STRIGA HERMONTHICA (Delile) Benth. RESISTANCE MECHANISMS IN KSTP'94 MAIZE VARIETY AND HOST INDUCED GENE SILENCING OF *CYSTEINE PROTEASE*

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(Biotechnology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2018

Striga hermonthica (Delile) Benth.

Resistance Mechanisms in KSTP'94 Maize Variety and Host Induced Gene Silencing of _CYSTEINE PROTEASE_

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A thesis submitted in partial fulfillment for the degree of Master of Science in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

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

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DEDICATION

This thesis is dedicated to my nephews Miguel Junior and Javier Murphy to encourage and cultivate the art of reading in them.

ACKNOWLEDGEMENT

My heart is filled with thankful praises and leaps with joy for every good and perfect gift is from above, coming down from God the father.

I am indebted to extend my heartfelt gratitude to several people, most of whom I will mention here. Sincere appreciation goes to my supervisors Dr. Amos Alakonya for granting me a chance to work on a funded project, supervision and his timely guidance all through. Dr. Steven Runo for allowing me to do my research in his laboratory –The Plant Transformation Laboratory (PTL) Kenyatta University, his continued support and guidance. I also thank Dr. Josaiah Mutuku (Biosciences eastern and central Africa-International Livestock Research Institute) for his unreserved support, supervision and guidance. You always helped me to find direction when I seemed to lose track.

I greatly appreciate Joel Masanga, the lab manager of Plant Transformation Laboratory, Kenyatta University, for his thorough training and truly exceptional work that exceeded expectations. The extra time, selfless effort and diligence you put in were certainly worthwhile. It was a joy and blessing for me to work with such a dedicated and enthusiastic young scientist. I would also like to convey my special thanks to Duncan Ogwenda for taking care of my explants in the farm as well as my transgenic maize plants in the glasshouse. My appreciation too goes to all members of the PTL; who in one way or another were very much helpful during the entire duration of the study program.

Special gratitude goes to my parents Benjamin Mwangangi and Evelyn Makau. My success is in large part due to the loving, financial, moral and spiritual support you gave me. I also appreciate my sister Carol Mutinda and brother Robert Mutinda. You always encouraged me even when things got tough and the motivation that has pushed me past the bumps in the road to be the best that I can be. My nephews Miguel Junior and Javier

murphy whose sunny smiles and joy for life gave me the zeal to work diligently and brightened my dark moments.

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ABBREVIATIONS AND ACRONYMS

2,4-D	2,4-Dichlrophenoxy acetic acid
ANOVA	Analysis of variance
BeCA	Biosciences eastern and central Africa
ILRI	International Livestock Research Institute
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CML	CIMMYT maize line
CIMMYT	International Maize and Wheat Improvement Center
DAP	Days after pollination
E. coli	*Escherichia coli*
FAO	Food and Agriculture Organization
g/L	Grams per liter
HIGS	Host induced gene silencing
IFAD	International Fund for Agriculture Development
IITA	International Institute of Tropical Agriculture
IM	Infection media
IZEs	Immature zygotic embryos
KU	Kenyatta University
KSTP'94	Kakamega *Striga* Tolerant Population
LB	Luria and Bertani

LGB	Larger grain borer
Mg/L	Milligrams per liter
mRNA	Messenger RNA
MS	Murashige and Skoog
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PTL	Plant Transformation Laboratory
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA-interference
RT-PCR	Reverse Transcriptase polymerase chain reaction
siRNA	Small interfering RNA
SSA	Sub-Saharan Africa
µL	Microliter

ABSTRACT

Striga hermonthica is a parasitic weed that causes yield losses in maize-producing countries throughout sub-Saharan Africa. A number of control methods against the noxious weed have been employed with limited success. There is therefore need to develop novel strategies for *Striga* control. The aim of this study was to develop technologies for using maize's natural resistance as well as engineering RNA interference host based resistance in maize against *Striga*. KSTP'94 maize variety has been known to be *Striga* tolerant but its utilization has been limited due to scarce knowledge on the genetic mechanisms underlying this resistance. Genetic engineering through RNA interference (RNAi) offers great promise to management of parasitic plants. However, its applicability to *S. hermonthica* management is limited due to lack of evidence on macromolecular trafficking between host and parasite. This study sought to investigate the movement of *CYSTEINE PROTEASE* messenger RNA (mRNA) – a key enzyme involved in haustoria formation was used. The study first sought to determine the mechanism of host resistance to *S. hermonthica* through histological analysis of a tolerant (KSTP'94) and susceptible (CML 144) maize genotypes. Secondly, movement of *CYSTEINE PROTEASE* mRNAs from *S. hermonthica* to maize was investigated using semi-quantitative Reverse Transcriptase polymerase chain reaction (RT-PCR). Finally, RNAi gene construct targeting silencing of *CYSTEINE PROTEASE* was transformed into maize using *Agrobacterium tumefaciens*. Consequently, second generation transgenic maize were subjected to *S. hermonthica* infection assays and data on the number of *S. hermonthica* plants germinating as well as those attaching onto the host plants analyzed and compared with non-transformed plants. Results showed that: i) KSTP '94 exhibited a higher level of post-attachment resistance to *S. hermonthica* compared to CML 144 owing to the lower number of parasite attachments, and smaller *Striga* seedlings with lower biomass; ii) there was trafficking of *CYSTEINE PROTEASE* mRNA from *S. hermonthica* to maize, and iii) transgenic plants expressing the *CYSTEINE PROTEASE* hairpin construct did not induce resistance against *S. hermonthica.* The resistance mechanism exhibited underscore the need to determine the genetic mechanisms underlying this resistance with the aim of integration of KSTP'94 into breeding programs.

CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Maize (*Zea mays L.*) is widely grown throughout the world in a range of agro-ecological environments and comprises the major staple diet in Sub-Saharan Africa (SSA) (FAO, 2017; IITA, 2011). It is the number one staple food crop on the continent with regards to cultivated area and total grain production (Reynolds *et al.*, 2015). In Kenya, apart from being used as food, maize is also used as animal feed with a per capita consumption estimated at 125kgs per year. (Yoshida and Shirasu, 2009; M'mboyi *et al.*, 2010). However, maize production is constrained by biotic and abiotic stresses including drought, salinity, insect pests and parasitic weeds that lead to a marked reduction of its yields (Diallo *et al.*, 2005; Anami *et al.,* 2008; Thomson, 2008). Among parasitic weeds, the root hemiparasite *S. hermonthica* is the most devastating and dominant constraint on cereal production including maize causing up to 100% yield losses (Khan *et al.*, 2014). Losses due to *S. hermonthica* infestation are estimated at US$7 billion annually, affecting over 100 million farmers (Spallek *et al.,*2013). In Kenya, key areas seriously affected by *S. hermonthica* are the Lake Victoria basin, the coastal strip and many parts of western Kenya (Khan *et al.*, 2006).

