a desired destination vector. Destination vectors contain a gene (ccdB) that is lethal to most strains of *E. coli*. 'Empty' destination vectors are therefore selected against upon transformation of *E. coli* cells with the recombination reaction. This negative selection, combined with positive selection for an antibiotic resistance marker, ensures that resulting colonies contain plasmids that have undergone recombination. The ease and speed with which a captured target sequence can be shuttled simultaneously into a variety of destination vectors are great advantages for high-throughput functional genomics/proteomics investigations.

Although topoisomerase-mediated cloning is used almost exclusively for capturing target sequences in entry vectors, there are other options. One option is to use traditional ligase-mediated insertion of a target sequence into an entry vector at a multiple cloning site that is flanked by attL sites. A second option is to use PCR primers that include attB sites when

amplifying the target sequence. The resulting PCR products can be recombined directly into a donor vector containing attP recombination sites using the BP clonase reaction mix (Invitrogen). This BP recombination reaction results in the target sequence being flanked by attL sequences, which allows subsequent recombination with a destination vector. These options, as well as detailed protocols, are described in the Gateway cloning manual(s) available from Invitrogen's website (http://www.invitrogen.com).

2.7 Putative genes responsible for haustoria development in parasitic weeds

There exist several parasitic weeds that are obligate holoparasites that attack the roots of many economically important crops in semi-arid and arid regions of the world. Thus they cause severe losses in crop yields and quality. These parasitic weeds are difficult to control by conventional means because of their life cycles which are closely synchronized with those of their hosts and are concealed underground undiagnosed until they have already inflicted irreversible damage (Aly *et al*., 2009). The parasitization of the host plant begins with the parasites developing haustoria that penetrate the host roots and forms physical and physiological bridges between the two species (Aly *et al*., 2009). Several genes have been reported to be responsible for haustoria development in various parasitic weeds such as; *Expansin* gene in parasitic angiosperms, *Cysteine protease* gene reported in *Cuscuta reflexa,* Mannose6-phosphate reductase gene in *Orobanche aegyptiaca*, *Tvpirin* and *quinone oxidoreductase* in *Triphysaria versicolor* (O'malley and Lynn, 2000; Aly *et al*., 2009; Bleischwitz *et al*., 2010; Bandaranayake *et al*., 2012). However, in *S. hermonthica* there is no report on such genes. Therefore, similar genes could be responsible for haustoriagenesis in *S. hermonthica* and thus such genes can be exploited in developing sustainable control strategies.

2.7.1 *Expansin* genes

Expansin genes have been reported to be responsible for cell wall loosening during cell expansion process in plants. They are a novel class of proteins that has been reported to induce cell enlargement by changing wall rheology in the network of intertwined saccharide polymers (McQueen-Mason, 1995; Shieh and Cosgrove, 1998; Cosgrove, 1999). At the low pH values associated with plant microfibrils and the polysaccharide matrix in which they are embedded. This alteration allows slippage of load-bearing cellulose microfibrils and results in cell wall expansion (McQueen-Mason and Cosgrove,

1992). *Expansin*s are both widely distributed across divergent plant species and highly conserved (Shcherban *et al*., 1995). Expansin expression is regulated by the same environmental and hormonal signals that increase the growth rate of rice internodes and the ripening of tomato fruit and other experiments have localized their expression to growing cells (Rose *et al*., 1997, Cho and Kende, 1998; Reinhardt *et al*., 1998).

Expansin has been argued to be a necessary component for cell enlargement and researchers suggested that it may even be a sufficient early stimulus for initiation of developmental commitments (O'Malley and Lynn, 2000). Parasitic strategies are widely distributed across the angiosperms and are estimated to have evolved at least eight different times. Within the obligate hemiparasitic and holoparasitic members, elaborate strategies for host selection have emerged. O'Malley and Lynn, (2000) demonstrated that in the parasitic Scrophulariceae, *S. asiatica*, for which signal-mediated host detection is critical, *Expansin* mRNA provides a reliable and accurate downstream molecular marker for the transition to the parasitic mode. Haustoria development is critically dependent on cellular expansion, and the regulation of the cell wall–loosening protein, *Expansin*, during signal exposure has been evaluated leading to a proposed mechanistic model for the control of developmental commitment in the vegetative/parasitic transition of *S. asiatica* (O'malley and Lynn, 2000).

2.7.2 *Mannose 6-phosphate reductase* genes

Mannose 6-phosphate reductase (*M6PR*) is a key enzyme in mannitol biosynthesis in plants. Aly *et al*. (2009) suggested that mannitol may be involved in osmoregulation in many harmful root parasites, a process which is essential for the parasite's uptake of water and nutrients from its host. He reported an increase in *M6PR* activity, observed when plants were subjected to drought conditions. The *Orobanche ramosa M6PR* gene has been isolated and characterized, and its expression was analyzed during early stages of the parasite's development, and it has been suggested as a potential target for efforts to control these parasitic plants (Robert *et al*., 1999; Delavault *et al*., 2002).

Aly *et al*. (2009) demonstrated that a pBin-IR-M6PR constructs engineered into plants can silence the expression of the *M6PR* genes in *Orobanche* tubercles growing on transgenic roots, as a result of reduction in *M6PR* mRNA transcripts, reduction in mannitol level, as

well as increase in the number of dead tubercles. The results were consistent with the hypothesis that mannitol accumulation in Orobanche plays an important role in this parasite's recruitment of water and nutrients from its host and involvement in Orobanche tubercles development. The role of M6PR in the parasitic weed orobanche could also be played in other parasitic weeds of the same plant family such as *S. hermonthica.*

2.7.3 *Cysteine protease* gene

During the parasite-host interactions both plant species act and react in order to invade, prevent, or tolerate invasion. Papain-like cysteine proteases have been identified at the surface of various interaction surfaces between plants and pathogens like bacteria, fungi, oomycetes, nematodes insects or herbivores (Shen *et al.*, 2006; Bleischwitz *et al*., 2010). Some of these are a component of a defense mechanism while others are implicated in the parasitic pathogen attack. Bleischwitz *et al*. (2010) identified cysteine protein as a component that may be important for successful infestation of the parasitic plant *Cascuta reflexa*, this could possibily open a new approach for development of parasitic plant blocking agents.

Bleischwitz *et al*. (2010) demonstrated that the encoded protein could also be significant for the host parasite interaction. The researchers experiment on the significance of cuscutain, a *cysteine protease* from *Cuscuta reflexa* in host-parasite interactions, demonstrated that there was a chance that a reduction of parasite-derived proteins weakens the parasite's infection efficiency and thereby strengthens host defense. Therefore the activity of *cysteine proteinase* could play a role in other parasitic plant interactions such as *Orobanche* or *S. hermonthica*. Inhibition of *cysteine proteases* could thus be of wider importance for antagonizing parasitic plants from different genera.

2.7.4 *Tvpirin* genes and *quinone oxidoreductase* genes

The potential of allelopathy to improve crop performance has been exploited, but little is known about them especially mechanisms by which plants detect and process chemical signals from other plants (Tomilov *et al*., 2006). Haustorium development in response to xenognosins provides a useful mode for investigating chemical signaling between plant roots in general. Both xenognosin response and allelopathy are mediated by similar

molecules: quinones and oxidized phenols. Depending on its concentration, 2, 6-dimethoxy-p-benzoquinone (DMBQ) can be either a developmental stimulant that induces haustorium development or a plant toxin (Tomilov *et al*., 2006). Parasite quinone oxidoreductases use both xenognosins and allelopathic phytotoxins as biochemical substrates (Wrobel *et al*., 2002; Bandaranayake *et al*., 2010). Furthermore, both allelopathy and haustorium induction are dependent upon the redox state of the chemical agent. Juglone, the active allelopathic agent from black walnut trees, is synthesized in the inactive hydro-juglone state that is then activated to the toxic state upon exposure to oxygen (Lee and Campbell, 1969).

Haustorium development is also dependent on the redox state of the inducer. Similarities between quinone associated allelopathy and haustorium initiation led to the hypothesis that the processes may share common molecular mechanisms (Tomilov *et al*., 2006). Haustorium development is a multistep process that requires the coordinated expression of a number of genes and pathways. Most of the processes associated with haustorium development have almost certainly been derived from autotrophic plant processes. O'Malley and Lynn (2000) reported that the *TvPirin* gene associated with haustorium development has homologs in autotrophic plants that must provide functions unrelated to parasitism. Other genes associated with haustorium development similarly have non-parasitic functions in autotrophic plants. *Expansin*s, non-enzymatic proteins that promote cell wall loosening, are used in both parasitic and non parasitic plant processes (O'Malley and Lynn, 2000; Wrobel and Yoder, 2001).

Crystallin quinone oxidoreductases catalyze similar biochemical reactions in many organisms but have specific functions in triggering haustorium development in parasites (Wrobel *et al*., 2002). *Tvpirin* provides both parasite specific and non-parasitic functions without apparent gene duplication. Bandaranayake *et al*., (2012) suggested that the role of *Tvpirin* is restricted to establishing basal levels of gene expression rather than xenognosin responsiveness. They proposed that *Tvpirin* is a generalized transcription factor associated protein that functions in the transcription of several different genes, some of which are needed for haustorium development.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Investigation of *Striga hermonthica* haustorium development in susceptible (*Namba nane*) and tolerant (*KSTP'94*) open pollinated maize varieties

3.1.1 Effect of *Namba nane* and *KSTP'94* maize varieties on *S. hermonthica* germination

Striga hermonthica infected soil was corrected from Alupe in Western Kenya, homogenized by mixing thouroughly and used to fill 200 pots (15cm in diameter). To increase the chances of germination, each pot was inoculated with 20 mg (approximately 20000 seeds) of *S. hermonthica*. Hundred pots were planted (one maize seed per pot) of *S. hermonthica* susceptible maize variety (*Namba nane*) and 100 pots with tolerant maize variety (*KSTP'94*). Fifty pots were filled with non-infected soil to serve as a control, and planted with *Namba nane* and *KSTP'94* variety. The experimental design was CRD and the experiment was conducted in the grasshouses located at Kenyatta University Plant Transformation Laboratory. Watering was done on need basis to maintain moderate soil moisture. The response of the two maize varieties to *S. hermonthica* was determined by scoring for Striga germination, number and appearance of haustoria over a period of time through purposive sampling of selected infected plants for upto 126 days period. The number of the maize plants with emergenged *S. hermonthica*, number of *S. hermonthica* plants per maize plant and number of haustoria connections per maize plant were also recorded over the study period. Photographs were of phenotypic reactions to *S. hermonthica* on the roots of the two maize varieties infected with the parasite were also taken.

3.1.2 Histological studies on effects of *Namba nane* and *KSTP'94* to *S. hermonthica* haustoria development.

3.1.2.1 Tissues collection, fixation and clearing

At 90 days after the planting, infected maize roots with *S. hermonthica* haustoria attached were collected and cleaned with tap water. The connection points of the haustoria and maize roots were excised and placed in pre-labeled tubes containing FAA (formalin 90%, acetic acid 5%, and alcohol 5%). Tissue fixation in FAA was done in a mechanical shaker

set at 50 revolutions per minute (RPM) at 37°C and incubated for two days. Tissue dehydration was done through different ethanol changes of 30%, 50%, 70%, 85%, and 95% of one hour. Tissues pre-staining was done using 5% Eosin in 95% of ethanol over night before dehydrating with 100% ethanol for two hours. Tissue clearing was done using histoclear to ethanol (1:1) for one hour, followed by 100% histoclear (National Diagnostics, Atlanta, Georgia, USA), for one hour as described by Bharathan *et al*. (2002).

3.1.2.2 Tissue infiltration and embedding

Tissues were treated in histoclear to wax ratios of 25%:75%, 50%:50%, 75%:25%, followed by 100% wax with one hour duration per change. Tissues were then infiltrated with 100% paraffin wax (Paraplast, Miccrormick Scientific, St.Louis, Missourri, USA) in an oven set at temperature of 60°C for 14 days, with changes of the wax after every eight hours. Tissues were then embedded in paraffin wax blocks and stored in the fridge at 4°C to await sectioning as described by Bharathan *et al*. (2002) and described in section 3.1.2.3.

3.1.2.3 Sectioning, staining and microscopic examination

Cross-sections of the tissues were done using a microtome (HM340E Microtome, Heldeberg Germany) fitted with a knife with sections thickness set at (5–10 µm). The sections were then mounted on slides and placed in an oven set at 37°C for 48 hours. The slides were then deparaffinised in histoclear for 10 minutes, and rehydrated in an ethanol series of 100%, 85%, 75%, 65%, 50%, 40 % and 30% followed by 100% water. The sections were separately stained with 1% Methylene blue or Ruthenium red for 15 minutes and mounted in Permount™ (Fisher Scientific, Pittsburg, Pennsylvania, USA) as described by Bharathan *et al*. (2002) and Kessler *et al*. (2001). The sections were then examined under a light microscope fitted with a camera. Photographs were taken and saved to photoshop for processing using Picasa version 3.1 software.

3.2 Determination and cloning of genes expressed in *S. hermonthica* haustorium tissues

3.2.1 Tissues collection and RNA extraction

Plant tissues were collected (90 day old plants from section 3.1.1) so as to have four RNA libraries as follows; 1. *S. hermonthica* roots not attached to maize roots, 2. *S. hermonthica* haustorium without any maize tissue, 3. Maize root tissues not colonized by *S.*

hermonthica and 4. *S. hermonthica* leaves. The plant tissues were excised with a clean sterile scapel and collected in tubes precooled with liquid nitrogen. The tissues were separately ground in liquid nitrogen using a motar and pestle. Approximately 20 mg of ground tissues, replicated twice was used for total RNA extraction as per the instructions of the RNeasy® mini kit mannual (Qiagen, Cat no 74104, Valencia. U.S.A). To disrupt the tissues, 350 µl of the RTL buffer was added to the 20 mg of tissue replicated twice and spinned for 3 minutes at 10,000 RPM. One volume of 70% ethanol was added to the lysate and mixed well by pipetting up and down. Up to 700 µl of the sample was transferred to RNeasy mini spin column placed in a 2 ml collection tube and spinned for 15 seconds at 10000 RPM. DNase 1 incubation mix was prepared by adding 10 µl of the DNase 1 stock solution to 70 µl of buffer RDD, mixed gently and spinned (Invitrogen, CAT 18068-015, Carlsbad, U.S.A).

The total RNA was subjected to DNAse treatment by adding 80 µl of the DNase 1 mix directly to the RNeasy column membrane, and placed on bench top at 20-30˚C for 15 minutes. Buffer RW1 was then added (350 µl) to the column and spinned at 10000 RPM for 30 seconds. The flow through was discarded and buffer RPE 500 µl added to the spin column and spinned at 10000 RPM for 15 seconds, and the same volume of RPE was added and spinned for 2 minutes at 10000 RPM. The RNeasy spin column was then placed in new 2 ml collection tubes and spinned at maximum speed for 1 minute to dry the membrane. The RNeasy spin column was then placed in new 1.5 ml collection tube and 30 µl of RNase –free water was added directly to the spin column membrane and spinned for 1 minute at 10000 RPM to elute the RNA.

3.2.2 cDNA synthesis

The total RNA extracted in section 3.2.1 was reverse transcribed to cDNA using Superscript™ III first stand synthesis sytem (Invitrogen, CAT 18080-051, Carlsbad, U.S.A). The first strand cDNA synthesis reactions were primed using random heximers and a replicate of the same was done using random heximers and oligo dT primers in a ratio of 9:1 respectively. For the cDNA synthesis using random heximers, the first mastermix was prepared by mixing 1µl of the10mM primers, 8 µl of the total RNA, and 1 µl of the 10 µM dNTPs to a total volume of 10 µl per reaction. The same was replicated using 1 µl of the mixture of random heximers and Oligo dT mixed in the ratio of 9:1,

respectively. The two mixtures were incubated separately at 65°C for 5 minutes, and then placed on ice for 1 minute. The second cDNA master mix was prepared by adding 2µl of RT buffer (10×), 4µl of $MgCl_2$ (25mM), 1µl of DTT (0.1M), 1µl of RNaseOUT™ (40 u/µl) and Superscript™ III RT(200u/ul) to a final volume of 10 µl. The second master mix was then added to the 10µl of the first maxter mix, and mixed gently before incubating as follows; 10 minutes at 25°C, 50 minutes at 50°C, and 5 minutes at 85°C, then chilled on ice. The mixture was then spinned briefly and 1 µl of RNase-H was added to each tube and incubated at 37°C for 20 minutes before being stored at -20°C.

3.2.3 Screening for genes expressed in *S. hermonthica* haustoria

The available literature on various genes reported to be involved in haustoria formation and development in other parasitc plants was used as guide on the putative genes expressed in haustorium. The genes expressed in *S. hermonthica* haustoria were predicted to be the *Expansin* gene as expressed in *Striga asiatica*, the *cysteine protease* gene in *Cuscuta reflexa*, *Mannose-6-phosphate reductase* in *Orobanche ramosa*, *Tvpirin* and *Quinone oxidoreductase* reported in *Tryphasaria versicor* (Bleischwitz *et al.*, 2010; Bandaranayake, 2010; Aly *et al.*, 2009; O'malley and Lynn, 2000). The vector NTI software program was used to perform sequence alignments and designing of gene specific primers (Table 1) from conserved regions of the putative sequences (Fig1-Fig5).

3.2.3.1 *Expansin* gene

The expansin gene sequences for *S. asiatica* (DQ442401) and *Arabidopsis thaliana* (U30478) were used to perform pairwise analysis with other homologous sequences available in the databases. The species which had 70-100% similarity upon tBlastx analysis were selected and used for multiple alignments using vector NTI software (Fig 1). These species were, *Cucumis sativus* (XP004141444) 84%, *Glycine max* (EP003554526) 82%, *Medicago trancatula* (XP003621258) 84%, *Solanum lycopersicum* (XP004228375) 80%, *Breonia chinensis* (AEQ55276) 80%, *Ziziphus jujuba* (ACK43223)78%, *Dyanthus caryophyllus* (AB542072) 80%, *Pyrus communis* (BAC67191) 77%, *Triphysaria versicolor* (AAF32409) 76%, *Vitis vinifera* (XP002284858) 75%, *Brassica rapa* (AGM16347) 94% and *Pyrus communis* (AB093032) 73% (Fig 1). *Expansin* gene specific primers (forward and reverse) were designed from the concensus sequences indicated by the red colour shading (Fig 1), using Oligocalculator software.

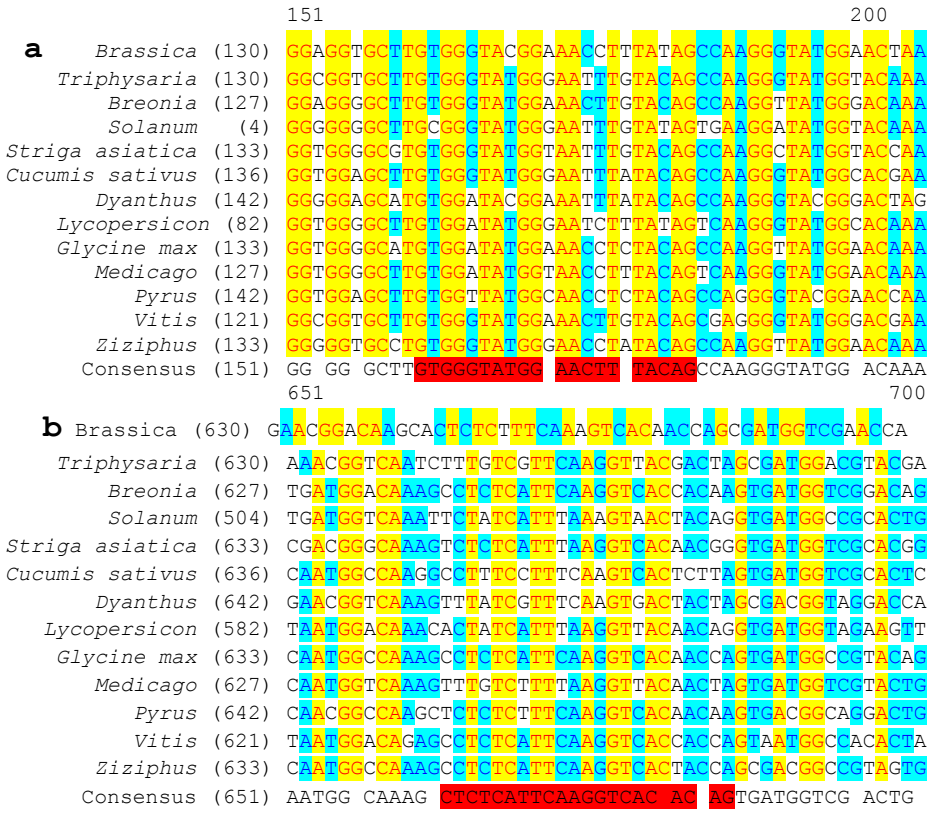

Figure 1: *Expansin* gene aligned sequences for primer design.
(a) Conserved region shaded with red was used for forward primer, while (b) was used for reverse primer design.