Host-parasite interaction begins with secretion of volatile chemical known as strigolactones by roots of the hosts. These cues then induce germination of parasite plant seeds (Matusova *et al.*, 2005). Upon germination, a haustorium (specialized feeding organ) is developed for host attachment and penetration (Yoder, 2001). The haustorium penetrates host roots where it establishes xylem connections for use in siphoning water, nutrients and organic solutes (Yoshida and Shirasu, 2009). Conventional management

strategies against *Striga spp* including cultural, mechanical, biological and chemical control methods have been reported but all have attained limited success in managing the menace (Teka, 2014). Genetic engineering offers an effective alternative for rapidly achieving resistance against *Striga spp* based on reports involving other parasitic plants. For instance, studies have shown that interspecific silencing of a *SHOOT MERISTEMLESS-like* gene in dodder disrupts dodder growth (Alakonya *et al.,* 2012). Efforts to employ Host induced gene silencing (HIGS) to control species from the Orobanchaceae, particularly the genus *Striga*, have yielded varying levels of success (Runo, 2011; Runo *et al*., 2011). RNA interference (RNAi) strategy has also been to develop maize resistant to *Striga asiatica by* targeting metabolic genes and generation of transgenic plants expressing silencing molecules against the target sequences (de framond, 2007). Aly *et al.* (2009) showed that gene silencing of *MANNOSE-6-PHOSPHATE* reductase by expression of double stranded Ribonucleic acid (dsRNA) sequences is effective in controlling *Orobanche aegyptiaca* in tomato. These studies on the selective silencing of target genes by small interfering RNA (siRNA) have paved the way for novel strategies for the control of plant pathogens (Prins *et al*., 2008). Potential host plants harbouring such a construct, designed to silence a gene that is necessary for an important metabolic activity of the parasite, may develop a significant level of resistance to parasitic weed.

Cuscutain, a *CYSTEINE PROTEASE*, is a gene that is activated concomitant to formation of haustoria in *Cuscuta pentagona* and could be a good target for genetic engineering approach towards management of parasitic plants. This is because *CYSTEINE PROTEASEs* are implicated in parasitic pathogen attack implying that it could play a role in parasitic plant interactions such as *S. hermonthica* (Bleischwitz *et al*., 2010). To add strength to this, studies on host-parasite interactions have shown that a reduction of parasite-derived proteins weakens the parasite's infection efficiency and thus strengthens host defense (Bleischwitz *et al*., 2010). The overall aim of this study was to explore host-induced gene silencing of *S. hermonthica CYSTEINE PROTEASE*, a

key enzyme involved in haustoria formation for *S. hermonthica* resistance in maize. The study hypothesized that activity of *CYSTEINE PROTEASE* could play a role in *S. hermonthica* interactions and that expressing small interfering RNA molecules, generated by hairpin constructs, into maize would silence expression of the *CYSTEINE PROTEASE* gene in the parasite upon translocation of signals from the host roots across the haustorium into the parasite in a similar fashion (Alakonya *et al.*, 2012).

For successful parasitism to occur, the haustorium acts as a channel of communication between host and its parasite. It is, however, not known whether macro and micro-molecules from parasitic plants cross this interface into the host. Exploring such trafficking mechanism would ensure delivery of silencing molecules into the parasite for resistance. In line with this, the present study sought to investigate possible movement of mRNA from *S. hermonthica* to maize. Since *Striga spp* establishes vascular connections with their host, it was hypothesized that if trafficking of the mRNA of the protein in context exists, then a silencing construct generating small interfering RNA (siRNAs) targeting the *S. hermonthica CYSTEINE PROTEASE* in host would lead to its downregulation in the parasite thereby thwarting its attachment, growth and development.

1.2. Problem statement

Kenya is home to 49.5 Million people (IFAD, 2017) and 70% of them largely depend on agricultural sector. Food insecurity is a key problem because the agricultural sector experiences decreased productivity due to climate change, lack of farm inputs among smallholder farmers, poor infrastructure as well as constraints from biotic factors (Andersson and Halvarsson, 2011). Maize production is lower than the demand due to biotic and abiotic constraints resulting in food insecurity (FAO, 2017). Key among these is infestation by *S. hermonthica* and *S. asiatica*. It has been estimated that an annual yield loss greater than USD 7 billion in sub-Saharan Africa alone occurs due to these

Striga spp infestation (Ejeta, 2007). Crop losses and the host range of this parasite have continued to increase in spite of the use of widely advocated control methods (Runo *et al.,* 2011). Losses of about 20% to 80% are experienced by the subsistence farmers (Gethi *et al.,* 2005). *Striga spp* reduces growth of their hosts more than it can be accounted for by loss of resources suggesting movement of a cytotoxic or pathogenic factor from parasite to host (Runo and Kuria, 2018).

1.3. Justification

Conventional management strategies against *Striga spp* comprising cultural (hand weeding, crop rotation, early planting and fallowing), biological and chemical (ethylene, herbicides) control methods have been reported but all have attained limited success in managing the menace (Teka, 2014). They are also laborious, time consuming and expensive for small scale farmers in developing countries. There is need, therefore, for a solution to the *Striga spp* menace that is practical, efficient and cost effective to farmers in such countries. In this case, developing *S. hermonthica*-resistant maize germplasm would help reduce losses due to this parasite infestation incurred by maize farmers in sub-Saharan Africa. Host-induced gene silencing of key genes involved in haustoria formation, such as the *CYSTEINE PROTEASE* could generate transgenic maize that enhances resistance to *S. hermonthica*. This is practical, given the successes in management of other parasitic plants.

Reports have demonstrated effective control of the root parasite *Orobanche* in transgenic tomato that expresses RNAi constructs for the parasite *MANNOSE 6-PHOSPHATE* reductase in the host (Aly *et al.,* 2009). Similar studies have shown that interspecific silencing of a *SHOOT MERISTEMLESS-like* gene in dodder disrupts dodder growth (Alakonya *et al.,* 2012). It is therefore vital to explore other genes not well studied but well known to play key roles in parasitism such as *CYSTEINE PROTEASE*. It is important to determine the resistance mechanism of KSTP’94 maize

for it could be used to donate genes for *S. hermonthica* resistance to susceptible maize genotypes. Investigating the trafficking of *S. hermonthica CYSTEINE PROTEASE* into maize is paramound because it could also be used to generate resistant germplasm through RNAi of key genes involved in parasite development.

1.4. Null Hypotheses

i. KSTP'94 maize genotype does not exhibit post-attachment resistance against *S. hermonthica.*
ii. *CYSTEINE PROTEASE* mRNA does not traffic across the *S. hermonthica* maize junction
iii. Transgenic maize expressing siRNAs against *CYSTEINE PROTEASE* does not have post attachment resistance to *S. hermonthica.*

1.5. Objectives

1.5.1. General objective

To determine *Striga hermonthic*a resistance mechanisms in KSTP'94 maize variety and determine host induced gene silencing of *CYSTEINE PROTEASE*

1.5.2 Specific objectives

i. To determine the *Striga* resistance in the KSTP'94 maize genotype.
ii. To determine mRNA movement of *CYSTEINE PROTEASE* from *S. hermonthica* into maize.
iii. To determine efficiency of *CYSTEINE PROTEASE* RNAi as a resistance mechanism against *S. hermonthica.*

CHAPTER TWO

LITERATURE REVIEW

2.1. Maize and its economic importance in Sub-Saharan Africa

Maize (*Zea mays*) comprises the major staple diet in Sub-Saharan Africa (SSA) and is grown widely throughout the world in a range of agro ecological environments (FAO, 2017; IITA, 2011). Maize is the leading crop in the continent both in cultivated area and total grain production (Reynolds *et al*.,2015). In the world, consumption of maize is more than 116 million tons per annum, with Africa consuming 30% while SSA accounts for 21% (IITA, 2011). In Africa, over 300 million people 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 has been reported (De Groote, 2002). Maize can be utilized in many ways, with all parts of the plant such as the grain, cob, stalk, leaves and tassel holding an economic value. The grain is used as food or fermented to produce a wide range of beverages, brews (Anami *et al.*, 2009), livestock feed, industrial inputs of starch, oil, sugar, protein and cellulose (Shiferaw *et al.*, 2011; M'mboyi *et al*., 2010). Maize is rich in essential minerals, vitamins A, B, C and E, carbohydrate, protein and iron, as well as dietary fiber (Rouf Shah *et al*., 2016; IITA, 2011). There has been increased demand in pursuit for alternative source of fuels worldwide. Maize has been used as a source of ethanol and biodiesel, which is a reliable, renewable and environmental friendly alternative for fossil fuel (Yacobucci and Schnepf, 2011;Beagle, 2013). Africa uses 95%, of its maize production as food compared to other world regions that use it as animal feed (IITA, 2011).

2.2. Maize production constraints in Kenya and Africa

There are various production constraints that adversely affect maize production in Kenya. These result from both biotic and abiotic stresses coupled with poor agronomic

practices by the farmers (Pathi *et al*., 2013). Abiotic stresses such as drought, unreliable rainfall and salinity cause reductions in maize yields therefore negatively affecting its economic importance (Thomson, 2008). Among these, drought and unreliable rainfall have a greater effect on production systems in Kenya since maize is grown almost exclusively under rain-fed conditions (Anami *et al.,* 2008). Other abiotic stresses are occurrence of floods in some maize growing areas and low soil nutrient levels (Cairns *et al.*, 2012; Zaidi *et al.,2010).* The major biotic constraints to maize production in SSA include *Striga* weeds, diseases such as maize streak virus (Mwangi and Ely, 2001) and ear rots and pests such as the larger grain borer (*Prostephan ustruncatus*), stem borers, maize weevil (*Sitophilus spp*) (Tefera *et al*., 2011) and the parasitic weeds of the genus *Striga* (Diallo *et al*.,2009).

Striga is commonly known as witchweed because it causes stunted growth and early discoloration of crop leaves before its emergence (Fischer, 2006). It is a noxious root hemi-parasite and most of its life cycle occurs underground. Among the five major *Striga* species (*S. hermonthica, S. asiatica, S. gesnerioides, S. aspera,* and *S. forbesii) S. hermonthica* and *S. asiatica* are the most important cereal weeds. *S. hermonthica* is the most devastating and dominant constraint on maize production that causes up to 100% yield losses leading to US$7 billion losses per annum (Ejeta *et al*., 2007). *S. hermonthica* is a serious constraint to cereal production in the Lake Victoria basin, the coastal strip, and many parts of western Kenya (Khan *et al.*, 2006a). The problem is more devastating in areas where both soil fertility and rainfall are low (Khan *et al*., 2001; Ejeta, 2007). *Striga spp* infestation continues to spread to new areas as farmers leave heavily infested fields for new ones (Khan *et al.*, 2002; Gressel *et al.*, 2004).

2.3. Origin, occurrence and distribution of *Striga spp*

The genus *Striga* has 30–35 species, 80% of these, are found in Africa while the rest occur in Asia and the United States (Runo *et al*.,2012). Studies have shown that *S.*

hermonthica and *S. asiatica* originated from the Nubian hills of Sudan and Semien mountains of Ethiopia (Atera *et al.*, 2014) and that sorghum and pearl millet also originated from these areas (Ejeta, 2007). *S. gesnerioides* may have originated in West Africa (Mohamed *et al.*, 2007). *S. hermonthica* is widespread throughout northern tropical Africa and extends from Ethiopia and Sudan to West Africa. It also extends from the western Arabian region southwards into Angola and Namibia. *S. asiatica* has extensive distribution and is found throughout semi-arid areas of tropical and subtropical Africa, Asia, and (Ejeta, 2007). Presently most parts of Africa are infested with this parasite with heavy infestation in some parts of east and West Africa. Moderate to light infestation is shown in central Africa and Southern Africa, has light infection except Botswana which is heavily infested as shown in Figure 2.1.

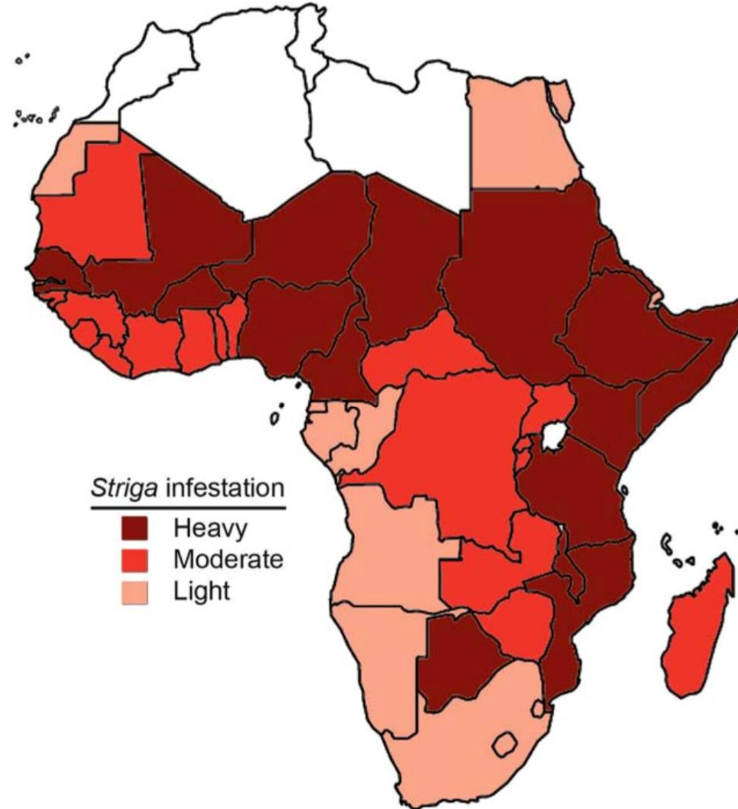

Figure 2.1: Distribution and infestation levels of *Striga spp* with in Africa (Ejeta, 2007)