3.2.3.2 *Cysteine protease* gene

The *cysteine protease* gene sequence for *Cuscuta reflexa* (FB701665) was used to perform pairwise analysis with other homologous sequences available in the databases. The species which had 70-100% similarity upon tBlastx analysis were selected and used for multiple alignments using NTI vector software. These species incude, *Actinidia deliciosa* 83% (EF530143), *Ipomea batatus* 84% (AF138265), *Lycopersicon esculentum* 77% (BT014429), *Solanum lycopersicum 77%* (AK322520), *Glycine max* 79% (NM001254521), *Gossypium hirsutum 80%* (AJ606072), *Ricinus Communis 75%* (XM002512917), *Populus trichocarpa* 78% (EF148275), *Vitis vinifera* 79% (FQ384071)(Fig 2). *Cysteine protease* gene specific primers (forward and reverse) were designed from the concensus sequences indicated by the red colour shading (Fig 2), using Oligocalculator software.

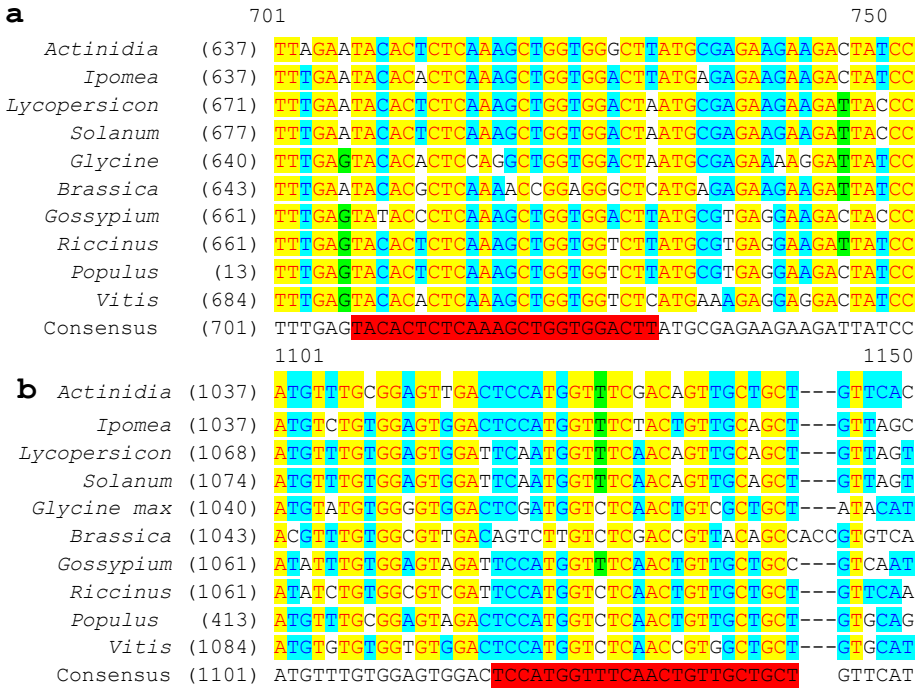

Figure 2: *Cysteine protease* gene aligned sequences for primer design.
(a) Conserved region shaded with red was used for forward primer, while (b) was used for reverse primer design.

3.2.3.3 *Mannose 6-phosphate reductase* gene

The *Orobanche ramosa* (AF055910) NADPH-dependent *Mannose 6-Phosphate reductase* (M6PR) gene sequence was used to perform pairwise analysis with other homologous sequences available in the databases. The species which had 70-100% similarity upon tBlastx analysis were selected and used for multiple alignments using NTI vector software. These species incude, *Glycine max* 85% (NP001240166), *Gossypium hirsutum 84%* (AF063538), *Ricinus Communis 85%* (XP002513006), *Medicago trancutula 87%* (XP003607080), *Zea mays 86%* (ACF83389), *Oryza sativa* 80% (BAD07953), *Prunus persica 84%* (EMJ21725), *Mulus domestica 68%* (AAV54113), *Apium graveolens 73%* (AAB97617)(Fig 3). The *M6PR* gene specific primers (forward and reverse) were designed from the concensus sequences indicated by the red colour shading (Fig 3), using Oligocalculator software.

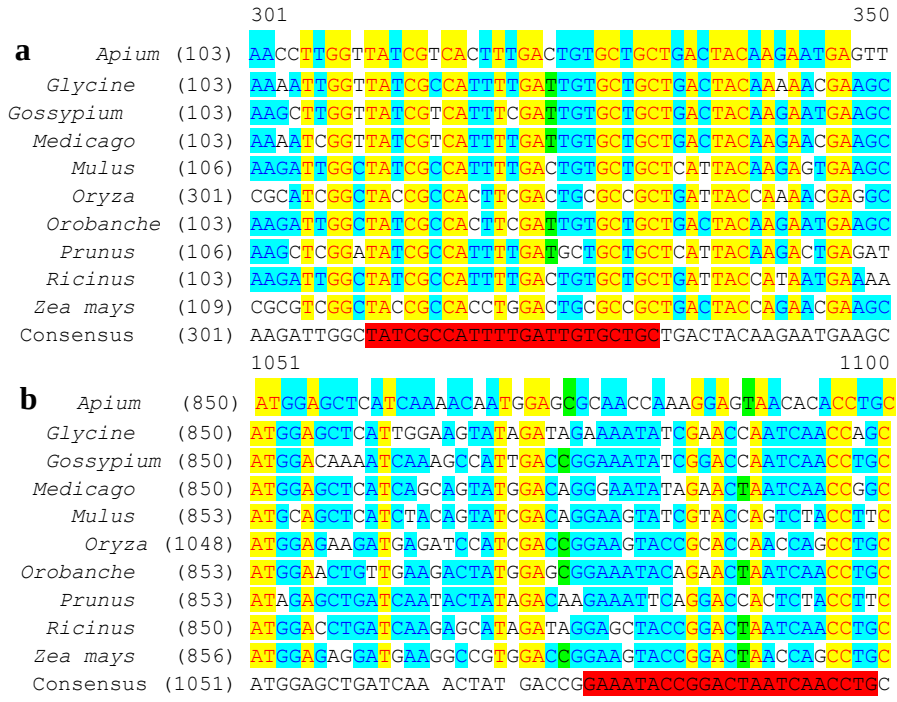

Figure 3: *Mannose 6-phosphate reductase* gene sequences aligned for primer design. (**a**) Conserved region shaded with red was used for forward primer, while (**b**) was used for reverse primer design.

3.2.3.4 *Tvpirin* gene

The *Trphysaria versicor* (JN606867) *Tvpirin* gene was used to perform pairwise analysis with other homologous sequences available in the databases. The species which had 50-100% similarity upon tBlastx analysis were selected and used for multiple alignments using NTI vector software. These species incude, *Glycine max 60%* (XP003552752), *Zea mays 59%* (ACG42629), *Oryza sativa 65%* (ABF99862), *Sorghum bicolor 52%, (XP00246044), Solanum lycopersicum 66% (XP004248000), Carica papaya 55% (AD124922), Populus trichocarpa 63% (XP002320452) and Arabidopsis lyrata 55% (XP002880027)*(Fig 4*).* The *Tvpirin* gene specific primers (forward and reverse) were designed from the concensus sequences indicated by the red colour shading (Fig 4), using Oligocalculator software.

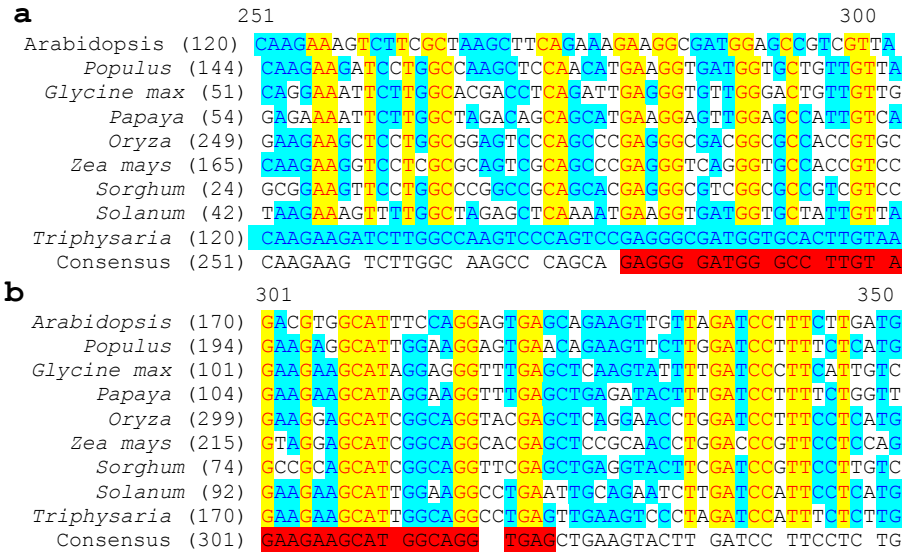

Figure 4: *Tvpirin* gene aligned sequences used for specific primer design.
(a) Conserved region shaded with red was used for forward primer, while (b) was used for reverse primer design.

3.2.3.5 *Quinone oxidoreductase* gene

The *Trphysaria versicor* (AF304461) *Quinone oxidoreductase* gene was used to perform pairwise analysis with other homologous sequences available in the databases. The species which had 70-100% similarity upon tBlastx analysis were selected and used for multiple alignments using NTI vector software. These species include; *Solanum lycopersicum* 71% (Ak325193), *Zea mays 64%* (EU959263), *Gosspium barbadense 64%* (AY429443), *Ricinus communis 69% (*XP002535145*) and Medicago trancatula* (Alfafa) 69% (XM003605634)(Fig 5). *Quinone oxidoreductase* gene specific primers (forward and reverse) were designed from the concensus sequences indicated by the red colour shading (Fig 5), using Oligocalculator software.

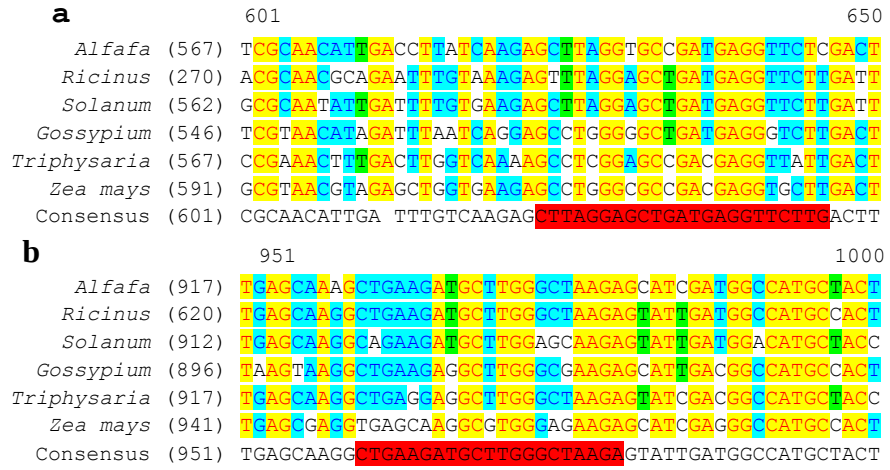

Figure 5: *Quinone oxidoreductase* gene aligned sequences used for specific primer. (a) Conserved region shaded with red was used for forward primer, while (b) was used for reverse primer design.

Table 1: Primers Sequences used in cloning of *S. hermonthica* haustoria genes

Primer ID	Putative Genes	Primer sequence	Annealing temperature	Expected fragment size
EXP F	*Expansin*	GTGGGTATGGTAACTTGTACAG	47.9˚C	527
EXP R	*Expansin*	CTAGTAGTGACCTTGAATGAGAG	46.8 ˚C	527
TVPF1	*Tvpirin*	GAGGGTGATGGTGCCATTGTTA	58.1˚C	444
TVPF2	*Tvpirin*	GAAGCATTGGCAGGTCTGAGA	56.5 ˚C	420
TVP R	*Tvpirin*	CAAGTACATGGTTGGTGTTCG	52.6˚C	444
CYS F	*Cysteine proteinase*	TACACTCTCAAAGCTGGTGGACTT	55.9 ˚C	400
CYS R	*Cysteine proteinase*	AGCAGCAACAGTTGAAACCATGGA	61.5˚C	400
M6PRR	*Mannose6-phosphate reductase*	TATCGCCATTTGATTGTGCTGC	60.6 ˚C	766
M6PRR	*Mannose6-phosphate reductase*	CAGGTTGATTAGTCCGG TATTTC	53.4 ˚C	766
QOR F	*Quinone oxidoreductase*	CTTAGGAGCTGATGAGGTTCTT	51.1˚C	356
QOR R	*Quinone oxidoreductase*	TCTTAGCCCAAGCATCTTCAG	53.6˚C	356

3.2.4 Amplification of cDNA using gene specific primers

The targeted genes were amplified in a 20µl reaction volume using 2µl of the synthesized cDNA (3.2.2) from both *S. hermonthica* and maize tissues. The Taq DNA polymerase and Taq DNA polymerase high fidelity enzymes were used in a ratio of 9:1, respectively (1µl), in addition to 10µl of the (2×) ready mix buffer containing mg^{2+} and 1µl of forward and reverse primer (0.5mM). The thermocycler was set at 94˚C denaturation temperature, 55˚C annealing temperature for the *Cystein protease*, *Tvpirin* and *mannose 6-phosphate reductase* genes, while the annealing temperature for the *Expansin* gene and *Quinone oxidoreductase* genes was set at 48˚C and 72˚C for 1 minutes as the extension temperature for 40 cycles. Gel electrophoresis was run using 1% agarose gel for 30 minutes at 100 volts, separately for each gene, and photographed under Ultraviolet light.

3.2.5 Isolation and purification of the putative gene fragments for cloning

The gel electrophoresis images in section 3.2.4 were used to determine specific fragments to cut for cloning. The gene fragments from *S. hermonthica* and maize cDNA were cut and purified using QIAquick® Gel extraction kit mannual (Qiagen. CAT no.28704, Valencia), followed by reamplification using gene specific primers. The reamplified fragments were then purified using QIAquick®PCR purification kit manual (Qiagen, CAT no.28104, Valencia), transformed into *E. coli* cells and used for cloning (section 3.2.5.2) into pCR®8/GW/TOPO® TA Gateway cloning vector (Invitrogen, CAT. K250020, Carlsbad, U.S.A) (Fig6).

3.2.5.1 Preparation of chemically competent *E. coli* cells

The DH5α *E. coli* cells were grown on plates with solid LB media without any antibiotic for two days in a 37˚C incubator. A colony was then picked using sterile loop and inoculated into 10 ml liquid LB media without antibiotic. The culture was incubated overnight at 37˚C with shaking at 200 RPM. On the following day, 1ml of the overnight grown cells was picked, inoculated in a fresh 10 ml liquid LB media and grown for 2 hours in an incubator at 37˚C with shaking at 200 RPM until they attained an optical density of 0.4-0.5. The cells were spinned at 12000 RPM for 2 minutes at 4˚C and chilled on ice for 1 hour. The cells were then resuspended in 1ml ice cold 0.1M $CaCl_2$ and incubated on ice for 1 hour before spinning at 12000 RPM for 2 minutes at 4˚C and resuspended again in 200 µl of ice cold 0.1M $CaCl_2$. The competent cells were stored at 4˚C.

3.2.5.2 Cloning of the purified PCR product into PCR® 8/GW/TOPO® TA vector

The PCR purified fragments of each of the five genes were cloned into PCR®8/GW/TOPO® TA gateway cloning vector (Fig 6), by adding 0.5 µl of buffer solutions and 0.5 µl of the vector into 2 µl of the PCR product. The cloning reaction was mixed gently by flicking the tubes and then incubated at room temperature for 30 minutes, before storing at -20°C to await transformation into competent *E. coli* cells. The PCR®8/GW/TOPO® TA recombinant vectors containing fragments from the putative gene were used to transform the chemically competent *E.coli* cells prepared in section 3.2.5.1. One µl of the recombinant vector was pipetted into 0.5 ml eppendoff tube and thawed on ice before adding 50 µL of the *E.coli* DH5α competent cells, and mixed quickly by flicking the tube. The mixture was incubated on ice for 40 minutes and heatshocked at 42°C for 40 seconds in a water bath, and then incubated back on ice for 2 minutes. SOC media was added (900 µl) and incubated for 1 hour in a shaker (200RPM) at 37°C. The 50 µl of the transformed cells was spread on LB solid media with spectinomycin100mg/ml and grown overnight.

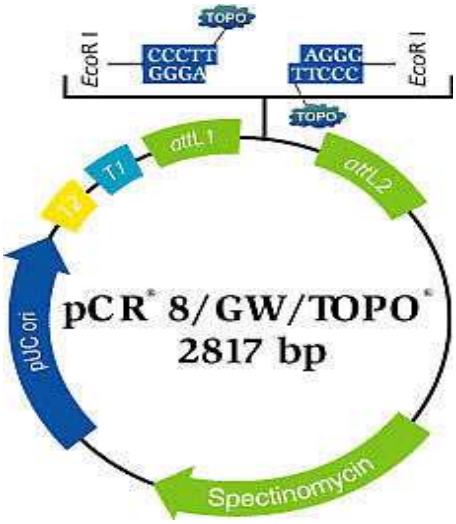

Figure 6: The PCR®8/GW/TOPO® TA cloning vector map, (Invitrogen, CAT. K250020, Carlsbad, U.S.A).

3.2.5.3 Plasmid extraction of the PCR®/GW/TOPO®TA recombinant vectors

Four colonies of each cloned gene fragment were selected and grown overnight in 10ml broth LB media at 37°C incubator with shaking at 200 RPM, aliquoted in Eppindorf tubes (6 ml) and spinned at 10000 revolutions per minute for 15 minutes. The cells were resuspended in 300 µl of buffer P1 (Tris –EDTA and glucose in addition to RNAse A) as per the instructions of Qiaprep spin miniprep kit (Qiagen, CAT. 27104, Valencia). The same volume was added for the lysis buffer P2, shaken vigorously and incubated at room temperature for 5 minutes. Neutralizing buffer N3 was then added and mixed immediately by inverting the tubes 4-6 times, and spinned for 15 minutes at 13000 RPM in a table top centrifuge.

The supernatant was applied to the Qiaprep spin column by pipetting and centrifuging for 1 minute at 13000 RPM before discarding the flow through. Half ml of the binding buffer PB was added and spinned for 1 minute and again the the flow-through was discarded. The wash buffer (PE) 0.75 ml, was then added onto the columns and spinned for 1 minute. The flow through was discarded and the columns were spinned again for an additional 1-minute to remove the residue wash buffer. The QIAprep spin columns were placed in clean eppindorf tubes and 50 µl of elution buffer added directly to the membrane and let to stand for 1 minute before spinning at 10000 RPM for 1 minute. The plasmid was then stored at -20°C to restriction digestion with *EcoR1*.