2.4. *Striga* lifecycle and its economic importance

The lifecycle of *Striga* is synchronized to that of its host (Figure 2.2) and involves mechanisms that coordinate the lifecycle of the parasite and that of the host (Bouwmeester *et al*., 2003). The *Striga* life cycle generally involves: germination, host attachment, formation of haustoria, penetration, and establishment of vascular connections, nutrients accumulation, flowering and production of seeds (Parker *et al*., 1993). *Striga* seeds only germinate in presence of certain hormones known as strigolactones, produced by the host and in other cases non-host species (Keyes *et al*., 2007). The parasite seedlings attach to the host and form vascular connections with the host, robbing it of its water, carbohydrates and minerals (Yoshida and Shirasu, 2009).

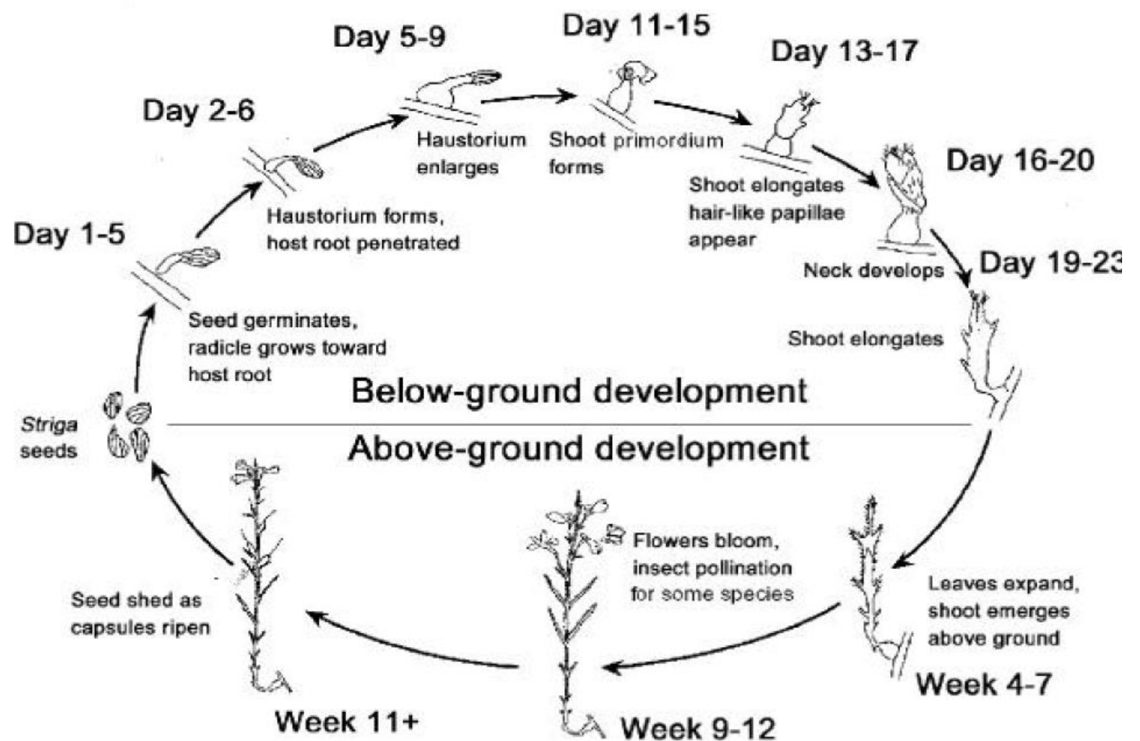

Figure 2.2: Major stages in the life cycle of Striga of development (Bouwmeester *et al*., 2003)

Economic losses due to *Striga spp* are vast. This is a parasite mainly for tropical cereal crops, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), and upland rice (*Oryza sativa*) (Press *et al*., 2001). These are parasitized by *S. hermonthica* and *Striga asiatica*, whereas legumes are parasitized by *S. gesnerioides.* The latter species is a serious constraint to cowpea production (Press *et al*., 2001). In

addition, it causes a reduced growth to the host because it disrupts its photosynthesis and utilizes its nutrients causing a deficit (Joel, 2007). The weed consumes a greater part of the host plants' solutes, causing wilting and early death of the plant (Ruyter-Spira *et al.*, 2011). It negatively affects host food resources which in most cases are usually already under considerable strain. It has been estimated that an annual yield loss greater than $7 billion in sub-Saharan Africa alone occurs due to *Striga spp* infestation (Spallek *et al.,* 2013). It has also been shown that 100×10^6ha of the African savannah zones are infested with *Striga spp* (Ejeta, 2007). The amount of yield loss depends on factors such as *Striga* density, species of the host, land use system, amount of soil nutrients and rainfall pattern (Atera *et al.*, 2012).

2.5. *Striga spp* control strategies

Striga is among the chief problems facing farmers in Africa hence there is need for an effective control method to combat it (Andersson and Halvarsson, 2011). This, however, requires understanding the physiological and metabolic interactions between host and the parasite (Ejeta, 2007). Most control strategies have aimed at disrupting the *Striga* life cycle hence causing death to the parasite. *Striga* is primarily a problem for small-scale subsistence farmers with limited options for external inputs. Over the past years, different control strategies have been recommended against *Striga spp.* Cultural practices such as crop rotation (Oswald and Ransom, 2001) with non-host intercrops (trap crops) (Udom, 2007), fertilizer application (Jamil *et al.*, 2011), soil and water management (Fasil and Verkleij, 2007), hand weeding (Ransom, 2000) and transplanting (Oswald and Ransom, 2001) have been attempted with limited success. Inter-cropping cereals with legumes is another low cost and viable strategy that has been reported to influence *Striga spp* infestation. For instance, (Khan *et al*. (2007) demonstrated that intercropping legumes with maize and sorghum reduces *S. hermonthica* but does not eliminate the weed. Other methods used include biocontrol using the fungus *Fusarium* (Ciotola *et al*., 2000) use of herbicides (imazapyr-treated maize seed and glyphosate-

resistant bean and sunflower) (De Groote *et al.*, 2007) and development of *Striga*-resistant germplasm (Ejeta, 2007). Limited knowledge of the biology of the parasite-host interaction limits *Striga spp* management practices since such information is vital for development of appropriate management strategies using both genetic modification (GM) and non-GM approaches (Runo *et al.,* 2011).