3.2.5.4 Confirmation of cloned gene fragments by restriction digestion

Identification of Topo clones with the gene fragments was done using 0.25 µl of *EcoR*1 restriction enzyme; in a 30 µl total reaction volume containing 3 µl of buffer, 5 µl of DNA and 22 µl of water. The mixture was incubated for one hour at 37°C and gel electrophoresis was done after one hour of incubation and the positive clones were sent for sequencing at the Intenational Livestock Research Institute (ILRI). The Sanger sequencing method was done using M13 forward primers that flank the cloning region of the PCR®/GW/TOPO®TA vector. Finally, cleaning of the sequences was done by editing off the PCR®/GW/TOPO®TA vector sequences both at the right and left border. The *S. hermonthica* sequences for each of the cloned gene were among themselves separately aligned to check for any similarity. Homology search was done for the resulting sequences

using tblastx aligorithm at NCBI, and the sequences of the species with more than 70% were selected for multiple alignment and phylogenetic analysis using Mega 6 software.

3.3 Developing VIGS protocol for *S. hermonthica* management

3.3.1 Vigs plasmids

Tobacco rattle virus (TRV)-derived vectors with the PDS insert and transformed into the agrobacterium were provided by Prof. Dinesh Kumar of the University of Califonia-Davis (Fig 7). The TRV contains bipartite positive-sense RNA genome (RNA1 and RNA2). The provided TRV1 vector that respresented RNA1 which encodes two viral replication proteins, a movement protein and a seed transmission factor. The provided TRV2 represented the RNA2 and encodes the coat protein and a nematode transmission factor (Verchot-Lubicz, 2002). The TRV2 contained Tomato phytoene desaturase (PDS) RNAi cassette (Fig 7a) while TRV1 (Fig 7b) did not contain the RNAi cassette. The two binary vectors were separately contained in the *Agrobacterium tumefaciens* strain GV3101 which was the delivery vector for viral vectors into plants through agro-inoculation. The *Agrobacterium* colonies carrying TRV1 and TRV2 vector with the PDS RNAi cassette were separately grown in LB liquid media containing Kanamycin 50 mg/l and rifampicin 1 mg/l overnight in a shaker set at 28°C. From the overnight culture, 1 ml was picked from each tube and again subcultured separately for 2 hours in 10 ml LB media containing Kanamycin 50 mg/l and rifampicin 1 mg/l and 150 µM of acetosyringone to induce the virulence genes.

The separate 10 ml liquid cultures were grown in darkness at 28°C with shaking at (200RPM) until they attained an optical density (OD) of 0.6. The two cultures were then spinned for 15 minutes at 10000 revolutions per minute (RPM) and resuspended in an induction buffer containing 150 µM acetosyringone, 10 mM of MES and 10 mM of $MgCl_2$ adjusted to PH 5.6 and grown again for 2 hours. *Agrobacterium* strain GV3101 containing TRV1 and TRV2 were then mixed in a 1:1 ratio and used in the agro-innoculation experiments. Agrodrench and agroinfiltration were the two agroinoculation techniques used.

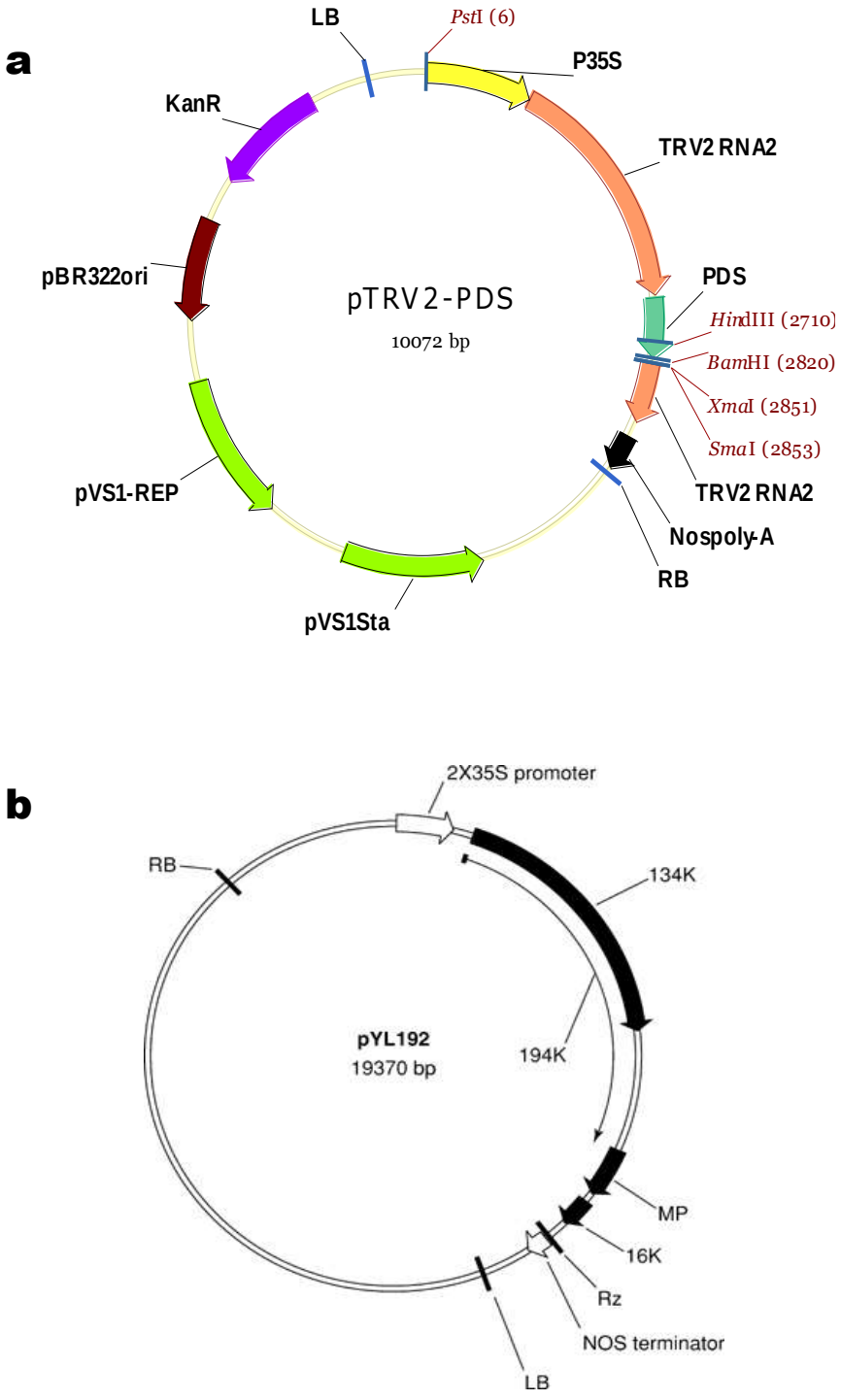

Figure 7: TRV vectors. (a) TRV2 vector map containing PDS gene fragment, (b) TRV1 vector map (empty). Using the TRV vectors details provided in the ABRC database (http://abrc.osu.edu), the maps were constructed using Vector NTI software, version 11.5.2.

3.3.2 Agrodrench

Agrodrench involved applying the mixture of GV3101/TRV2-PDS and GV3101/TRV1 (1:1) ratio directly onto the soil adjacent to the crown part of 3-4 week old *S. hermonthica* plants as described by Choong *et al*. (2004). The experiments involved six plants and was repeated three times. The negative controls were six plants treated with GV3101/TRV1only, six plants treated with GV3101 only and six plants treated with the induction media only. All the negative controls were replicated thrice as well. Pictures were taken after every seven days and the number of plants showing photobleaching effects were recorded.

3.3.3 Agroinfiltration

For leaf infiltration, tobacco (*N. tabacum*) was used as the positive control since VIGS for the PDS gene has succeded in tobacco (Choong *et al.,* 2004). Two to three fully open leaves in 4 weeks old plants were pricked on the lower side with a wire brush. Using a needleless syringe and applying little pressure, the leaves were infiltrated with GV3101/TRV2 PDS and GV3101/TRV1 mixture gently while pressing on the upper part of the leaf, until fully wet. Eight plants were infiltrated and the experiment was repeated three times. In *S. hermonthica*, all the young leaves on the upper part of the plant were infiltrated by pricking the lower side of the leaves with a wire brush. Using cotton wool the GV3101/TRV2-PDS and GV3101/TRV1 mixture was gently applied on the pricked leaves until they became fully wet. Eight *S. hermonthica* plants were used and the experiment was repeated three times. The control experiments for tobacco and *S. hemonthica* were treated with GV3101 empty, GV3101/TRV1 and plants uninfiltated with the water only. In tobacco, photos were taken after every two days and the number of photobleached plants recorded while in *S. hermonthica* the photos and recording was done after every seven.

3.3.4 Screening for silencing of *Phytoene desaturase* gene through RT-PCR

Leaf tissues of 3 plants of each treatment were collected and ground in liquid nitrogen using a motar and pestle. Approximately 20 mg of ground tissues was used for total RNA extraction as per the instructions of the RNeasy® mini kit (Qiagen, Cat no 74104, Valencia) as described in section 3.2.1. The total RNA was subjected to DNAse treatment and incubated at 37˚C for 15 minutes. The total RNA extracted was then reverse transcribed to

cDNA synthesis using Superscript^{TM} III first stand synthesis sytem (Invitrogen, CAT 18080-051, Carlsbad, U.S.A) as described in section 3.2.2. The first strand cDNA synthesis reactions were primed using random heximers. The synthesized cDNA was then amplified using PDS primers and TRV primers. The PDS primers were (Forward primer 5´-GAGAAACATGGTTCAAAAATGG-3´and reverse primer 5´-AACACAAAAGCATCTCCCTC-3´). The PDS primers were designed to prime outside the region of homology between the VIGS vector and the target mRNA. The TRV primers were (Forward 5'-ACTCACGGGCTAACAGTGCT-3' and reverse primer 5'-GACGTATCGGACCTCCACTC-3'. The thermocycler conditions were set at 94˚C for denaturation, 55˚C annealing and 72˚C Extension for 40 cycles. The 20 µl of PCR product was mixed with laoding dye and sybergreen and loaded in 1% of agarose gel before running the electrophoresis at 100 volts for 30 minutes. The gel was viewed under UV light and the photos were then taken.

CHAPTER FOUR

4.0 RESULTS

4.1 *Striga hermonthica* haustorium development in susceptible (*Namba nane*) and tolerant (*KSTP'94*) open pollinated varieties of maize

4.1.1Effects of maize genotypes on *S. hermonthica* germination

The two maize cultivars revealed striking differences in their ability to support growth and development of *Striga hermonthica*. There was emergence of *S. hermonthica* from the infected pots planted with susceptible variety (*Namba nane)* as well as the tolerant variety (*KSTP'94*). In both cases, maize that emerged from pots that were infested with *S. hermonthica* appeared stunted, chlorotic and in some cases death was observed (Fig 8a and 8b). The *S. hermonthica* haustoria appeared hemispherical in shape (Fig 8d) in the both varieties. Further, the roots infested by *S. hermonthica* in the *Namba nane* variety developed intense hairly roots (Fig 9a) compared to uninfected roots of the same variety (Fig 9b). Additionally, accumulation of a viscid substance at the parasite host interface was observed under a light microscope (Fig 9c). *Striga hermonthica* infected roots in the *KSTP'94* variety also had more intense development of hairly roots (Fig 10a) compared to uninfected roots (Fig 10b). However, little or no accumulation of the viscid substance was observed in the case of *KSTP'94* variety (Fig 10c).

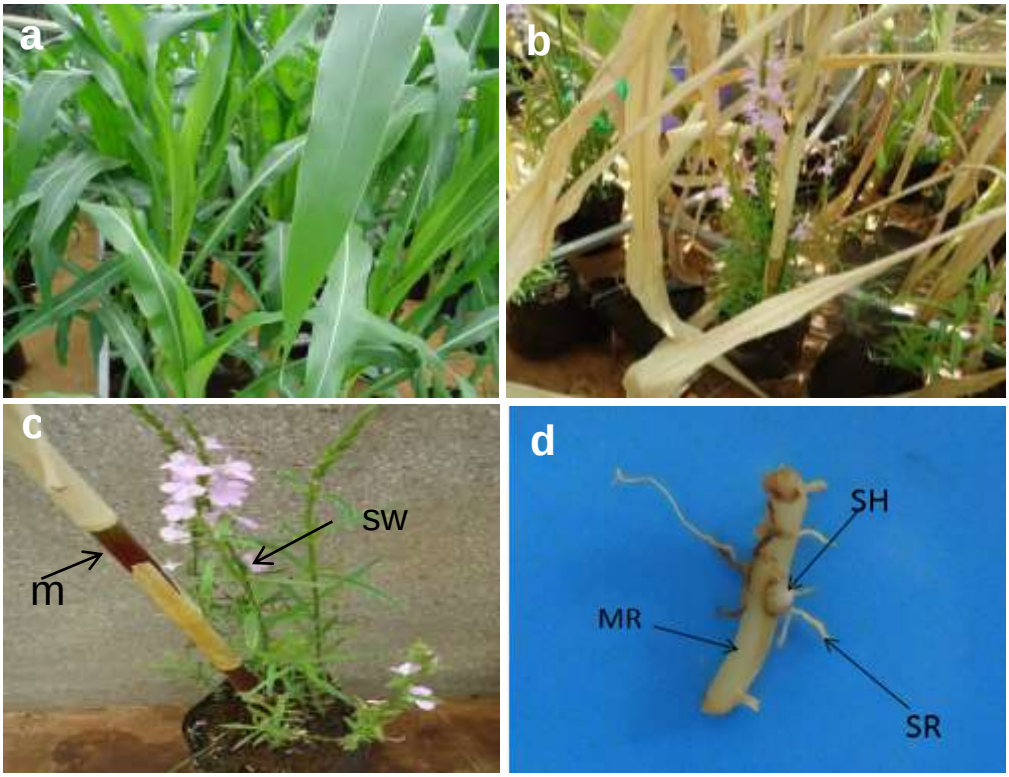

Figure 8: Comparison of *S. hermonthica* uninfected maize and infected maize at 63 days after planting. (a) Uninfected healthy maize, (b) *S. hermonthica* infected maize, (c) *S. hermonthica* (sw) infected maize (m), (d) *S. hermonthica* root (sr) with haustoria (sh) attached to maize root (mr).

Figure 9: Comperative appearance of *Namba nane* roots infected with *S. hermonthica* to non-infected roots. (a) Namba nane infected roots (b) Namba nane roots not infected(c) S. hermonthica haustoria infecting Namba nane variety maize root (The arrows show a reddish substance secreted at the host and parasite interface).

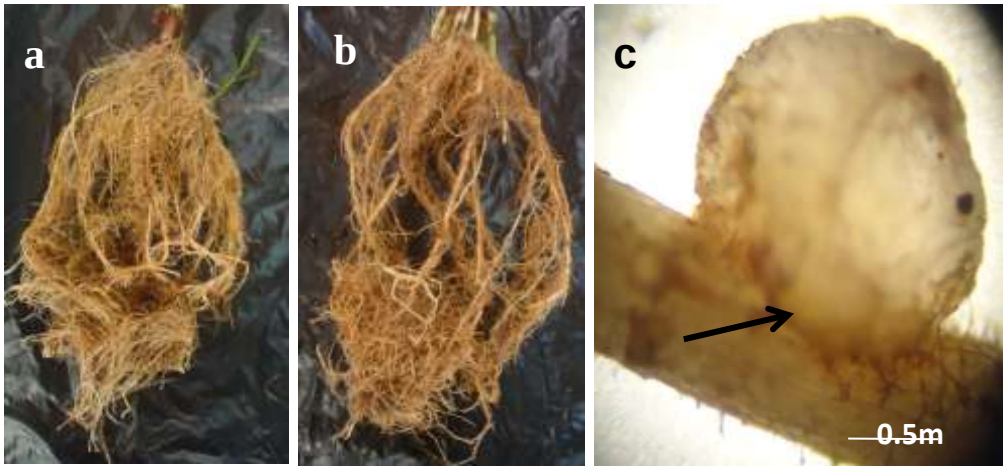

Figure 10: Comperative appearance of *KSTP´94* roots infected with *S. hermonthica* to non-infected roots. (a) KSPT´94 infected roots (b) KSPT´94 roots not infected(c) S. hermonthica haustoria infecting KSPT´94 variety maize root (The arrows show a reddish substance secreted at the host and parasite interface).

The rates of *S. hermonthica* emergence on the two infected maize varieties were varied. In the susceptible variety (*Namba nane*), *S. hermonthica* emerged from the soil 50days after planting (Fig 11). In the tolerant (*KSTP'94*) variety, the parasite took 90 days to emerge from the soil (Fig 11). The number of *S. hermonthica* plants that infected maize plants was also varied between the varieties. The susceptible variety (Namba nane) had an average of 26±0.6 Striga plants/maizeplants at 90 days after planting, compared to the tolerant variety (*KSTP'94*) which only had an average of 5±0.5 Striga plants at 105 days after planting (Fig 11). The percentage *S. hermonthica* emergency also varied between varieties. In *KSTP'94* for instance, only 10.7±0.9% of the infected maize had *S. hermonthica* growing compared to 50.3±0.9% *Namba nane* variety maize (Fig 12). On the other hand, the number of *S. hermonthica* haustorium attached to the roots of *KSTP'94* variety roots were observed to be lower 18±2.6 in comparison to *Namba nane* variety 42±2.3 at 112 days after planting (Fig 13).

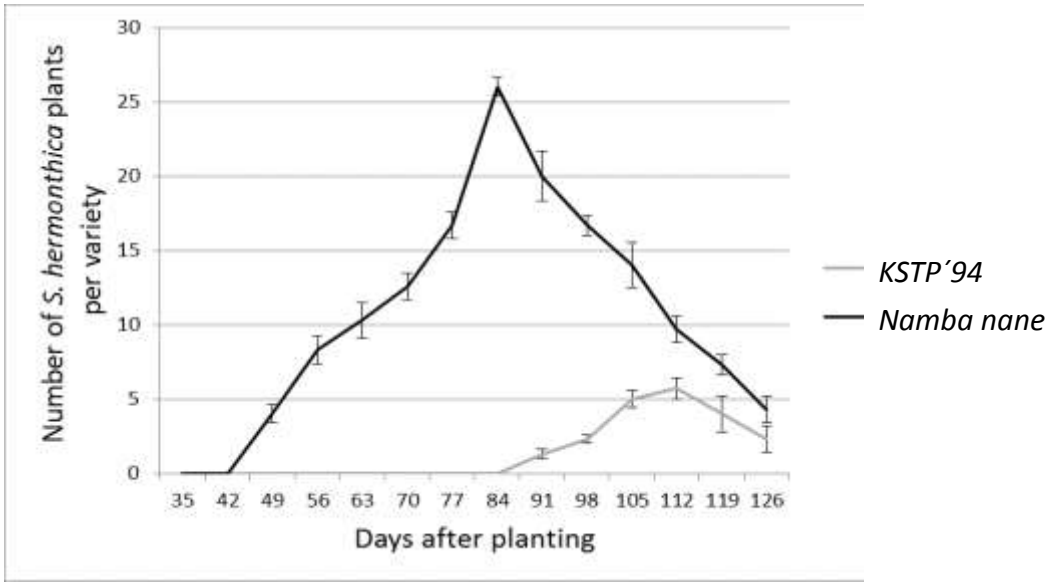

Figure 11: Average *S. hermonthica* infestation of the two maize varieties up to 126 day's period after planting.

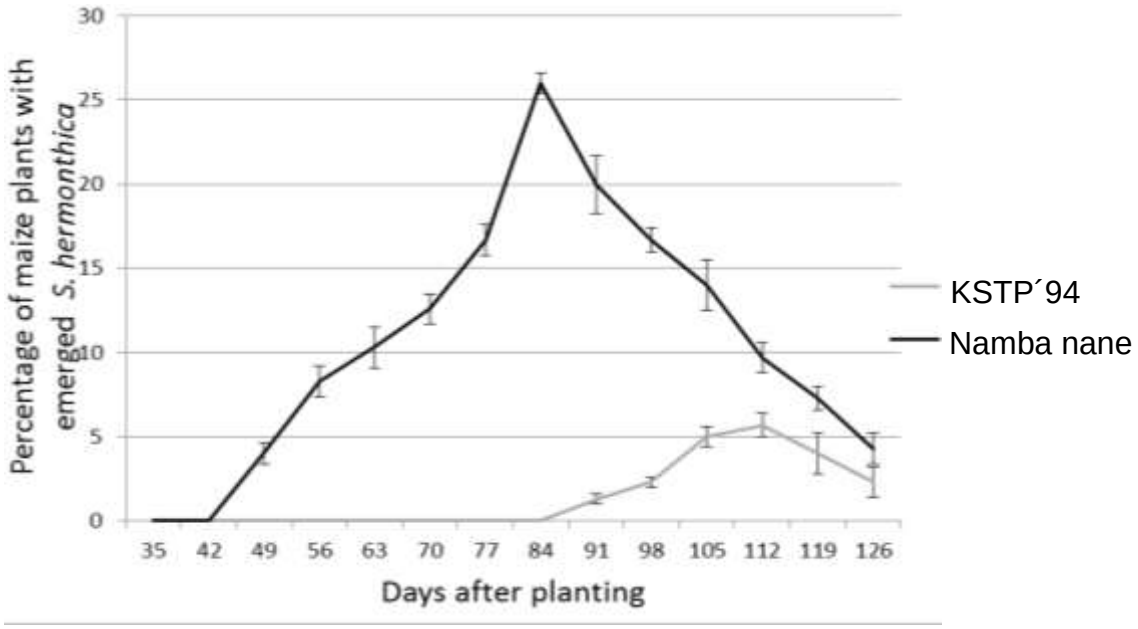

Figure 12: Percentage of N*amba nane* and *KSTP' 94* maize varieties with emerged *S. hermonthica*.

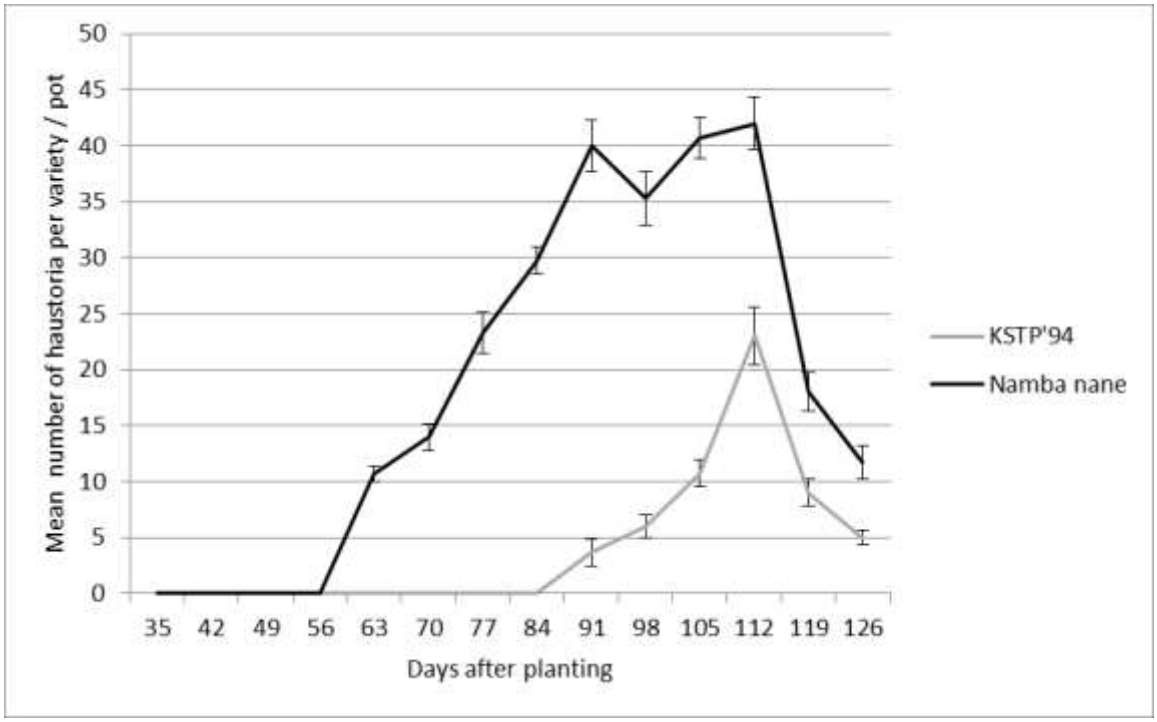

Figure 13: Number of *S. hermonthica* haustoria attached to *Namba nane* and *KSTP'94* maize varieties up to 126 days after planting

4.1.2 Histological analysis of *S. hermonthica* haustorium development in *KSTP'94* and *Namba nane* maize varieties

Histological examination of *S. hermonthica* haustoria attached to both *KSTP'94* and *Namba nane* varieties indicated that the early stages of parasite development were similar. There was evidence of high cell division of the haustoria at the connection point with the host in both *Namba nane* (Fig 14a) and *KSTP'94* (Fig 14b) varieties. The haustorium intrusive cells appeared to push between the host epidermal cells to the cortex (hc) in *Namba nane* (Fig 14c) and in *KSTP'94* (Fig 14d). At the cortex, subsequent growth was supported by continued cell division and elongation of cells at the apex of the haustorium intrusive cells. In the *Namba nane* variety, 53±1.7 % of the sections indicated that haustorium intrusive cells pushed through the cortex (hc) with ease and through the endodermis to the stele (Fig 14e). *Striga hermonthica* haustorium was observed to encounter some resistance at the host cortex and endodermis (he) in the *KSTP'94* variety in 20±1.5% of the sections, as evidenced by the branching of the haustorium tip forming searching hyphae (sh) that spread along the endodermal layer (Fig 14f). At the stele,

30±1.3% of the sections indicated that the haustorium intrusive cells in the *Namba nane* variety formed few contact hyphae that made connections to the host xylem (hx) (Fig 14g). Subsequently, the haustoria cells were observed to differentiate and form continuous bridge (px) with the host xylem in the *Namba nane* variety (Fig 14i). At this stage the haustoria tissues were fully developed and differentiated to have the xylem and the vascular core (vc) encircled by the hyaline body (hb). In the *KSTP'94* variety only in few cases of the histologically examined tissues had evidence of connections to the vascular elements (Fig 14j).

In the *KSTP'94* variety, the intrusive cells inside the stele were diminished (Fig 14j). There was accumulation of unidentified substance at the parasite host interface in the two varieties, which stained darkly. This was clearly evidenced in the tissues stained with the methylene blue dye (Fig 15). Methylene blue is known to stain the phosphate groups of nucleus, sulphate groups of glycosaminoglycans (found at the cellular surfaces and in between the cells) and the carboxyl groups of proteins. On the other hand, Ruthenium red stains the mucopolysaccharrides, pectins, intracellular lipids and a large group of proteins. The haustoria penetration resistance at the cortex in the *KSTP'94* variety was clearly shown in the sections stained with methylene blue dye, forming searching hyphae (sh) that spread independently in the host cortex (Fig 15d).

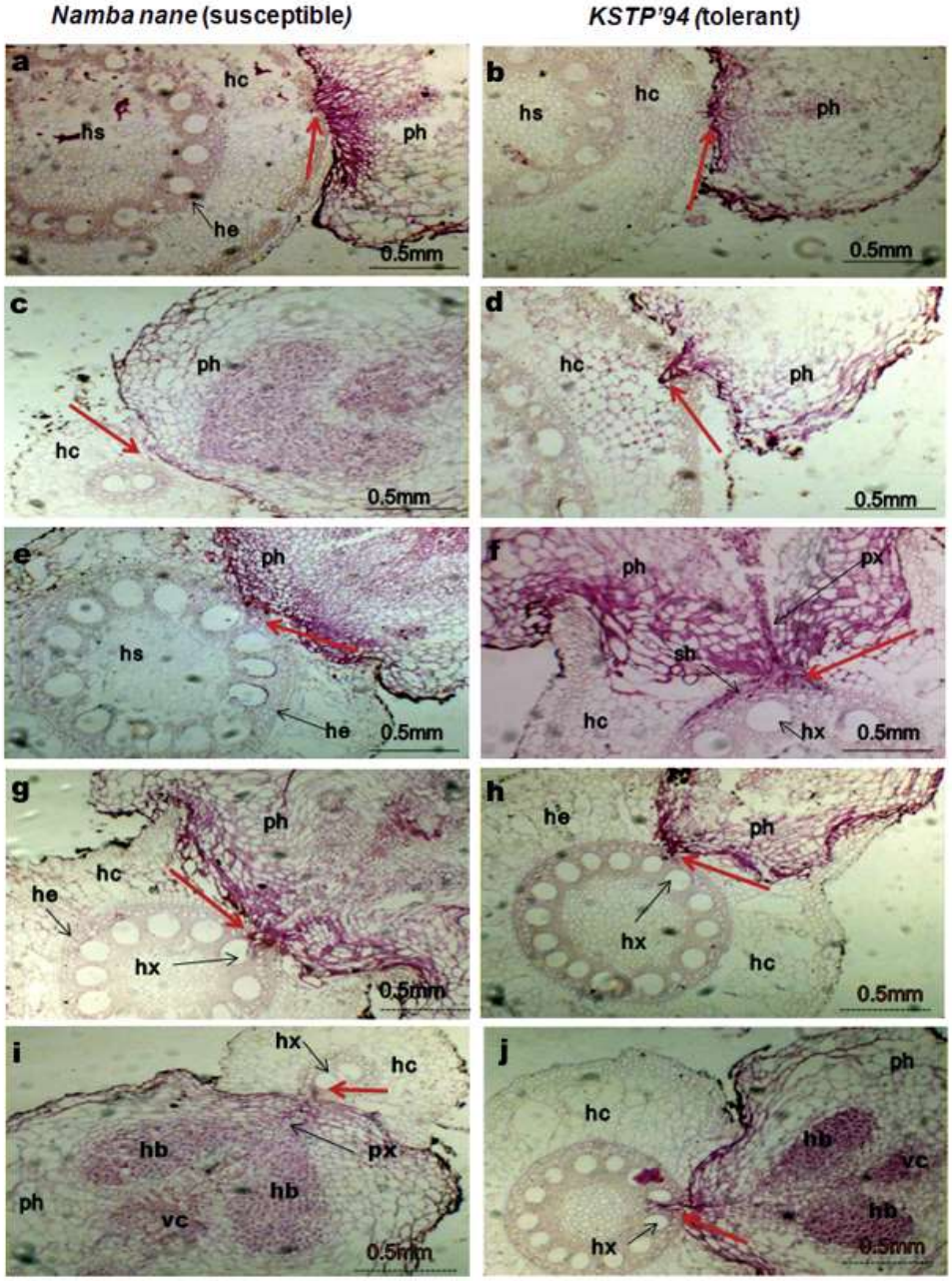

Figure 14: Histological characterization of haustoria development in Namba nane and KSTP'94 maize varieties (Sections stained using Ruthenium red dye). a) *S. hermonthica* haustoria (ph) connecting to Namba nane maize variety, **b)** *S. hermonthica* haustoria (ph) connecting to *KSTP'94* maize roots, **c)** *S. hermonthica* haustoria (ph) penetration into Namba nane variety (host) cortex (hc), **d)** *S. hermonthica* haustoria (ph) pushing through into *KSTP'94* variety (host) cortex (hc), **e)** *S. hermonthica* haustoria (ph) penetrating the Namba nane variety endodermis (he) layer, **f)** S. *hermonthica* haustoria (ph) along the *KSTP'94* variety endodermis (he). **g)** *S. hermonthica* (ph) through the *Namba nane* endodermis (he) to the stele, **h)** *S. hermonthica* haustoria (ph) facing resistance at the *KSTP'94* endodermis (he) **i)** *S. hermonthica* haustoria (ph) making connections to *Namba nane* host xylem (hx) and the haustoria differentiating to form host-parasite xylem connections (px), **j)** Parasite haustoria (ph) attaching to the *KSTP'94* host xylem cell walls (hx) The red arrows indicates the stage of attachment.

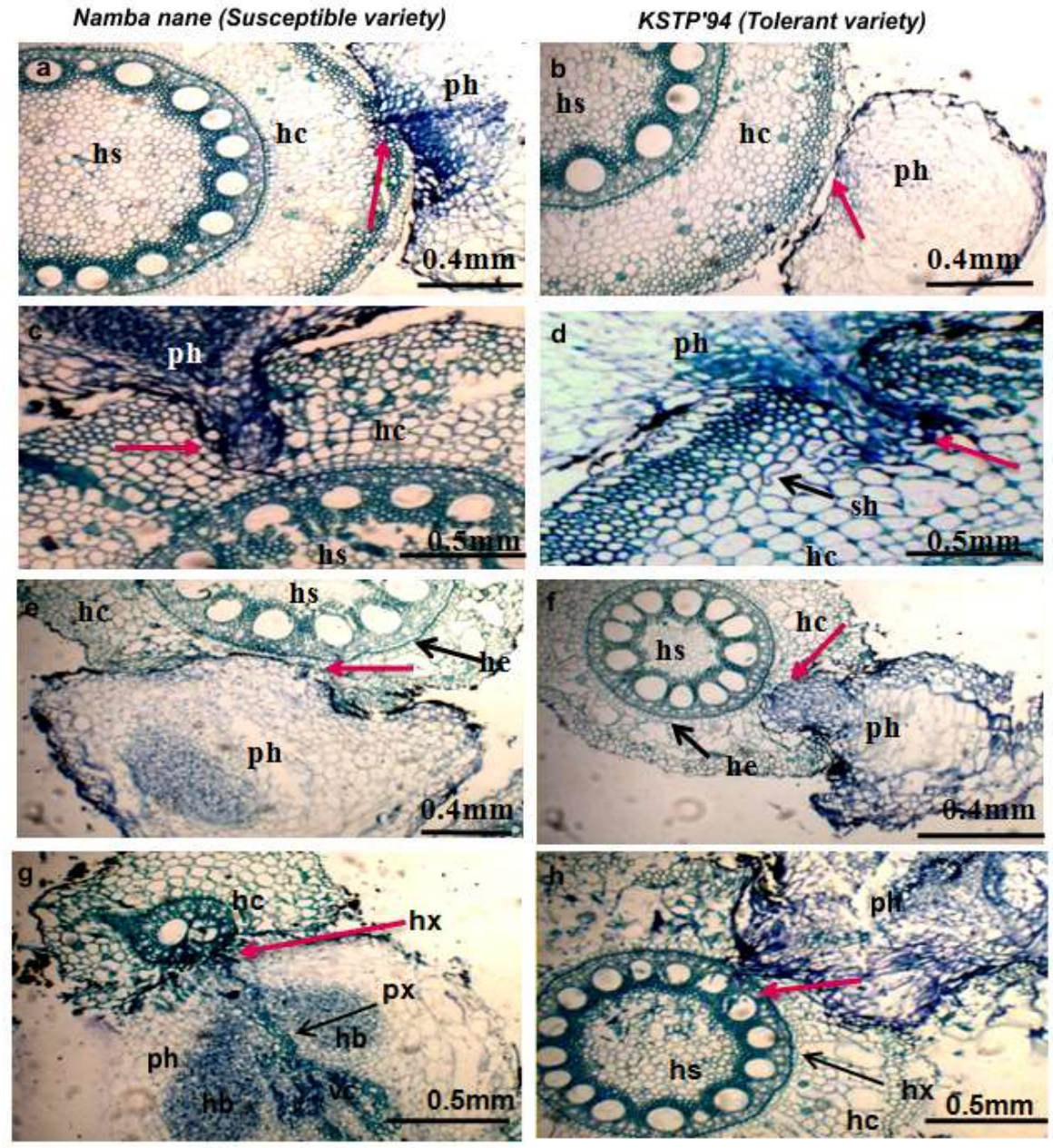

Figure 15: Histological characterization of haustoria development in KSTP'94 and Namba nane maize varieties (Sections stained using Methylene blue dye)

a) Haustoria connection with the *namba nane* variety, **b)** Haustoria connection with the *KSTP'94* variety, **c**: Haustoria penetration into the *Namba nane* cortex (hc), **d**) Haustoria penetration into the *KSTP'94* cortex (hc) and forming searching hyphaes (sh). **e)** Haustoria getting into contact with the endodermis (he) in *Namba nane* variety, **f**) Haustoria getting into contact with the host endodermis in KSTP'94 variety, **g**) Haustoria making connections with the host vascular elements and forming vessels (px) that form bridges with the host in Namba nane variety, hyaline body (hb) and the vascular core (vc), **h**) Haustoria penetrating into the host stele in the *KSTP'94* variety. The red arrows show the stage of haustoria attachment.

4.2 Cloning of putative genes involved in *S. hermonthica* haustoria formation

4.2.1 *Expansin*

Amplification of the synthesized cDNA from *S. hermonthica* and maize tissues using *Expansin* specific primers revealed expression of the gene in both tissues (Fig 16a). However, the expression of the gene in *S. hermonthica* tissues (haustoria, roots and leaves) had a different profile from that of maize roots revealing three fragments of sizes 700bp, 400bp and 300bp (SH1, SH2 and SH3) (Fig16a) that were used for cloning. The quality of the cDNA was confirmed as indicated by amplification with primers for the Actin a house keeping gene which revealed a fragment of 400bp (Fig 16b). The three fragments from *S. hermonthica* tissues were excised from the gel and purified using the gel purification kit (Qiagen.CAT no.28704, Valencia) (Fig16c). In the maize roots, five fragments were identified but only two unique ones (MR1-400bp and MR2-300bp) were cloned (Fig 12C). Cloning of the three fragments from *S. hermonthica* and two from maize roots was successful as confirmed by restriction digestion using EcoR1 (Fig 16d).

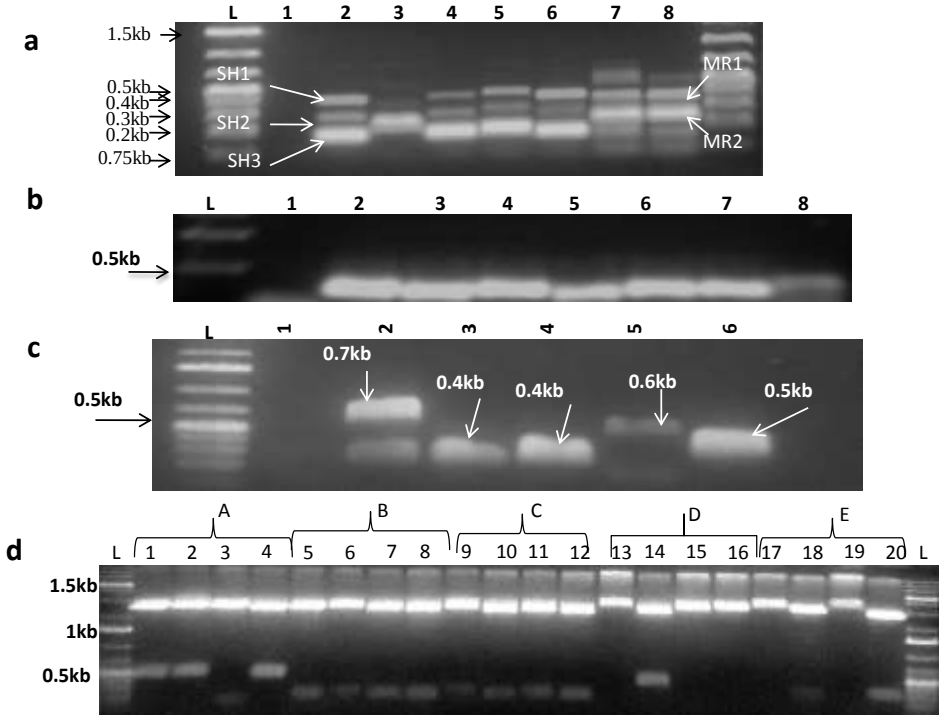

Figure 16: Amplification, purification and cloning of *Expansin* gene
(a) Amplification of cDNA using *Expansin* gene specific primers, Lanes 1 is the negative control, 2 and 3 is cDNA from *S. hermonthica* haustoria, 4 and 5 is the cDNA from *S. hermonthica* roots, 6 and 7 is the cDNA from *S.hermonthica* leaves and 8 and 9 is the cDNA from maize roots. (b) The internal control with Actin primers. (c) Gel purified fragments for cloning into topo TA vector; lane L is 1kb plus gene ruler ladder,1 is negative control, 2 purified SH1 fragment, 3 and 4 are SH2 and SH3 respectively,and 5 and 6 are MR1 and MR2 respectively. (d) Screening for positive clones through restriction digestion with *EcoR1* restriction enzyme. Lane L is 1kb plus gene ruler ladder, A, is SH1 fragment. B, is SH2 fragment, C is SH3 fragment, D is MR1 fragment and E is MR2 fragment.