Genetic engineering offers promise of rapidly achieving resistance against *Striga spp*. Recent findings have shown that RNAs freely translocate between parasitic plants and their hosts (Kim and Westwood, 2015; Roney *et al.*, 2007). This translocation suggest a possibility that RNA-interference (RNAi) could be used as a potent tool to interfere with vital processes in the parasite by transforming the host with an RNAi construct that targets gene sequences specific to the parasite. Tomilov *et al*. (2006) demonstrated that interfering hairpin constructs transformed into host plants could downregulate expression of the targeted genes in the parasite. Recently, researchers have developed transgenic sorghum expressing dsRNAs against *Striga* (Runo *et al.*, 2011). A similar strategy that targeted *Striga* metabolic genes to engineer resistance in transgenic maize did not yield events with a discernable resistance phenotype, possibly owing to lack of translocation of the siRNAs through the haustorium or low transgene expression (de Framond *et al.*, 2007).

2.6. Genetic transformation of maize

Genetic engineering involves isolation and transfer of genes with well-known roles into a plant genome (Hansen, 2000). This entails making constructs with the target gene in a suitable vector in between an ideal promoter and terminator. *Agrobacterium*-mediated transformation is widely used in transformation of plants although other gene transfer methods such as electroporation and particle bombardment have also been used in the past (Ishida *et al.*, 2007). *A. tumefaciens*-mediated technique is often preferred because it results to transfer of large fragments of DNA with minimal rearrangements into the

genome hence providing a stable low copy number transformants (Ishida *et al*., 2007). Immature zygotic embryos are widely employed explants in maize transformation due to their high efficiency in transformability (Frame *et al.*, 2000; Negrotto *et al.*, 2000; Ishida *et al*., 2007).

2.7. RNA trafficking in parasitic plants

Most parasitic plants establish vascular connections with their hosts aiding in transfer of not only nutrients and water but also macromolecules such proteins, mRNA, metabolites and pathogen such as viruses (Kim and Westwood, 2015). The mRNA molecules are not restricted to the cells in which they are synthesized but move from cell to cell and even over long distances through the phloem (Lucas and Lee, 2004) with information that coordinate plant development (LeBlanc *et al*., 2012). Symplastic connections allow transfer of mRNA from the parasite to the host and vice versa (Roney *et al*., 2007). For instance, host mRNA was detected 30 cm far from the point of infection in the parasite phloem in tomato-*Cuscuta pentagona* interaction (David-schwartz, 2008). Tomato-*Philepanche aegyptiaca* interaction in which dextrans up to 70 kDa in size were detected in the parasite show that macromolecules are also transferred from the host to the parasite through the xylem (Aly *et al*., 2011).

Macromolecular transfer is bidirectional as shown by the transfer of different mRNAs between *Cuscuta* spp. and its hosts (Roney *et al*., 2007). The mobility of macromolecules has been used in control of parasitic weeds in which silencing constructs targeting essential genes are transformed to host plant producing siRNA that suppress parasite growth (Tomilov *et al*., 2008). Their study demonstrated that the silencing signal move from one host to another using the parasite as a bridge and that the siRNA signals are bidirectional and can travel over long distances.

Host induced gene silencing (HIGS) has been used to silence genes, for instance, *MANNOSE-6-PHOSPHATE* in *P. aegypitiaca* (Aly *et al*., 2009) while RNAi of *KNOTTED-like* homeobox transcription factor *SHOOT MERISTEMLESS–like* in *Cuscuta pentagonia* was shown to significantly reduce parasite growth (Alakonya *et al.*, 2012). Results from these research show that successful translocation of siRNAs is effective for controlling parasitic plants. This technology is, however, limited by lack of knowledge on candidate genes to target for use in genetic transformation.

2.8. Host induced gene silencing against parasitic plants

Parasitic plants are difficult to control by chemical methods because their life cycle is synchronized with that of their host plant (Bouwmeester *et al*.,2003). Identification of long-distance movement of RNA molecules between parasitic plants and their hosts by Westwood and Bouwmeester (2009), set up a stage for use of HIGS strategy targeting specific gene sequences in parasitic plants in development of host resistance. RNAi strategy involves genetic modification of plants to express double stranded ribonucleic acid (dsRNA) molecules with sequences derived from the gene of interest (Wesley *et al*.,2001). To make the RNAi construct, a partial or full-length coding sequence is cloned in the sense and antisense orientations, separated by an intron with a suitable promoter. The dsRNA is cleaved into short 21–25- nucleotide RNAs by DICER-like enzyme. The dicer cleavage products are referred to as short interfering RNA or siRNA (Zamore *et al.*, 2000). One strand (passenger strand) is degraded and the other strand (guiding strand) of the siRNA is incorporated into an RNA-induced silencing complex (RISC) in conjunction with the Argonaute multi domain protein, which contains an RNAse H-like domain responsible for guiding the complex to bind and destroy homologous transcripts (Martinez *et al.*, 2002). Subsequently, the cleavage of this strands brings about silencing of target genes in the infecting parasite (Bakhetia *et al.*, 2005).

Several studies have shown translocation of dsRNA and/or siRNAs from plants to various pathogens through HIGS (Koch and Kogel, 2014). Different levels of success of host resistance have been achieved using this strategy (Runo *et al.*, 2012). Recently, studies have shown that interspecific silencing of *STM* (*SHOOT MERISTEMLESS*) gene disrupt dodder (*Cuscuta pentagona*) parasitism in Transgenic tobacco expressing an RNAi directed against the dodder (Alakonya *et al.*, 2012).

2.9. Role of *CYSTEINE PROTEASEs* in parasitism

Proteases are endopeptidases that cut the internal bonds of polypeptide chains (Rawlings and Barrett, 1994). Proteases are classified into four groups based on their active site and mechanism of action; including serine proteases, *CYSTEINE PROTEASEs*, aspartic proteases, threonine proteases and metallo-proteases (Rawlings *et al.,* 2004). *CYSTEINE PROTEASEs* are found in all organisms with diverse functions including programmed cell death (PCD) in response to both developmental cues and pathogen, regulation of epidermal cell fate among others (Niño *et al.,* 2014). Papain and cathepsin belong to the most abundant family of *CYSTEINE PROTEASEs* (van der Hoorn, 2008). There are two clans of *CYSTEINE PROTEASEs* implicated in plant diseases and resistance including clan A and clan E (CA and CE) (Rawlings *et al.* ,2014). *CYSTEINE PROTEASEs* in these two clans have a Papain-like fold with the CA being the most abundant in species of Bacteria, protozoa, fungi, plants , animals and viruses (Rawlings *et al.* ,2014).

Papain like *CYSTEINE PROTEASEs* (PLCP) play crucial roles in plant-pathogen interactions. For preventing unwanted digestion, *CYSTEINE PROTEASEs* are synthesized as zymogens, and contain a prodomain (regulatory) and a mature domain (catalytic). The prodomain acts as an endogenous inhibitor of the mature enzyme and for activation of the mature enzyme, removal of the prodomain is necessary and achieved by different modes (Bleischwitz *et al.,* 2010). *CYSTEINE PROTEASEs* have a His-Cys-Asn triad in their active site. The histidine residue acts as a proton donor and enhances the

nucleophilicity of the cystine residue. This enzyme has been shown to play a role in the successful infection process, possibly by weakening host structures through protein degradation in *Cuscuta* (Bleischwitz *et al.,* 2010). Cuscutain, an ortholog of *CYSTEINE PROTEASE* is a gene that is activated concomitant to formation of haustoria in *Cuscuta pentagona*. It encodes a pre-pro-protein, with each protein subunits having a different role. The pre-peptide targets the Cuscutain primary protein to the extracellular space. In the extracellular space unprocessed translation product is cleaved and deleted from the pre- and pro-peptide. Deletion of the inhibitor pro-peptide converts Cuscutain from an inactive form to an active enzyme with a similar function as *CYSTEINE PROTEASE.* When the enzyme is released it plays a role of infection by weakening host structures through protein degradation (Bleischwitz *et al*., 2010). *CYSTEINE PROTEASEs* have been identified on surfaces of plants and various pathogens like bacteria, fungi, oomycetes, nematodes insects or herbivores as they interact. *CYSTEINE PROTEASEs* are implicated in parasitic pathogen attack (Bleischwitz *et al*., 2010). This implies that *CYSTEINE PROTEASE* could play a role in parasitic plant interactions such as *S. hermonthica.*

Several genes involved in haustorial development have been identified in *S. hermonthica* (Kirigia *et al*., 2014). Key among them is *CYSTEINE PROTEASE*, and in Cuscuta, *CYSTEINE PROTEASE* enzymes have been shown to play a role in successful infection, possibly by weakening host structures through protein degradation (Bleischwitz *et al.,* 2010). In the current study, I hypothesized that this enzyme play a similar role in *S. hermonthica* infection and therefore can be targeted for downregulation through host induced gene silencing in maize.

2.10. Sources of natural resistance to *Striga* parasitism in maize

The most effective and sustainable control strategy against *Striga* is an integrated approach that utilises innate host-derived resistance. Therefore, identification of new

sources of *Striga* resistance has been prioritised in *Striga* resistance breeding programs. Sources of resistance to *Striga* have been identified in maize (Amusan, *et al*., 2008), rice (Gurney *et al*., 2006), sorghum (Mohamed *et al*., 2003; Haussmann *et al*., 2004; Mbuvi *et al*., 2017) and cowpea (Menkir, 2006). Such host based *Striga* resistance mechanisms act either before (pre-attachment resistance) or after infection (post-attachment resistance). Pre-attachment resistance occurs when a host produces low amounts of strigolactones or when *Striga* receptors that perceive germination stimulants are insensitive to the strigolactone produced by the host. This is because for *Striga* germination to occur, strigolactone binds to HYPERSENSITIVE TO LIGHT (HTL) receptors in *Striga*. Binding causes degradation of an F-box protein, which in turn activates gene regulatory processes that lead to *Striga* germination (Lumba *et al*., 2017). Pre-attachment resistance can also be due to production of low haustorial initiation factors whose effect is failure by *Striga* to develop haustorium effectively (Rich *et al*., 2004). In contrast, post-attachment *Striga* resistance mechanisms act after *Striga* has attached and attempted to penetrate the host. These mechanisms result in physiological or biochemical barriers, which prevent the *Striga* haustorium from connecting to the host xylem. Host plants can also produce secondary metabolites that block parasite ingression or induce a hypersensitive immune response at the host-parasite interphase (van Dam and Bouwmeester, 2016). In some instances, *Striga* produces enzymes that degrade host tissues and barriers before making xylem connection (Rogers and Nelson, 1962; Maiti *et al*., 1984).

Maize is alien to Africa and as a result, it is generally more susceptible to the weed. Therefore, *Striga* resistance in maize could only be sourced from wild grass relatives like *Zea diploperennis* (Lane *et al*., 1997; Amusan *et al*., 2008) and in *Tripsacum dactyloides* (Gutierezz-Marcos *et al*., 2003). Such efforts have led to development of *Striga* resistant inbred line ZD05 suitable for integration in breeding programs in Western Africa (Amusan *et al*., 2008). In Eastern Africa, the open pollinated maize variety KSTP'94 has since 1995 been used as *Striga* tolerant maize variety especially in

Western Kenya, a *Striga* prone region. It exhibits remarkable resistance to *Striga* under field conditions; a characteristic that has made it a subject of intense research in the region. Such research has found the resistance of KSTP'94 to be due to production of low amounts of the strigolactone sorgomol (Yoneyama *et al*., 2015). Sorgmol is a strigolactone that does not efficiently induce *Striga* germination and therefore the resistance of KSTP'94 was concluded to be due to pre-attachment resistance.

2.11. Screening for *Striga* resistance

Development of *Striga*-resistant cultivars has been limited by lack of dependable screening techniques (Yagoub *et al.*, 2014). Some of the screening techniques that have been employed in the past include laboratory assaying methods, pot screening and field trials (Rodenburg *et al.*, 2015). Though field screening practice helps generate statistics on *Striga* infestation under natural conditions, the method is limited by the existing environmental effects. To circumvent this and initiate a reliable post-attachment screening, rhizotron screening system is ideal (Rodenburg *et al*., 2015). Rhizotrons are transparent root observation chambers which enable *Striga* attached to the host plant to be counted, evaluation of phenotype of the resistance mechanisms and determination of the effect of *Striga* on host biomass over a period of time and with minimal disturbance (Cissoko *et al.*, 2011; Gurney *et al.*, 2006; Runo *et al*., 2012; Rodenburg *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Validation of Post-attachment *S. hermonthica* resistance mechanisms in KSTP'94 and CML 144 maize genotypes

3.1.1. Preconditioning for *S. hermonthica* seeds

Striga hermonthica seeds (obtained from maize growing fields in Kibos, Western Kenya in 2015) were used for post-attachment resistance assays. Seeds were preconditioned as described by Gurney *et al.* (2003) before germination. Firstly, *S. hermonthica* seeds (25mg) were surface sterilized using 10% (v/v) NaOCl for 10 minutes with gentle agitation, rinsed three times with sterilized distilled water then spread on a glass fiber filter paper (Whatman GFA) placed in sterile petri dishes. Seeds were then incubated for 11 days at 29ºC. Finally, seeds were germinated by treating with 3ml of 0.1ppm GR24 (Chirax, Amsterdam), which is a synthetic strigolactone and incubated overnight at 29ºC. Germination efficiency of the *S. hermonthica* seedlings was determined using Leica MZ7F microscope (Leica inc.) and only plates showing >70% germination were used to infect maize roots.