A total of nine positive clones from *S. hermonthica* and two from maize were sequenced (Fig17). The two fragments from maize were choosen because they were prominent and besides, maize expansin had been cloned. Multiple alignments of the 9 sequenced clones from *S. hermonthica* haustoria revealed four *Expansin* genes (Fig 17). The *S. hermonthica Expansin* 1 was unique upon pairwise comparison and was named (*Striga Expansin 1*). *Expansin* 2 and 3 were similar and were named (*Striga Expansin 2*), while *Expansin* 4, 6, 7 and 9 were similar and were named (*Striga Expansin 3*), Expansin 5 and 8 were similar and was named (*Striga Expansin 4*). A total of four *Expansin* genes were identified in *S. hermonthica* and none of the four sequences was identical to the *S. asiatica Expansin* gene (DQ442401) available at the NCBI data base (Fig 17).

The four *S. hermonthica* expansin genes identified were compared with the other known *Expansin* sequences at the NCBI database using tblastx pairwise analysis aligorithm. The analysis revealed sequences with greater than 70% similarity. The Striga *Expansin*1 and 2 had 80-94% gene homology with various species, including; *Pyrus communis* 86% (AB093032), *Triphysaria versicolor* 85% (AF32409), *S. asiatica* 94% (AF 291658), *Prunus avium* 86% (AF 297522), *Eriobotrya japonica* 86% (AB0096604), *Ricinus communis 8*1% (XM002523388), *Solanum domissum* 84% (AAU90318). *Dimocarpus longan* 87% (EU416314), *Vitis vinifera* 80% (XP002284858), *populus trichocarpa 86*% (XP002312253), *Glycine max* 85% (XP003529476), *Gossypium hirsutum* 85% (AAM47002) and *Breonia chinensis* 85% AEQ5528 among others. The *S. hermonthica* expansins 3 and 4 showed similarity with other plants spp peroxidase superfamily genes such as; *Nicotiana tabacum* 85% (AAD33072), *Nelumbo nucifera* 85% (ABN46984), *Camellia oleifera* 86% (ACT21094), *Ipomea batatsas* 82% (AFY26877), *Hevea brasiliensis* 83% (AEK87128) and *Avicennia marina* 86%(BAB16317) among others.

The phylogenetic analysis of the *Expansin* gene in *S. hermonthica* was conducted and the results indicated that the gene shares the most recent ancestor with other parasitic weeds such as *S. asiatica* and *Triphysaria versicolor* (Fig 18). *Striga hermonthica Expansin*1 gene is in the same clade with that of *S. asiatica*, and further with *T. versicolor*, with the same branching pattern indicating the same history of common ancestry, *Striga hermonthica Expansin* 3 and 4 are in the same clade with the sequenced maize *Expansin* 2 as indicated by a common node and share a past common ancestor with *S. hermonthica Expansin* 1. However, the *S. hermonthica Expansin 2* gene share a recent ancestor with peroxidases of various plant *spp* clustering at the base of the tree and acts as a basis for the analysis that produced the tree. *Striga hermonthica Expansin* genes seems have evolved very fast as evidenced by long branch lengths indicating higher number of evolutions (Fig 18).

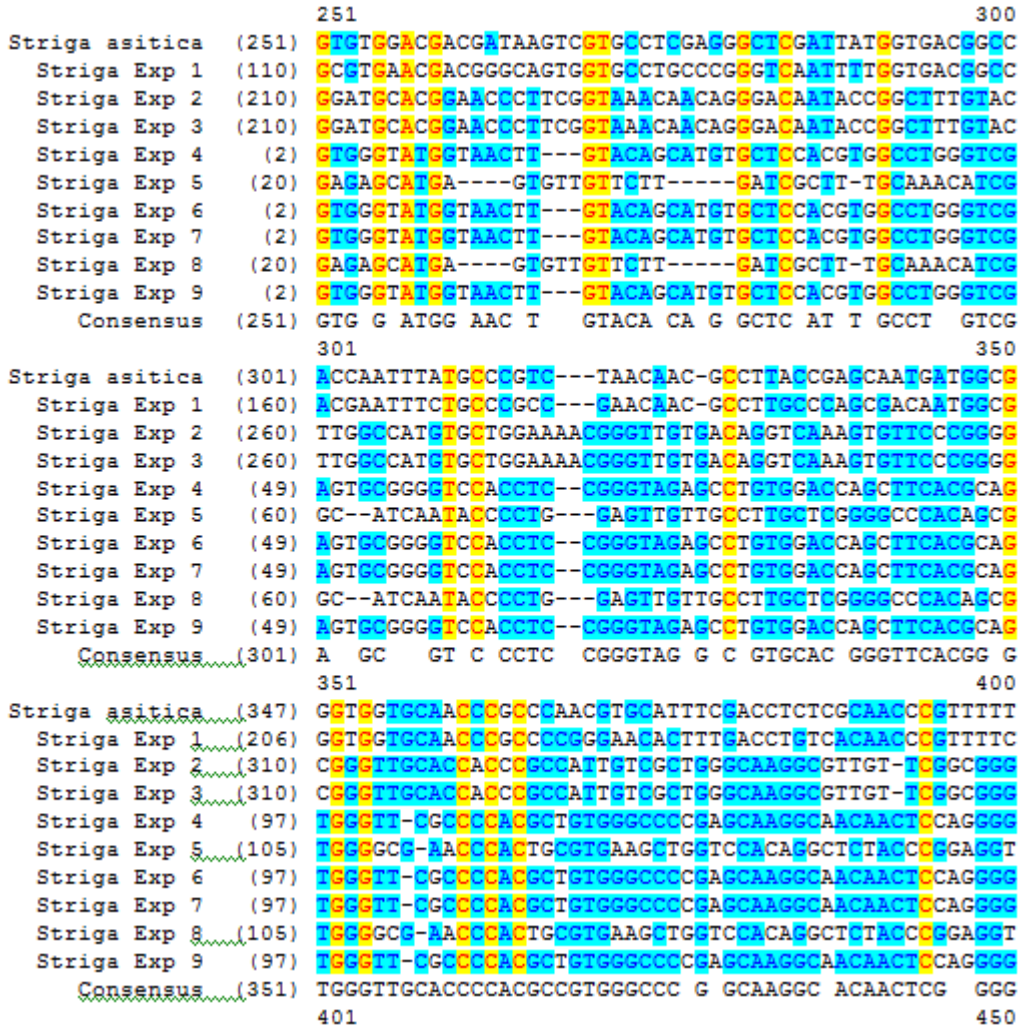

Figure 17: A section of aligned sequences of the Expansin genes expressed in *S. hermonthica* haustoria.

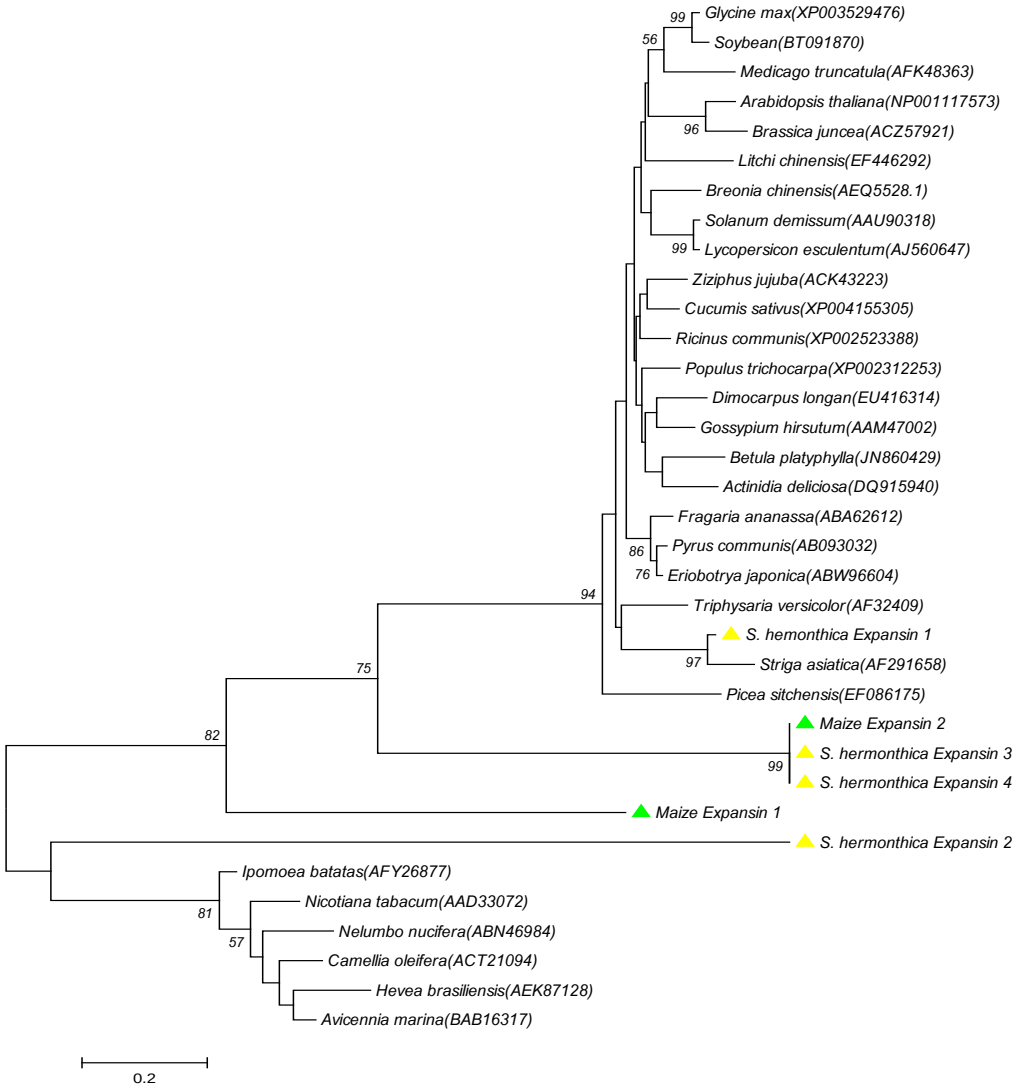

Figure 18: Evolutionary relationships of *S. hermonthica Expansin* gene

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 6.42763424 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura and Kumar, 2004). The analysis involved 35 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 162 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The Expansin gene from *S. hermonthica* is indicated by yellow colour while that of maize is indicated by light green colour.

4.2.2 *Cysteine protease* gene

Cysteine protease gene expression in *S. hermonthica* haustoria had the same band size (600bp) with that of the roots, while the leaves had two bands of approximately 700bp and 400bp in size (Fig 16A). In general, the bands in *S. hermonthica* tissues were different in size from those of maize. The maize roots revealed an expression of two bands of different sizes, 1000 and 500bp (Fig 19a). The quality of the cDNA was confirmed by amplification with primers for the Actin a house keeping gene that revealed a fragment of 0.4kb (Fig 19b). The fragments from *S. hermonthica* and maize tissues were excised from gel purified and cloned into TOPO TA cloning vector (Fig 19c). Cloning of the fragments from *S. hermonthica* and from maize roots was successful as confirmed by restriction digestion using EcoR1 (Fig 19d).

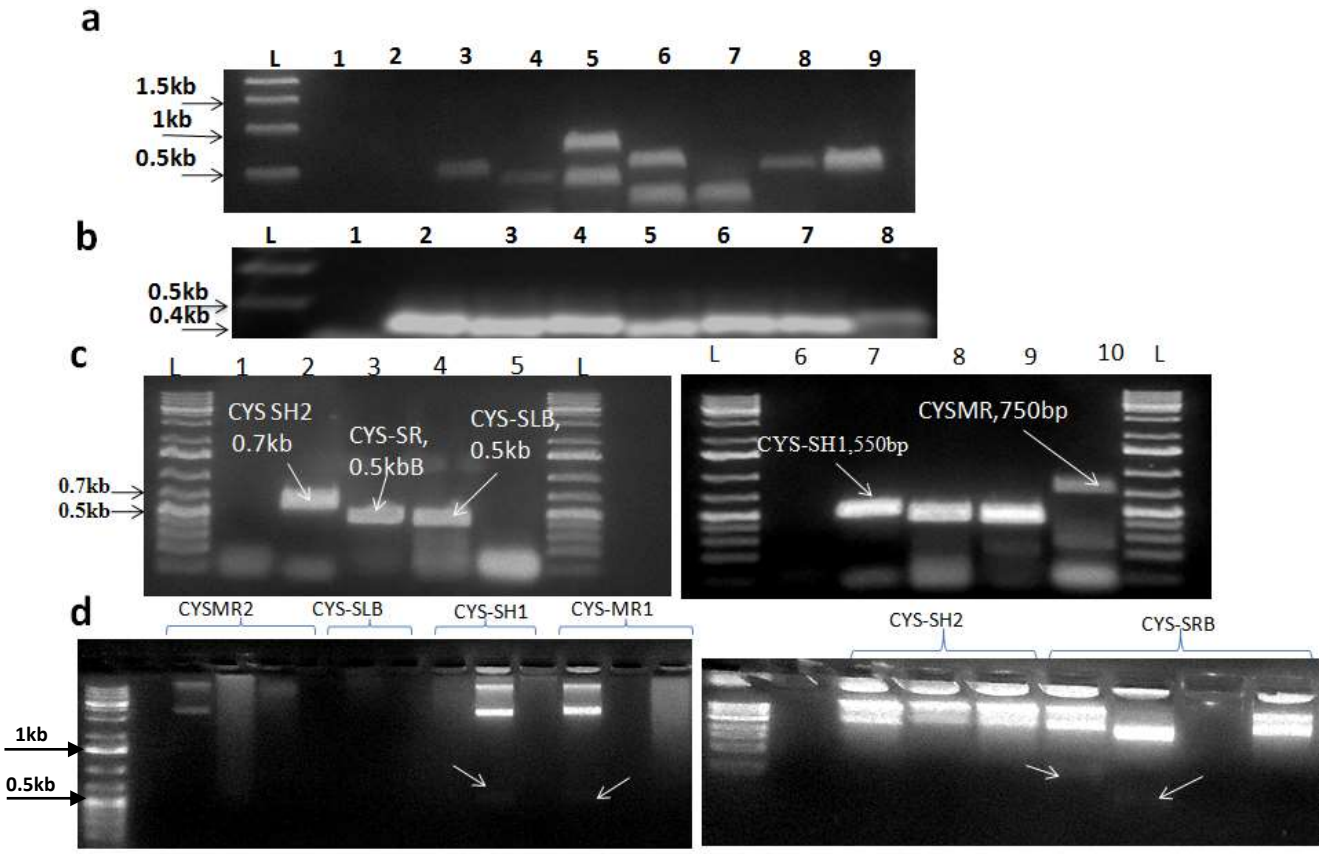

Figure 19: Amplification, purification and cloning of *Cysteine protease* gene
(a) Amplification of cDNA from *S. hermonthica* and maize tissues using Cysteine protease gene specific primers, Lanes 1 is the negative control, 2 and 3 is cDNAfrom *S. hermonthica* haustoria, 4 and 5 is the cDNA from *S. hermonthica* roots, 6 and 7 is the cDNA from *S. hermonthica* leaves and 8 and 9 is the cDNA from maize roots, (b) The internal control using Actin primers.(c) purified fragments for cloning into topo TA, (d) Screening for positive clones through restriction digestion with *EcoR1*.

Multiple alignments using NTI vector software of the sequenced *Cysteine protease* gene revealed 4 unique genes in *S. hermonthica* (Fig 20). From the alignments, *CYS* 1, *CYS* R1 and *CYS* R2 share 100% homology and were named (*Striga Cysteine protease* 1), while *CYS*2, *CYS*3, and *CYS* 4 are different from each other and were named (*Striga Cysteine protease* 2, 3 and 4 respectively). Pairwise analysis of the sequenced *S. hermonthica Cysteine protease* genes was done using tBlastx alogorithim at the NCBI. The results of *Striga CysteIne protease* 1 revealed similarity with species such as *Ricinus communis* 92% (XM002512963), *Vitis vinifera* 90% (NP001267989), *Populus trichocarpa* 88%

(AAX19661), *Ipomea batatas* 90% (AAF40414) *Gossypium hirsutum* 90% (CAE54306), *Lotus japonica* 83% (CAE45588), *Medicago trancatula* (XP003589348) and *Glycine max* 86% (XP003539008) among others. *Striga cysteine protease* 2 and 3 revealed similarity with *Picea sitchensis* 75% (ACN40679), *Pachysandra terminalis* 76% (DQ403257), *Capscum chinensis* 80% (NP001267989), *Fragaria vesca* 81% (XP004291404), *Nicotiana tabacum* 81% (AAK07731) and *Cicer arietinum* 75% (XP004507484). The *Striga cysteine protease 4* had no significant similarity with other species most probably because it was too short. The phylogenetic analysis gave two groups of *S. hermonthica cysteine protease* genes. *Striga cysteine protease* 3 and 4 genes share a recent common ancestor with *Cuscuta reflexa* with almost equal number of evolutions and same branching pattern (Fig 21).

Figure 20: Aligned sequences of the *S. hermonthica Cysteine protease* gene

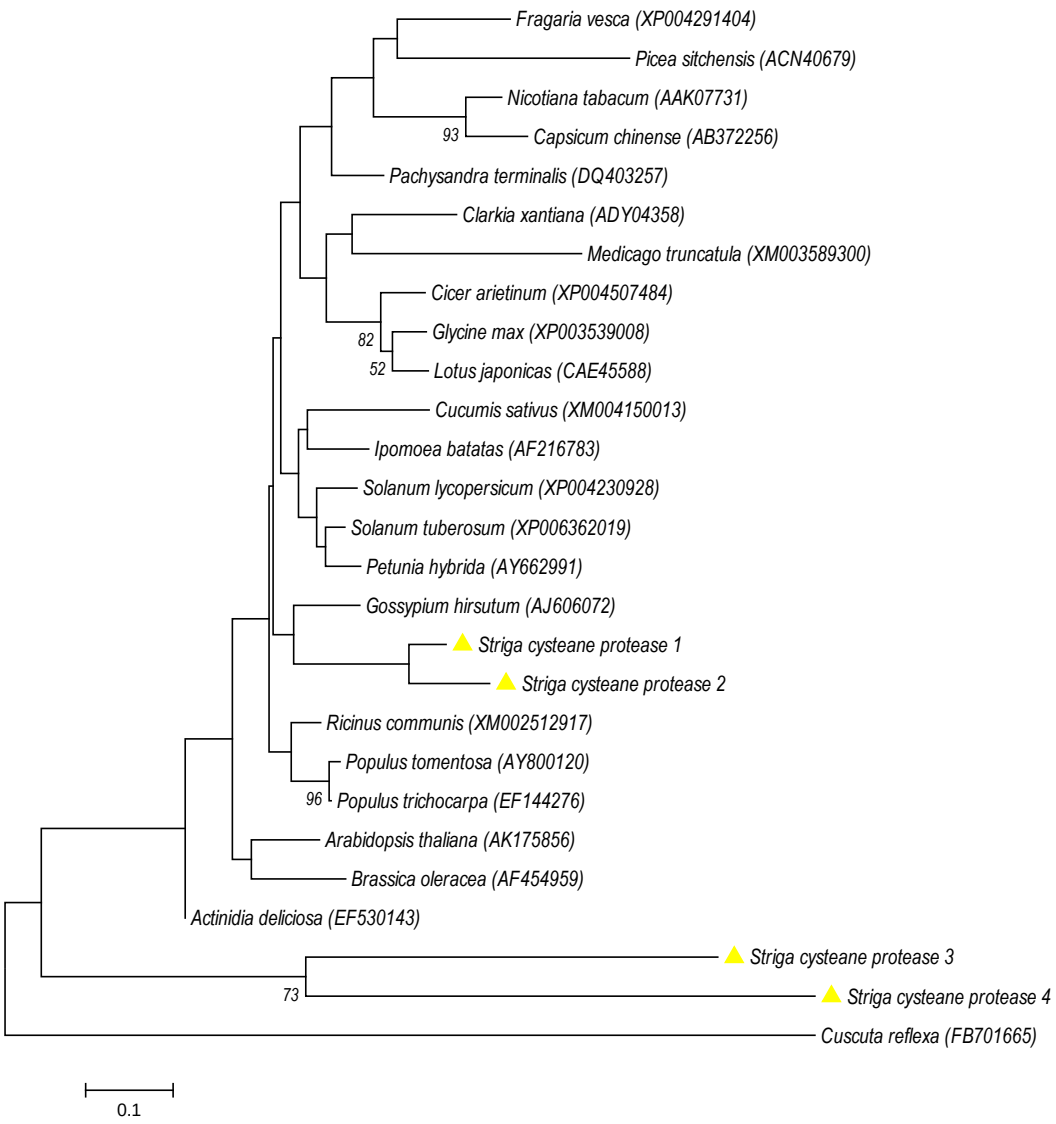

Figure 21: Evolutionary relationships of *Cysteine protease* gene
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 5.22998591 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura and Kumar, 2004). The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 67 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). The *S. hermonthica cysteine proteise* genes are indicated by yellow colour.

4.2.3 *Mannose 6-phosphate reductase* gene

Mannose 6-phosphate reductase (M6PR) gene expression in *S. hermonthica* tissues was different from those of maize roots. The parasites haustoria and roots tissues had the same band of approximately 600 bp, while the leaves had two bands of 1000bp and 600bps (Fig 22a). The maize roots had a unique band of approximately 800bp totally different from that in the *S. hermonthica* tissues (Fig 22a). The quality of synthesized cDNA was indicated by the primers for internal control Actin gene (Fig 22b). The purified fragments (Fig 22c) were successifully cloned as indicated by confirmation through restriction digestion with EcoR1 enzyme (Fig 22d).

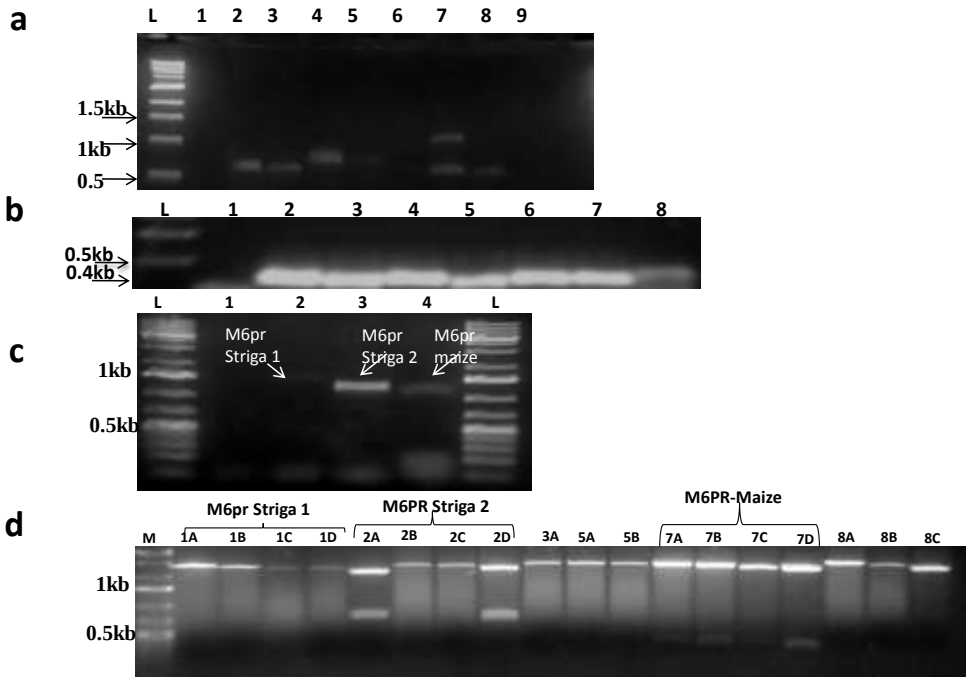

Figure 22: Identification, purification and cloning of *Mannose 6-phosphate reductase* gene.
(**a**) Amplification of cDNA from *S. hermonthica* and maize tissues using *M6PR* gene specific primers, Lanes 1 is the negative control, 2 and 3 is cDNA from *S. hermonthica* haustoria, 4 and 5 is the cDNA from *S. hermonthica* roots, 6 and 7 is the cDNA from *S. hermonthica* leaves and 8 and 9 is the cDNA from maize roots, (**b**) The internal control using Actin primer, (**c**) purified fragments for cloning, (**d**) Screening for positive clones through restriction digestion with *EcoR1*.

Alignment of the sequenced *mannose 6-phosphate reductase* gene revealed three unique genes in *S. hermonthica* (Fig 23). The *M6PR* 1 and *M6PR* 2 were 100% similar and were named *Striga M6PR* 1. The sequenced *M6PR*3 and *M6PR*4 were unique from each other and were named *Striga M6PR* 2 and 3, respectively (Fig 23). The pairwise analysis with other sequences at the NCBI data base for *Striga M6PR 1* revealed 70-90% similarity with malate dehyrogenase genes sequences of various plant species. These species include; *Solanum lycoperscon* 82% (NP001234056), *Solanum tuberosum* 81% (XP006361712), *Genlisa aurea* 80% (EPS69170), *Medicago trancatula* 78% (XP003638165), *Cicer arietinum* 78% (XP004496135), *Glycine max* 78% (XP003536287), *Zea mays* 77% (AEG42731), *Ricinus communis* 78% (XP002532626), *Oryza sativa* 77% (XP001062517), *Arabidopsis thaliana* 75% (NP974958) and *Picea sitchensis* 72% (ABK25224) among others. Striga *M6PR* 2 and *3* indicated uncharacterized proteins with *spp* such as *Morus notabilis* 49% (EXC33992), *Populus trichocarpa* 45% (XP00637945), *Capsella rubella* 49% (XP006300750) and *Solanum lycopersicum* 56% (XP004245684) among others. The phylogenetic analysis of the sequenced *mannose 6-phosphate reductase* genes of *S. hermonthica* indicated sharing of common ancestry with that of *Orobanche ramose* with the later being the root of the tree (Fig 24).

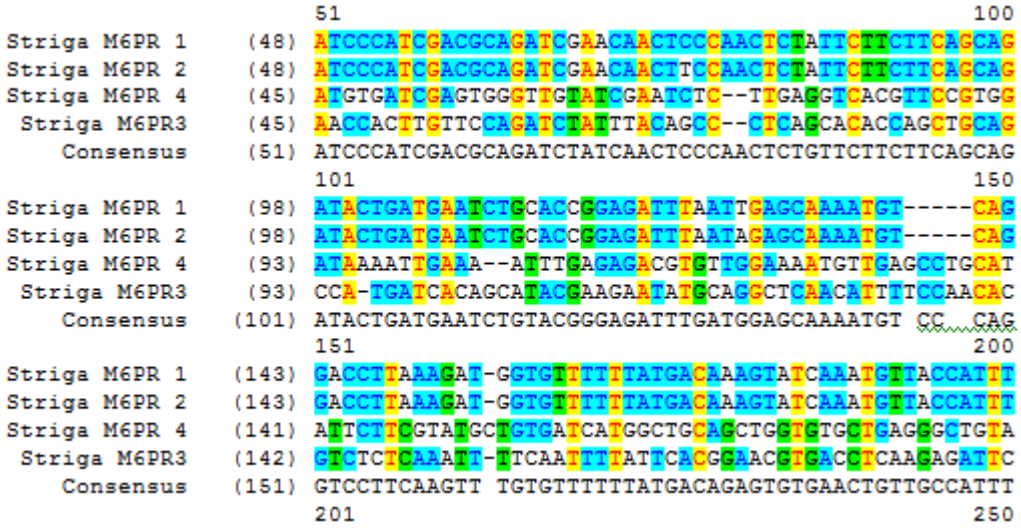

Figure 23: Aligned sequences of the *S. hermonthica* M6PR gene

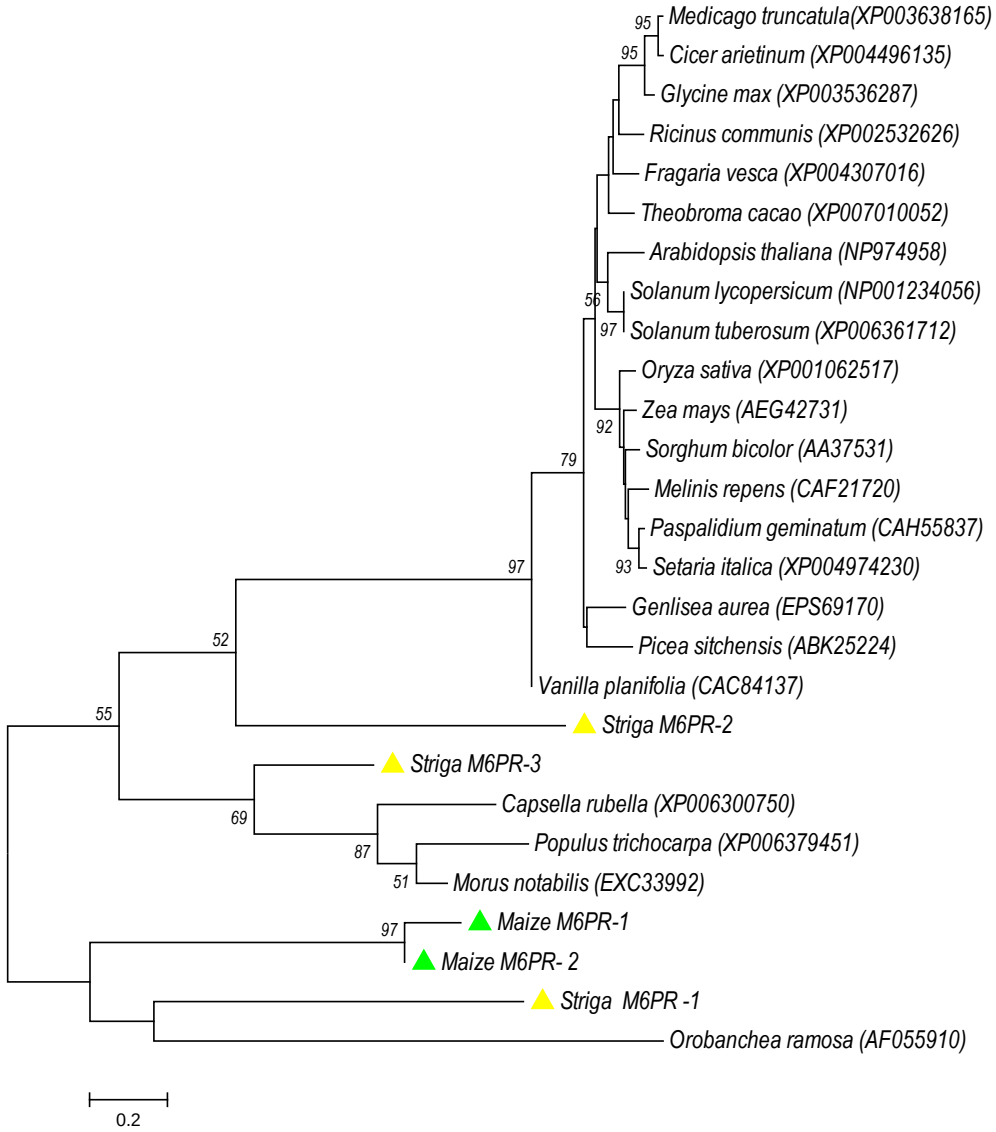

Figure 24: Evolutionary relationships of Mannose 6-phosphate reductase gene
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 8.66939672 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura and Kumar, 2004). The analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 125 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The *S. hermonthica M6PR* genes are indicated by yellow colour and green for maize.

4.2.4 *Tvpirin* gene

Tvpirin gene was expressed in both *S. hermonthica* and maize tissues. Four unique bands were observed in maize roots (TVP-MR1, 2, 3 and 4) different in sizes as to the four fragments in *S. hermonthica* leaves (Fig 25a). The *S. hermonthica* haustoria indicated one unique fragment (Fig 25a). The quality of the cDNA was confrmed by amplification with primers for the Actin gene (Fig 25b). The Tvpirin gene fragments were not successfully cloned (Fig 25c).

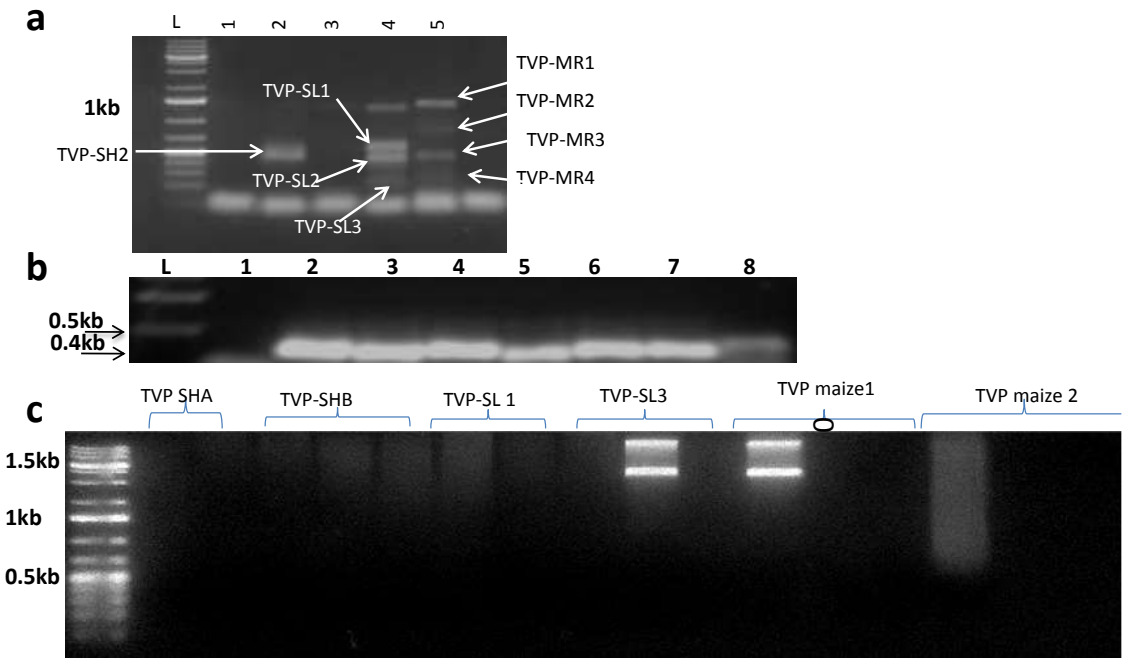

Figure 25: Amplification, purification and cloning of Tvpirin gene.
(**a**) Amplification of cDNA from *S. hermonthica* and maize tissues using Tvpirin gene specific primers, Lanes L is 1kb plus gene ruler ladder, 1 is the negative control, 2 is cDNAfrom *S. hermonthica* haustoria, 3 is the cDNA from *S. hermonthica* roots, 4 is the cDNA from *S. hermonthica* leaves and 5 is the cDNA from maize roots, (**b**) The internal control using Actin primers, (**c**) Screening for positive clones through restriction digestion with EcoR1.

4.2.5 *Quinone oxidoreductase* gene

There was a high expression of *Quinone oxidoreductase (QOR)* gene in *S. hermonthica* roots and haustoria revealing a band sizes of 1.3kb and 0.5kb and sizes of 0.7 and 0.4kb in the leaves. In maize, the *QOR* gene seemed to have a high expression also with band sizes of 0.5kb and two faint ones between1 and 1.5kb (Fig 26a). However, the fragments could be purely isolated from gel hence the QOR gene was not cloned.

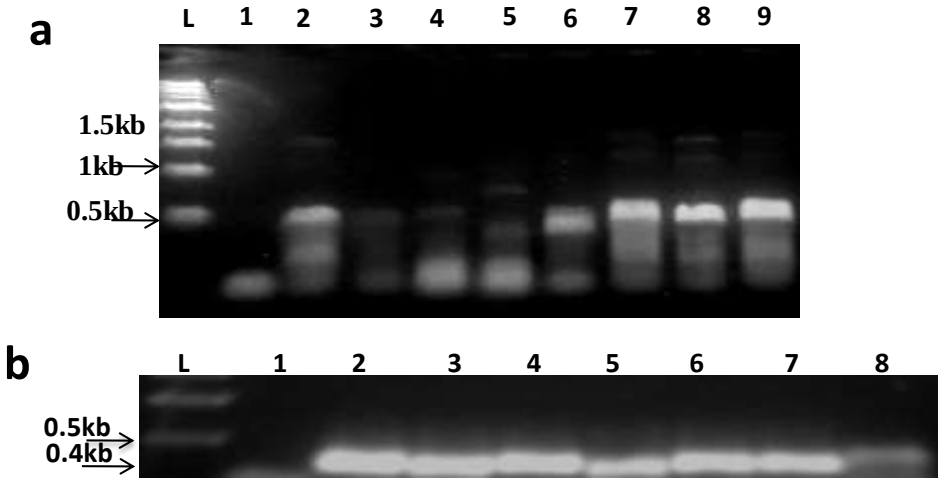

Figure 26: Identification, purification and cloning Quinone oxidoreductase (QOR) gene. (**a**) Amplification of cDNA from *S. hermonthica* and maize tissues using QOR gene specific primers, Lanes L is 1kb ladder, 1 is the negative control, 2 and 3 is cDNA from *S. hermonthica* haustoria, 4 and 5 is the cDNA from *S. hermonthica* roots, 6 and 7 is the cDNA from *S.hermonthica* leaves and 8 and 9 is the cDNA from maize roots. (**b**) The internal control using Actin primers.

4.3 Development of VIGS protocol for *S. hermonthica* using *Phytoene desaturase* RNAi construct

4.3.1 VIGS induced RNAi on *S. hermonthica* PDS causes chlorosis

A viral induced technique through agro-drench method was effective in *S. hermonthica* plants. This was evidenced by down regulation of the PDS gene resulting into photobleached phenotypes on the leaves of *S. hermonthica* plants agro-drenched with the *Agrobacterium* strain GV3101 habouring the TRV1 and TRV2 vector containing the PDS insert (Fig 27a, b, c, d, and e). The bleaching effects appeared on the 14th day after agro-drench but the plants recovered on the 28th day post infection (Fig 27c) and (Fig 27e), respectively. There was no chlorosis in the plants under control experiments (Fig 27f-Fig 27t).

Similarly agro-infiltrated *S. hermonthica* plants developed chlorotic phenotypes with mosaic like appearance on the leaves of PDS targeted plants. The yellowing and the mosaic effects appeared on the 7th day after agro-infiltration (Fig 28b) and the plants recovered on the 28th day post agro-infiltration (Fig 28d). The negative control plants showed no chlorotic symptoms (Fig 28f-28o). Agro-infiltration of one month old wild type tobacco plants led to photo-bleaching of leaves at 6 days after infiltration. These plants recovered 12 days post agro-infiltration (Fig 29a, b, c, d, e). All the tobacco negative control plants did not show chlorotic symptoms (Fig 29f-Fig 29t).