3.1.2. Infection of *S. hermonthica* seedlings on maize

Maize inbred line CML 144 (CIMMYT) and open pollinated variety (OPV) KSTP'94 (KALRO) were screened for post-attachment *S. hermonthica* resistance. Seeds were first germinated in 10×10×7 cm pots filled with vermiculate and 5 days post planting, seedlings were transferred to root observation chambers, rhizotrons – (25×25×5cm) perspex chambers (Gurney *et al.*, 2006) prepared as follows: Chambers were first packed with vermiculite and lined with a 25×5×5cm foam strip at the bottom to absorb excess water and a 50-micron thick mesh placed on top. A germinated maize seedling

was placed on the mesh, chamber closed and wrapped with aluminium foil. Plants were then maintained in the glasshouse at 12-h photoperiod with 60% humidity and day and night temperatures of 28ºC and 24ºC respectively. During growth on rhizotrons, plants were drip irrigated with 25ml of 40% Long Ashton nutrient solution for 3 minutes (Hudson, 1967). Maize seedlings with well-developed roots (10 days on rhizotrons) were then infected with 25mg of pre-germinated *S. hermonthica* seeds by aligning the *S. hermonthica* seeds along the maize roots with a soft paint brush. Five plants per genotype were screened and the experiment replicated three times.

3.1.3. Analysis of post-attachment *S. hermonthica* resistance in maize

3.1.3.1. Measures of *S. hermonthica* resistance

Infected maize roots were screened for *S. hermonthica* resistance at 9 and 21 days after infection (DAI). At 9 DAI, *S. hermonthica* seedlings attached on maize roots were observed and documented using a Stereomicroscope (Leica MZ4 fitted with DFC320FX camera (Leica, Germany). After 21 days, *S. hermonthica* attached to maize roots were harvested, placed on 90mm petri plates and photographed. Image analysis using ImageJ, v. 1.45 (http://rsb.info.nih.gov/ij/) was then carried out to determine the length and the number of *S. hermonthica* parasitizing each host plant. To determine the total *S. hermonthica* biomass attached on maize roots, harvested *S. hermonthica* seedlings were oven dried for 7 days at 45ºC and weighed.

3.1.3.2. Histological analysis of *S. hermonthica* resistance in maize

To determine the extent of parasite development within the host root, microscopic screening of the connection point between *S. hermonthica* and maize roots was carried out according to Gurney *et al.* (2003). Tissues at the point of host-parasite infection were collected from rhizotrons, 9 DAI and fixed using Carnoy's fixative (4:1 ethanol:acetic acid). This was followed by dehydration with 100% absolute ethanol for 30 minutes,

pre-infiltration in ethanol-Technovit (Haraeus Kulzer GmbH) solution for 2 h, and a further pre-infiltration step in 100% Technovit solution for 1 h. These tissues were then left in fresh 100% Technovit for 3 days. For embedding, samples were placed in Eppendorf lid molds containing 1 part Technovit 1 and 15 parts hardner2 and left to set. Embedded tissues were then mounted on wooden blocks using the Technovit 3040 kit following the manufacturer's instructions (Haraeus Kulzer GmbH). Small sections (5 micron-thick) were cut using a microtome (Leica RM 2145) and transferred to glass slides. The sections were stained using 0.1% Toluidine Blue O dye in 100Mm phosphate buffer for 2 minutes, washed in distilled water and dried at 65°C for 30 minutes. Microscope slides were then covered with slips using DePex (BDH, Poole, UK), observed and photographed using a Leica microscope mounted with a DFC camera (mm).

3.2. Determination of mRNA trafficking of *S. hermonthica CYSTEINE PROTEASE* in maize

Maize (CML 144 and KSTP) was germinated and transferred to rhizotrons packed with vermiculite as earlier described. After 11 days of growth, maize roots were infected with pre-germinated *Striga* seeds as described in section 3.1.1 above and these were feed with Long Ashton nutrient solution for 21 days. The infected tissues were collected at 5cm, 10cm and 15cm from the point of attachment including controls; uninfected maize and *S. hermonthica* for RNA extraction.

3.2.1. RNA Extraction

RNA was extracted from maize roots infected with *S. hermonthica*, unifected maize and *S. hermonthica* using the Qiagen RNeasy kit according to the manufacturer's instructions. In brief, 25mg of the tissues was ground under liquid nitrogen then disrupted using 350µl of RLT buffer and 450µl of 70% ethanol followed by

centrifugation at 10,000 rpm. 700µl of the sample was transferred to an RNeasy mini spin column placed in a 2ml collection tube and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. DNase 1 stock solution was prepared by injecting 550µl of RNase-free water into the DNase vial using a needle and syringe. The total RNA subjected to DNase I treatment by adding 80µl of the DNase I mix directly to the RNeasy column membrane and allowing it to stand at room temperature for 15 minutes. 350µl of the binding buffer (RW1) was added to the column and centrifuged at 10,000 rpm for 15 seconds. The flow through was discarded and 500µl of RPE buffer (washing buffer) added to the RNeasy spin column followed by spinning at 10,000 rpm for 15 seconds. The flow through was discarded and another 500µl of RPE buffer added followed by spinning at 10,000 rpm for 2 minutes. The spin column was placed in a new 2ml collection tube and centrifuged at 10,000rpm for 1 minute to dry the membrane. The column was placed in a new 1.5ml collection tube and 50µl of RNase-free water added directly to the spin column membrane and centrifuged at 10,000 rpm for 1 minute to elute the RNA. The RNA was quantified using a nanodrop and 5µg used for cDNA synthesis.