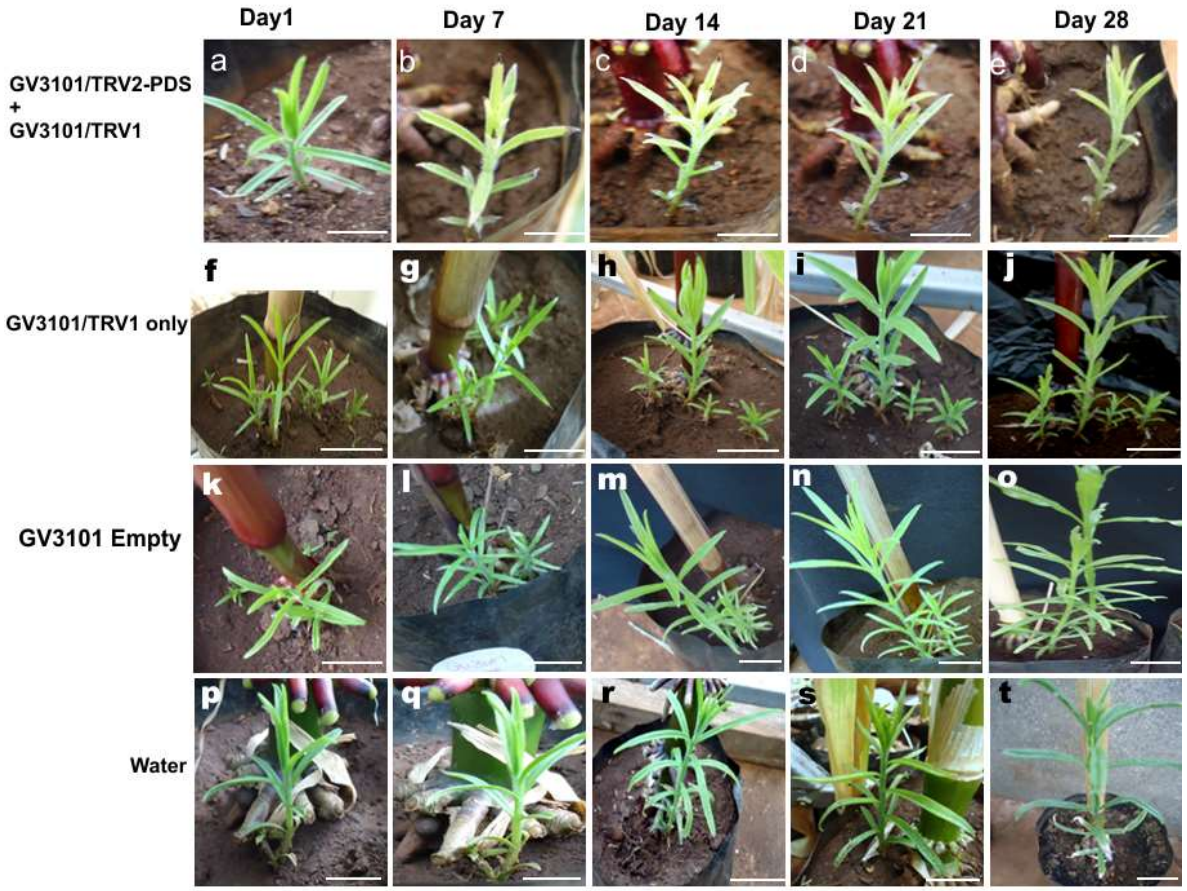

Figure 27: Viral induced gene silencing via Agro-drench method on *S. hermonthica*. **a, b, c, d**, and **e** are plants agro-drenched with Agrobacterium strain GV3101 with TRV2-PDS (GV3101/TRV2-PDS), and mixed in 1:1ratio with GV3101 containing TRV1 empty vectors (GV3101/TRV1), in days 1, 7, 14, 21 and 28 after agro-drench respectively; **f, g, h, i** and **j** were agro-drenched with GV3101 vectors containingTRV1 empty (GV3101/TRV1) in days 1,7,14,21 and 28 respectively. **k, l, m, n**, and **o** plants were agro-drenched with GV3101 only, while **p, q, r, s** and **t** were only watered in days 1, 7, 14, 21 and 28 after agro-drench respectively. All scale bars represent 5cm.

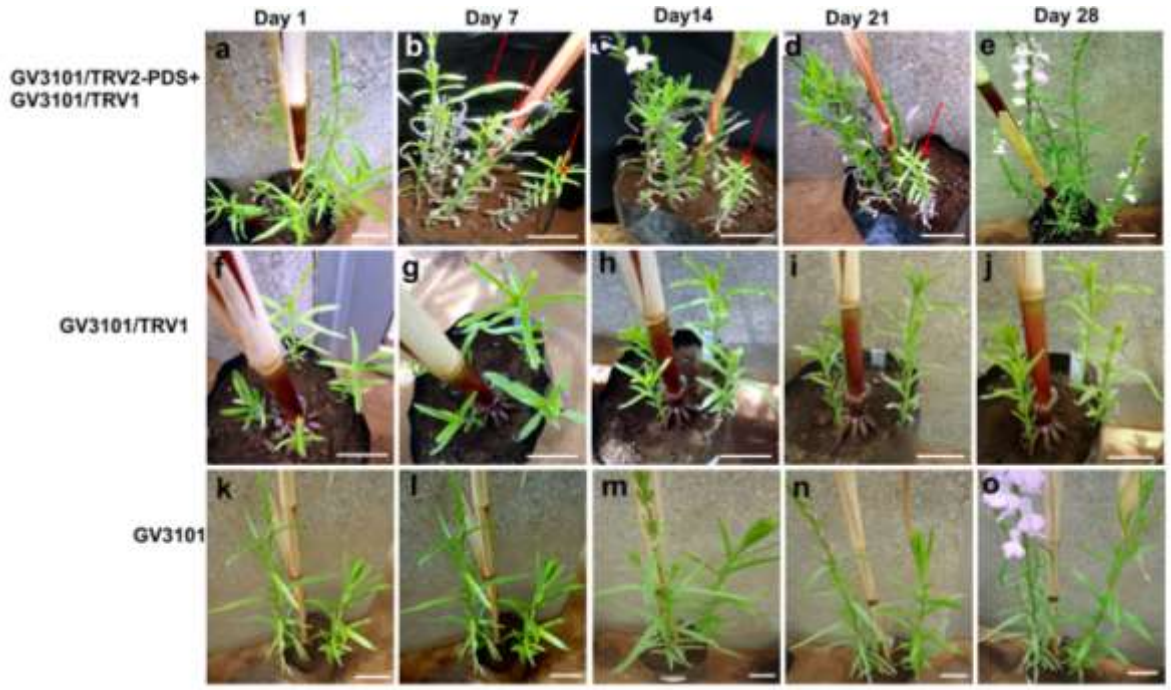

Figure 28: Viral induced gene silencing via agro-infiltration method on *S. hermonthica*. a, b, c, d, and **e** are plants agro-infiltrated with *Agrobacterium* strain GV3101 habouringTRV2-PDS (GV3101/TRV2-PDS), and mixed in 1:1 ratio with GV3101 containing TRV1 empty vectors (GV3101/TRV1), in days 1, 7, 14, 21 and 28 after agro-infiltration respectively; **f, g, h, i** and **j** are plants agro-infiltrated with GV3101 vectors containing TRV1 empty (GV3101/TRV1) in days 1,7,14,21 and 28 after infiltration respectively**; k, l, m, n,** and **o** are plants agro-infiltrated with GV3101 only, in days 1, 7, 14, 21 and 28 after agro-infiltration respectively. All scale bars represent 5cm.

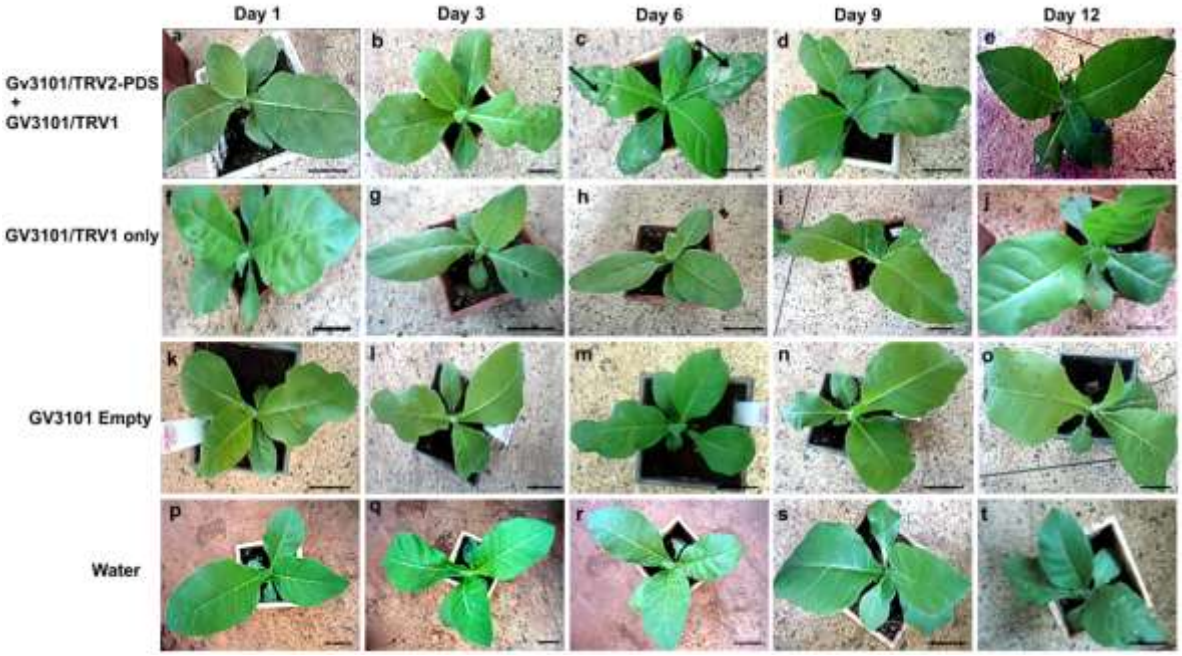

Figure 29: Viral induced gene silencing via Agro-infiltration in *N. tabacum*.
a, b, c, d, and **e** are photobleached plants agro-infiltrated with PDS construct within TRV2 viral vectors in *Agrobacterium* strain GV3101 (GV3101/TRV2/PDS), and mixed in 1:1 ratio with GV3101 containing TRV1 empty vectors (GV3101/TRV2), in days 1, 7, 14, 21 and 28 after agro-infiltration respectively; **f, g, h, i** and **j** were agro-infiltrated with GV3101 vectors containing TRV1 empty (GV3101/TRV1) in days 1, 7, 14, 21 and 28 after infiltration respectively; **k, l, m, n**, and **o** plants were agro-infiltrated with GV3101 only, while P, Q, R, S and T were infiltrated with water in days 1, 7, 14, 21 and 28 after agro-infiltration respectively. All scale bars represent 3cm.

4.3.2 VIGS induced RNAi is because of down-regulation of *S. hermonthica* PDS

To confirm if silencing of the PDS had occurred in *S. hermonthica* due to infiltration and agrodrench, RT-PCR analysis was done using PDS and TRV gene specific primers. The PDS primers were designed to prime outside the region of homology between the VIGS vector and the target mRNA. The RT-PCR of the cDNA from chlorotic *S. hermonthica* plants, using PDS primers amplified a 250 bp fragment as expected (Fig 30A). However, the fragment was extremely faint due to down reguration of the PDS gene in these plants. The negative control plants treated with GV3101/TRV1, GV 3101 empty and those which were only watered had a sharp band indicating the PDS was highly expressed in these plants because there was no downregulation (Fig 30B, 30C, 30D, respectively). Additionally, the cDNA amplification with TRV primers indicated the success of VIGS by amplifying a 400 bp fragment on plants treated with GV3101/TRV2 with the PDS insert (Fig 30E). Amplification of the cDNA from plants treated with; GV3101/TRV1 empty, GV3101 empty and water only did not have the 400bp fragment amplification with TRV primers (Fig 30F, 30G, 30H respectively). The *S. hermonthica* DNA amplification with primers for Actin gene indicated the 426 bp fragment indicating the quality of the cDNA of plants from all the four treatments (Fig 30I, J, K, L).

The RT-PCR of *Nicotiana tabacum* chlorotic plants targeted for PDS gene silencing, with GV3101/TRV2-PDS+GV3101/TRV1 construct was also done using PDS primers. The result indicated an amplification of a very faint band of 250bp in size due to down regulation of the PDS gene (Fig 31A). The negative control plants infiltrated with GV3101/TRVI, GV3101 empty and water had an amplification of a prominent 250bp band (Fig 31B, 31C and 31 D, respectively). The cDNA amplification with TRV primers indicated the success of VIGS by amplifying a 400 bp fragment on plants treated with GV3101/TRV2-PDS +GV3101/TRV1 (Fig 31E). Amplification of the cDNA using TRV primers from plants treated with; GV3101/TRV1 empty, GV3101 empty and water only did not have the 400bp fragment amplification (Fig 31F, 31G and 31H respectively). The *N. tabacum* cDNA amplification with primers for Actin gene showed the 426 bp fragment indicating the quality of the cDNA (Fig 31 I, J, K, L).

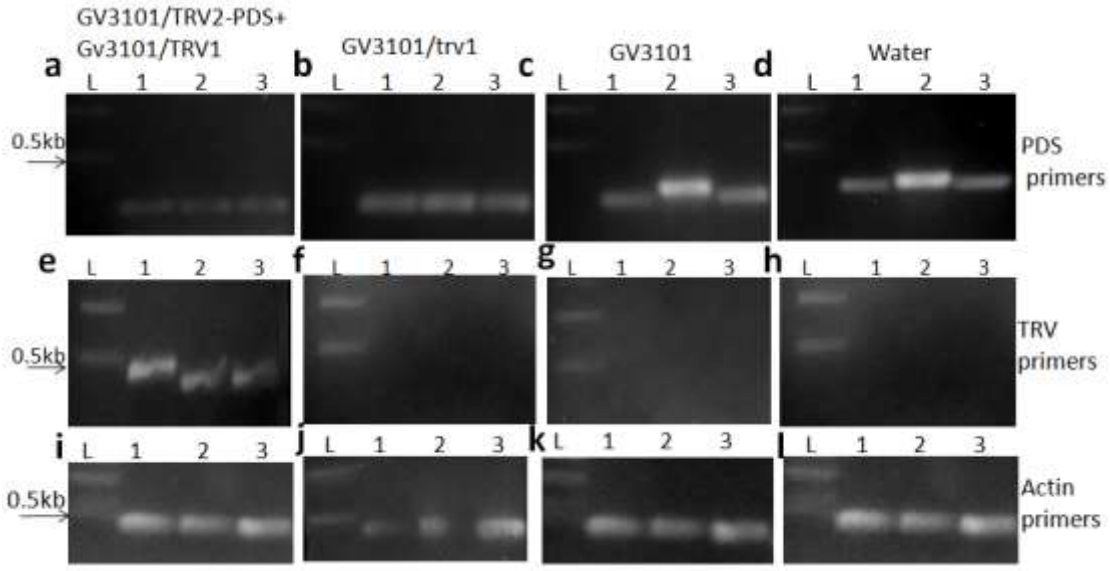

Figure 30: RT-PCR to confirm silencing of *PDS* gene on *S. hermonthica*.
a) *S. hermonthica* treated with GV3101 with the TRV2 vector containing the PDS gene insert (GV3101/TRV2-PDS) +GV3101/TRV1) and amplified with PDS primers. Lane (L) represents 1kb ladder while 1, 2, and 3 are the replicates (**b**) The cDNA plants treated with GV3101 containing TRV1 empty vectors (GV3101/TRV1) with three replicates (1, 2 and 3) and amplified with PDS primers (**c**) Three replicates (1, 2, 3) of plants treated with GV3101 empty and amplified with PDS primers (**d**) represents plants treated only with water. and amplified with PDS primers (**e**) *S. hermonthica* cDNA amplified with TRV primers from plants treated with Gv3101/TRV2-PDS+ GV3101/TRV1 vectors with three replicates (1, 2, 3). (**f**) *S. hermonthica* cDNA from Plants treated with GV3101/TRV1 empty vectors, three replicates (1,2, and 3) amplified with TRV primers,(**g**) Plants treated with GV3101 empty, three replicates (1, 2, 3) amplified with TRV primers.(**h**) Plants treated with water only, three replicates (1,2,3), cDNA amplified with TRV primers. Images (**i, j, k** and **l**) are the internal control using Actin primers for the Striga cDNA from plants treated with GV3101/TRV2-PDS+GV3101/TRV1, Plants treated with GV3101/TRV1 only, Plants treated with GV3101 only and plants treated with water only, respectively.

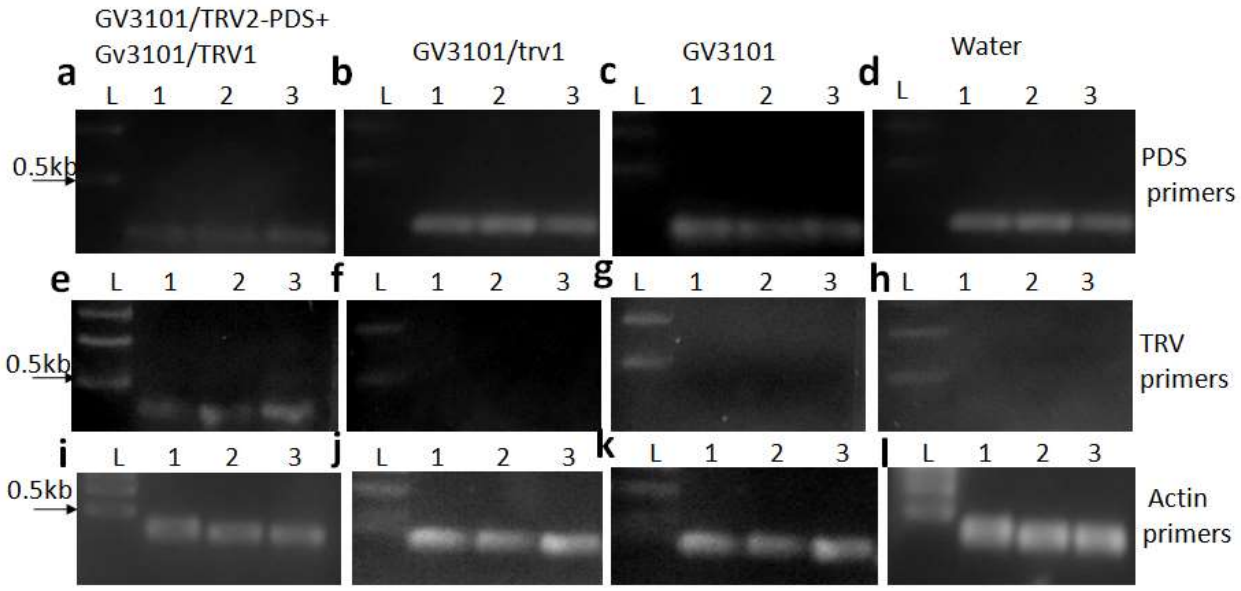

Figure 31: RT-PCR to confirm silencing of *PDS* gene on *Nicotiana tabacum*
a) *N. tabacum* plants treated with GV3101 with the TRV2 vector containing the PDS insert (GV3101/TRV2-PDS) and mixed in 1:1 ratio with GV3101 containing TRV1 vectors (GV3101/TRV1) and amplified with PDS primers. Lane (L) represents 1kb ladder while 1, 2, and 3 are the replicates. **(b)** The cDNA of plants treated with GV3101 containing TRV1 empty vectors (GV3101/TRV1) with three replicates (1, 2 and 3).and amplified with PDS primers **(c)** Three replicates(1, 2, 3) of plants treated with GV3101 empty and amplified with PDS primers **(d)** represents plants treated only with water. and amplified with PDS primers **(e)** *N. tabacum* cDNA amplified with TRV primers from plants treated with GV3101/TRV2/PDS gene insert and mixed in 1:1 ratio with GV3101/TRV1 vectors with three replicates (1, 2, 3). **(f)** *N. tabacum* cDNA from Plants treated with GV3101/TRV1 empty vectors, three replicates(1,2, and 3) amplified with TRV primers**,(g)** Plants treated with GV3101 empty, three replicates (1, 2, 3) amplified with TRV primers **(h)** Plants treated with water only, three replicates (1,2,3), cDNA amplified with TRV primers. Images (**i, j, k** and **l**) are the internal control using Actin primers for the tobacco cDNA from plants treated with GV3101/TRV2/PDS+GV3101/TRV1, Plants treated with GV3101/TRV1 only, Plants treated with GV3101 only and plants treated with water only, respectively

4.3.3. VIGS induced RNAi efficiency on *S. hermonthica*

Statistical analysis using student's t-test revealed that VIGS was successful on plants targeted for PDS silencing (treated with GV3101/TRV2/PDS+GV3101/TRV1) in *S. hermonthica* plants. There was photobleaching by 60.2±2.9 percentage of *S. hermonthica* plants targeted for PDS silencing via Agro-infiltration method. In Agro-drench method, only 10±1.5 indicated photobleaching characteristics. *Nicotiana tabacum* plants were used as positive control because VIGS sytem has earlier been reported to be successful. The PDS targeted *N. tabacum* plants indicated 100% photo-bleaching. None of the negative control treatments indicated photo-bleaching effects (Table 2).

Table 2: VIGS efficiency in *S. hermonthica* and *N. tabacum*

Treatment	**No of transformed plants**	**% of pds transformed Plants (Photo-bleached)**	**% of pds negative Plants(Not photo-bleached)**
S. hermonthica			
GV3101/TRV2PDS+GV3101/TRV1			
Agro-infiltration	12.9±2.9	60.2±2.9*	39.8±5
Agro-drench	12.0±0.0	10±1.5	90±1.0
GV3101/trv1	8.0±1.5	0.0	100.0*
GV3101	8.0±1.0	0.0	100.0*
N. tabacum			
GV3101/TRV2PDS+GV3101/TRV1	8.0±0.0	100.0*	0.0
GV3101/TRV1	8.0±0.0	0.0	100.0*
GV3101	8.0±0.0	0.0	100.0*

* Significant values; $P \leq 0.05$

CHAPTER FIVE

5.0 DISCUSSION

5.1 *Striga hermonthica* haustorium development in susceptible (*Namba nane*) and tolerant (*KSTP'94*) open pollinated varieties of maize

The chlorosis and death observed on Striga infected maize could be attributed to the negative effects of parasitism that involve syphoning of water and nutrients. This is consistent with previous reports (Gurney *et al*., 2006). The enhanced root growth that resulted in development of hairy roots on the upper profile of the maize root system infected with S. *hermonthica* could be due to Striga-host trafficking of auxin enzymes through haustoria connection. Increased auxin levels have been reported to increase root proliferation (Kim *et al.*, 1999). These high auxin levels could have originated from the Striga and could have traficked into maize through the haustoria. Micro and macromolecular trafficking betwen parasistic plants and their host has widely been reported (Rakefet *et al*., 2008, Alakonya *et al*., 2012). This auxin trafficking from Striga to maize could be one of the many Striga's evolutionary mechanisms to stimulate root growth in its host after initial contact so as to increase surface area for more haustoria connections.

The unknown secretion at the Striga host interface of the two maize varieties could be as a result of accumulation of cell wall components such as pectins, proteins and lignin. These compounds have also been reported to be acting to aid invasion of the parasite, tolerate invasion or act as a defense mechanism (Yoder, 1999). The role of chemical barriers such as phenols, phytoalexins and pathogenesis related proteins to parasitism, at the host-parasite interface has been shown to account for resistance in some varieties (Hood *et al*., 1998; Westwood *et al* 1998; Goldwasser *et al*., 1999). Since accumulation of such unknown substances occurred in both varieties, it is not clear whether the nature of the substance aided successful parasitism in susceptible variety while those secreted by tolerant variety acted as chemical barriers. If the substances were meant to be for resistance against the parasite, then *S. hermonthica* has evolved to resist such mechanisms from its hosts. This further suggests that the compounds are not the main cause of resistance in the

tolerant variety. On the other hand, if the substances were cellwall-degrading enzymes to aid in successful parasitism, then other barriers could be the cause for the resistance to parasitism in the tolerant variety. Analysis of these chemical compounds' composition at the parasite host interface in both tolerant and susceptible variety could help in determining if they are similar or different, and if they confer resistance in *KSTP´94* or not.

Losher-Goshen *et al*., (1998) reported that enzymes such as pectin methyl esterase (PME), which are of parasite origin, de-esterifies pectins in the host cellwall and in the middle lamellar near the intrusive cells. Yoder, (1999) also reported pectin methyl esterases detected cytological in endophytic *Orobanche* haustoria, leading to host cellwall pectin's changes at the site of haustorium invasion. This, with other enzymes presumably polygalacturonase, results in complete degradation of host cellwall pectins that allows separation of cells and smoothens the penetration of the parasite intrusive cell between host cells without rupturing them (Yoder, 1999). Accumulation of such substances or enzymes could further account for the dark staining at the parasite-host interface and explains the easy penetration of the *S. hermonthica* haustoria in the susceptible variety.

The resistance to parasitism by the tolerant variety (*KSTP'94*) through delayed emergence and less numbers of attached *S. hermonthica* could be attributed to production of low amounts of parasite germination signalling compounds. *Striga hermonthica* seeds only germinate in response to specific chemical stimulants (Strigolactones) that are present in the root exudates of the host (Graves *et al*., 1989; Graves *et al*., 1990; Dörr 1997; Amusan *et al*., 2008; Runo *et al*., 2012; Akiyama *et al*., 2005; Besserer *et al*., 2006; Joel *et al*., 2007). This mechanism therefore could be some of the pre-parasite attachments resistance mechanism by the tolerant variety (Rispail *et al*., 2007). The phased increase in the number of haustoria attached to each maize plant in both susceptible and tolerant maize indicated that the Striga haustoria is host regulated by some signal that may be of host origin. These signals may also habour key information on the host development stages for instance at some point a high number of haustoria formation was observed and was closely followed with flowering. This observation could be attributed to trafficking of florigen a conserved flowering protein that is usually produced in maize root but moves through the vasculature to the meristem where it stimulates flowering. The protein has been shown to be a long-range signal that is localised in the vasculature (Jaeger and Wegge, 2007).

Given that the Striga plant has a connection to the roots, the signal must have acted as a warning to the parasite that the host was preparing to flower. This signal must have been delivered through Striga-maize haustoria connection. Upon reaching the parasite the signal was interpreted to induce more haustoria formation so as to increase intake of water and nutrients needed in seed formation a mechanism that the parasite uses to spread (Ejeta, 2007). It is also possible that florigen increases production of germination signals in Striga hosts and could be the parasites survival technique for synchrony with the host so that it can complete its cycle in advance. Striga has been reported to have their lifecycles intimately sychronized with those of their hosts (Ejeta, 2007).

Histological analysis revealed high cell division in haustoria at the connection point between the parasite and the host and the haustoria intrusive cells apex pushed through the epidermis to the host cortex in both susceptible and tolerant varieties of maize. Yoder (1999) reported that haustorial cells at the parasite–host interface divide and elongate, pushing between rather than through the epidermis cells and cortex of the host root. This Striga resistance mechanism against haustoria observed in the tolerant variety could be attributed to mechanical or physiological barriers against the parasite. The endodermis is generally considered a substantial barrier to vascular penetration by the root parasitic weeds. This layer has been reported to be the site of resistance expression in many varieties of several crops including; rice to *Striga hermonthica* (Gurney *et al*., 2006), Sorghum to *Striga asiatica* (Maiti *et al.*, 1984) and sunflower to *Orobanche cumana* (Dörr *et al*., 1994). Penetration of the host root cortex by haustoria has been reported to be largely a mechanical process aided by the voluminous air space in the host root cortex (Joel and Losner Goshen, 1994; Dörr, 1997).

The high haustoria's penetration resistance in *KSTP'94* may be associated with high lignification of the endodermal cell walls, suggesting that the endodermis possessed tough mechanical tissues, sclerenchyma, serving as a barrier to haustoria penetration (Amusan *et al*., 2008). Selection for cultivars offering barriers at the cortex and endodermis would hence be of importance in enhancing resistance to successful parasitism. The genetics behind these barriers is however yet to be uncovered, but advancement of the intrusive cells penetration into the host cortex is reported to be remarkably responsive to host-specific factors (Hood *et al*., 1998). This could therefore explain the ease of haustoria

penetration in the susceptible variety due to recognition of such host-specific factors as opposed to the tolerant variety. Additionally, *KSTP'94* variety might be producing low amounts of chemical stimulants, which apart from stimulating *S. hermonthica* germination aids subsequent haustoria development, attachment and penetration into the host cells (Graves *et al*., 1989; Graves *et al*., 1990; Dörr 1997; Amusan *et al*., 2008; Runo *et al*., 2012).

Secretion of unknown substance was observed at the parasite-host interface of the two maize varieties as evidenced more in the section stained using methylene blue. Accumulation of such substances or enzymes in the parasite-host interface could further explain the reason for the dark staining. However, despite accumulation of these substances in the *Namba nane* variety, *S. hermonthica* parasitism was successful with no evidence of barriers. The parasites haustoria penetrating *Namba nane* variety indicated successful differentiation making parasite-host xylem to xylem connections, developing the vascular core and the hyaline body. The hyaline body is thought to metabolize the solutes withdrawn from the host, or at least regulate the supply of nutrients to the developing parasite (Gurney *et al*., 2006). Only in few cases was the haustoria observed to make vascular connections with the tolerant variety.

5.2 Cloning of *S. hermonthica* putative gene involved in haustoria formation

Expansin, cysteine proteinase and *Mannose 6-phosphate reductase* genes cloned and analysed in this research have shown that these genes have diverged in *S. hermonthica* probably to help the parasite colonise its many hosts in the savanna. The *expansin* gene for instance play important roles in plant cell growth, fruit softening, abscission, and emergence of root hairs, pollen tube invasion of the stigma and style, meristem function, and other developmental processes where cellwall loosening occurs (Cosgrove, 1999). Further, expansins are responsible for cellwall loosening during cell enlargement, a transition to the parasitic mode in which the haustorium is formed and regulated by xenognostic quinones (Mc-Queen-Mason, 1992; O'Malley and Lynn, 2000). The *expansin* evolution analysis also indicated that the S. *hermonthica expansin*1 has evolved the same number of times as that of *S. asiatica* (Fig 18). The *expansin* gene in *Triphysaria vercolor, S. asiatica* and *S. hermonthica* share a recent common ancestor. On the other hand, some

of the sequenced *S. hermonthica* genes have evolved more than those of the other species. This could be a parasite survival mechanism to overcome the host.

The *cysteine protease* gene has been reported to play a role in parasite –host interaction in *Cuscuta reflexa* (Bleischwitz *et al*., 2010). *Cysteine proteinases* also referred to as thiol proteases have been known to play an essential role in plant growth and development, senescence and programmed cell death, accumulation of storage proteins such as in seeds, storage protein mobilization, and participate in both anabolic and catabolic processes. In addition, they are involved in signalling pathways and in the response to biotic and abiotic stresses accompanying the hypersensitive response to pathogen and parasite attack (Hoorn and Jones, 2004). The enzyme has been reported to play a role in successful parasite infection process possibly by weakening host structures through protein degradation. The expression of *cysteine protease* gene in the *S. hermonthica* roots and haustoria was the same in band size hence could be playing the same role in the two tissues, while in the leaves, the gene had two signals indicating that there are two *cysteine protease* genes expressed there.

The two signals in maize roots were different in sizes from those of the parasite hence could be concluded that the cysteine proteins in maize are different from those of *S. hermonthica*. The role of *cysteine proteases* in other parasitic weeds such as *Orobanche spp* or *Striga spp* could be for parasite-host interactions as reported in *C. reflexa*. Although *S. hermonthica* colonizes roots of host, there exists a possibility of consistencies at the molecular level. The evolution analysis showed that the genes evolved almost the same number of times, while another group shares a more common ancestor with *Gossypium hirsutum* and *Ipomea batatas* (Fig 21). Inhibition of *cysteine proteases* could thus be of wider importance for antagonizing parasitic plants from different genera.

Mannose 6-phosphate reductase (*M6PR*) is a key enzyme in mannitol biosynthesis, and it has been suggested that mannitol accumulation may be very important for *Orobanche spp* development (Aly *et al*., 2009). The expression of the *M6PR* gene in *S. hermonthica* roots and haustoria indicates that the gene could also be playing an important role in Striga parasitism. However, there was an expression of *M6PR* gene in leaves of *S. hermonthica* therefore the gene could be playing the same or different role in the leaves. Increased

M6PR activity has been observed when plants are subjected to drought conditions (Aly *et al*., 2009). The evolution analysis revealed that the cloned *S. hermonthica M6PR 3* gene shared a recent common ancestor with *Orobanche ramose* (fig 24). Therefore, the *S. hermonthica M6PR* gene is a potential target for efforts to control this parasite. The parasitism genes appears to be constantly evolving and as already been reported to have evolved nine times (Rubiale, 2003).

Tvpirin genes and *quinone oxidoreductase* genes have been reported in parasitic plants such as *Triphysaria vericolor* with a role of host factor recognition and haustorium signalling (Bandaranayake *et al.,* 2010). The expression of these two genes in *S. hemonthica* tissues indicated the presence of the genes in the parasite, hence could be playing the same role as in *T. vericolor* (Fig 25 & 26). Although the two genes were not successifully sequenced, they could also be potential targets for genetic manipulations in efforts to control *S. hermonthica.*

5.3 Development of VIGS protocol targeting the *PDS* gene in *S. hermonthica*

Virus-induced gene silencing is a powerful, easy and efficient functional genomics tool for gene function analyses (Liu *et al*., 2002a). The technique is based on post-transcriptional gene silencing (PTGS) which plants employ as their innate antiviral defense mechanism in order to counter viral multiplication. The PTGS defense mechanisms employed by VIGS are very similar to the pathways of RNAi (Jaberolansar *et al*., 2010). In this study, the virally delivered PDS in both *S. hermonthica* and *N. tabacum* resulted in inhibition of the carotenoid biosynthesis. This was evidenced by down-regulation of the *phytoene desaturase* gene in *S. hermonthica* and *N. tabacum* plants resulting in photobleached phenotypes. These PDS downreguration affects were observed to appear 7 and 14 days post inoculation in agro-infiltrated and agro-drenched plants respectively (Fig 27 & 28).

The early appearance of silencing in agro-infiltrated *S. hermonthica* as compared to agro-drench could be ascribed to high efficiency in the delivery of the signals that initiate the innate silencing machinery of PGS in the leaves. In this case the VIGS vector was introduced in *S. hermonthica* via *A. tumefaciens* infection. It is possible that *A. tumefaciens* was more efficient at transferring theT-DNA containing the viral genome and pds into the

host genome in the cells of the leaves than in the root cells; this could have therefore delayed all the down stream silencing steps in agro-drenched plants.

Further, these results show that a better mechanism in spreading the silencing signal after introduction exist in *S. hermonthica* leaf tissues than in the root system or stems. The translocation of PTGS silencing factor can utilize both short-range cell-to-cell movement through plasmodesmata and phloem-associated long-range transport mechanisms (Himber *et al.,* 2003; Voinnet *et al.,* 1997). The RNA-dependent RNA Polymerase 6 (RDR6), designed for long-distance transport possibly by amplification of the silencing signal (Kalantidis *et al*., 2008) is required. From this experiment, it was not possible to check whether RDR6 in leaf, root and stem cells of *S. hermonthica* is responsible for the difference in efficacy. The silencing efficiency was, however, reported to be proportional to the number of silencing molecules in cells (Kalantidis *et al*., 2008). *Nicotiana tabacum* plants were used as the positive control because VIGS targeting the PDS had earlier been tested successfully (Jaberolansar *et al*., 2010). The photo-bleaching was exhibited in the tobacco leaves within 6 days post Agro-inoculation.

Phytoene desaturase (PDS) is a key enzyme involved in carotenoid biosynthesis pathway (Lu *et al*., 2003a). It's known that reduced levels of photo-protective carotenoids leads to rapid destruction of chlorophyll by photo-oxidation which results to white or bleached phenotypes (Becker and Lange 2003). Plants induce homology dependent defense mechanisms in response to attack by virus, therefore engineering a virus into a plant to target a gene of interest results in silencing of the gene through post transcriptional gene silencing (PTGS) (Robertson, 2004). The PTGS mechanisms are similar to those of RNA interference. Since VIGS is a transient transformation and not stable, the phenotypical characteristics of the silencing started disappearing within 28 days after the symptoms appeared in *S. hermonthica* leaves and in 12 days for *N. tabacum* plants. The negative control plants treated with GV3101/TRV1, GV3101 empty and those treated with water did not indicate any downreguration effects of carotenoid both phenotypically and in molecular analysis.

RT-PCR to confirm silencing of PDS gene, in both *S. hermonthica* and *N. tabacum* resulted in very faint bands on the cDNA from chlorotic plants due to down regulation of

PDS gene (Fig 30 & 31). On the other hand, bands from negative control plants had very intense bands indicating high expression of PDS gene. Amplification with TRV primers was seen only on the PDS silenced plants because of the TRV2 presense. The PDS insert must be contained in TRV2 expression vectors which encode genes for the virus coat protein responsible for viral replication for any silencing to occur (Choong *et al*., 2004). The TRV1 on the other hand encodes for genes responsible for viral transmission. The two vectors were separately harboured in the *Agrobacterium* T-DNA region which serves to deliver the expression vectors into plants. Mixing the two vectors in 1:1 ratio after inducing the virulence gene led to the success of VIGS in *S. hermonthica*. This explains why there were no PDS downreguration on the negative control experiments.

The *S. hermonthica* and tobacco silencing of the PDS was therefore as a result of the infected plants employing the post transcription gene silencing as defense mechanism against the virus. This study has a VIGS protocol that can be used for reverse genetics or functional genomics studies in *S. hermonthica* established. This approach also ensures that the gene validation can go without working with stable transformation of harmful parasites or their recalcitrant monocot hosts. Although there is limited evidence that genetic material can be exchanged between *S. hermonthica* and its host (Hiei *et al.,* 1994; Yoshida *et al*., 2010) the developed tools could independently without having to worry about delivery enough of the silencing molecules from the host to parasite via haustoria. Infact, once the factors that enable *S. hermonthica* to uncontrollably colonize its host are identified, the parasite could be basted by boasting host defense mechanisms through gene over-expression techniques along the identified pathways. In such a case delivery of resistance molecules will not be in question, as the host will directly exhibit resistance to *S. hermonthica* on attachment.

CHAPTER SIX

6.0 CONCLUSIONS

i. The *S. hermonthica* haustorium development process on *KSTP'94* and *Namba nane* is different in that for KSTP´ 94 there is poor host-parasite interraction, mechanical barriers and poor haustoria connection to the host vasculature, while the reverse was observed in *Namba nane*.

ii. Cloning and sequencing of genes expressed in *S. hermonthica* haustoria was successful and further analysis revealed that most of the genes had evolved more that once probably to suit their many hosts in the savanna.

iii. The presence of many genes expressed in haustoria also indicates that haustoria development is a coordinated multistep and multi-gene process probably involving several pathways.

iv. The VIGS mechanism exists in *S. hermonthica* and could be used in above ground functional genomics in the parasite using either agrodrench or agroinfiltration as gene delivery systems.

v. VIGS is more efficient in tobacco than in *S. hermonthica*.

6.1 RECOMMENDATIONS

i. Low chemical signaling mechanisms existing in tolerant varieties can be exploited in developing Striga resistance varieties.

ii. Further studies are required to distinguish the relative role of enzymes and other substances produced at the parasite –host interface in both susceptible and tolerant varieties.

iii. Transformation by stable RNAi constructs can be explored to target the identified *S. hermonthica* genes as a management strategy against the parasite.

iv. The role of the genes at the Parasitic Plants Resources Database can be studied for any above ground phenotypes in Striga using the developed VIGS protocol.

v. Validation of the genes for haustoria formation in *S. hermonthica* through functional mutants is proposed as opposed to VIGS.

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APPENDICES

Appendix 1: Publications

Kirigia D, Runo S and Alakonya A (2014). A viral- induced gene silencing system for functional geneomics in parasitic plant *Striga hermonthica*. *Plant methods*, **10**:16.

Kirigia D, Runo S and Alakonya A: Characterization of haustorium development Process in *KSTP'94* (tolerant) and *Namba nane (susceptible) open pollinated* maize varieties. *Weed research journal, Wiley (Under review).*

Kirigia D, Runo S, Matheka J, Omar R and Alakonya. Cloning and Sequencing of Genes key in *S. hermonthica* haustorium formation, *3 Biotech*, Springer (Under preparation).

The sequences of Cloned genes key in haustoriagenesis in *S. hermonthica* were submitted in the genebank. NCBI accession Numbers (KP027304, KP027302, KP053296, KP053300, KP027303, KP053297, KP053298, KP053299)