3.2.2. Complimentary DNA (cDNA) synthesis and Reverse transcriptase polymerase chain reaction

First strand complementary DNA (cDNA) synthesis was performed using SuperscriptIII Reverse transcriptase polymerase chain reaction (RT-PCR) kit (Invitrogen, CAT 18080-051, Carlsbad, U.S.A) in Bio-Rad thermocycler. Following the manufacturer's instructions, 5µg/µl of the total RNA was first primed using 50ng/µl of random hexamers, 10mM dNTP mix and diethyl pyrocarbonate (DEPC)-treated water to a 10µl volume. This mixture was first incubated at 65°C for 5 minutes and then transferred to ice for 1 minute. A master mix was prepared by adding 2µl of 10X RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT, 1µl of 40U/µl RNaseOUT and 1µl of 200U/µl Superscript III reverse transcriptase enzyme in a separate tube. This was mixed by finger

flicking and then 10µl added to the RNA/primer mixture above and mixed gently. This was incubated for 5 minutes at 25°C followed by 50 minutes at 50°C, then heated at 85°C for 5 minutes. Finally, 1µl of RNase H was added to the mixture and incubated for 20 minutes at 37□. The cDNA was stored at -20ºC to await PCR amplification. PCR amplification was done using specific primers for *S. hermonthica CYSTEINE PROTEASE* based on NCBI gene bank sequence accession no KP027303. The oligonucleotide primers were; F: 5' GTA CGG GTA ATC TTC GGA ATC3' and R: 3' TGT GGG AGT TGC TGG GCG TTC 3. Polymerase chain reaction amplification was carried in a 20µl reaction mixture using a Bio-Rad thermocycler. The reaction mixture comprised 5µl of 5 X Taq polymerase reaction mix (New England Bio-labs Inc., MA, USA), 1µl of 0.25µM of each primer (forward and reverse) and 1µl of the template cDNA synthesized above. The PCR conditions included pre-heating at 94□ for 5 minutes, 35 cycles of denaturation at 94□ for 30 seconds, annealing at 63□ for 30 seconds, a 68□ extension for 1 minute and then a 10 minutes final extension at 72□. The PCR product was stained with 3µl of Gel red and marked with DNA loading dye then electrophoresed on 1% agarose gel in Tris-acetate-EDTA (TAE) buffer. The gel was run at 110 volts for 45 minutes and then visualized under UV light in a trans-illuminator.

3.2.3. Sequence analysis, siRNA prediction and search for off targets in maize

The *S. hermonthica CYSTEINE PROTEASE* gene sequence (accession no KP027303) was obtained from National Center for Biotechnology Information (NCBI) and a nucleotide blast (blastn) carried out. Related *CYSTEINE PROTEASE* sequences from *Pisum sativum* (Z68291.1), *Nicotiana tabacum* (AB032168.1), *Arachis hypogaea* (DQ011884.1), *Phaseolus vulgaris* (AJ224766.1), *Carica papaya* (JN689334.1), *Solanum lycopersicum* (LN736308.1), *Sorghum bicolor* (XM002447255.2), *Zea mays* (EU117211.1), *Oryza sativa* (X80876.1) and *Rattus norvegicus* (U19866.1) were also identified and downloaded. Sequence alignment was performed using the default

parameters of MUSCLE in MEGA software version 7.0. A phylogenetic analysis was then done and presented as a tree using the Neighbor-Joining (NJ) algorithm (Tamura *et al.*, 2011) with bootstrap test of phylogeny of 1,000 replicates. The *S. hermonthica CYSTEINE PROTEASE* sequence was used as a query to search for putative effective siRNAs and potential off-target candidates against the maize mRNA database with the RNAi scan tool (http://bioinfo2.noble.org/cgi-bin/RNAiScan/RNAiScan.pl). The summary of the query had 5' antisense strand, starting with an A or U base, a 5' sense strand, starting with a G or C, the first seven bases of antisense strand (5' to3') have at least 5A or U bases and the percentage GC content 30-70% (Appendix I).

3.3. Generation and evaluation of transgenic maize expressing siRNAs against *S. hermonthica CYSTEINE PROTEASE*

3.3.1. Generation of a silencing construct

3.3.1.1. Growth of *Striga* and RNA Extraction

This work was carried out at the plant transformation laboratory Kenyatta University. Maize seeds (CML 144) was grown in soil infected with preconditioned *S. hermonthica* seeds. *S.hermonthica* plantlets were allowed to emerge above the soil and the roots were harvested for RNA extraction using the Qiagen RNeasy kit according to the manufacturer's instructions. RNA Extraction was done as described in section 3.2.1.

3.3.1.2. Complimentary DNA (cDNA) synthesis

First strand cDNA synthesis was performed using Superscript III Reverse transcriptase polymerase chain reaction (RT-PCR) kit (Invitrogen) in Bio-Rad thermocycler. The total RNA extracted in section 3.3.1.1 was reverse transcribed to cDNA as earlier described in section 3.2.3 above. Five (5) µg/µl of the total RNA was used for cDNA synthesis. The cDNA was stored at -20ºC to await amplification by PCR.

3.3.1.3. PCR amplification of *S. hermonthica CYSTEINE PROTEASE* and PCR purification

PCR amplification was carried out using designed primers based on NCBI gene bank sequence described above (accession no KP027303, AppendixIII). Polymerase chain reaction amplification was carried out in a 50µl reaction mixture using a Bio-Rad thermocycler. The reaction mixture composed of 25µl of X5 Taq (New England Bio-labs Inc., MA, USA), 2.5µl of 0.25µMof each primer (forward primer and reverse), 5µl of template cDNA synthesized above. The PCR conditions included pre-heating at 94℃ for 5 minutes, 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 63℃ for 30 seconds, a 68℃ extension for 1 minute and then a 10 minutes final extension at 68℃. The PCR product was stained with gel red and marked with DNA loading dye then electrophoresed on 1% agarose gel in TAE buffer. The gel was run at 110 volts for 45 minutes and then visualized under UV light in a trans-illuminator. The DNA was purified by ethanol precipitation by adding 10µl of 3M sodium acetate to 30µl of the PCR product. To this mixture, 300µl of 100% ice cold ethanol was added and incubated at -20℃ for 1h. The mixture was centrifuged at 10,000 rpm for 15 minutes and the supernatant discarded. Pelleted DNA was then washed with 700µl of 70% ethanol and centrifuged again for 15 minutes at 10,000 rpm after which the pellet was air-dried and resuspended in 50µl of deionised water. A gel was run at 110 volts for 45 minutes and then visualized under UV light in a trans-illuminator.

3.3.1.4. Preparation of competent *E. coli cells*

Disarmed empty *Escherichia coli* cells, strain DH5α, were used in cloning according to Tu *et al.*(2005). The DH5α cells were grown on plates with solid Luria and Bertani (LB) media for two days in an incubator at 37℃. A colony was picked and inoculated in 40 ml liquid LB media without antibiotics and incubated overnight at 37℃ with shaking. The overnight culture was inoculated in a fresh 30 ml liquid LB media and incubated at

37□ for two hours with shaking. A 0.1M solution of $CaCl_2$ was prepared and chilled at -20ºC. The cells were centrifuged for 2 minutes at 4ºC and the pellet transferred into 2ml tubes and chilled on ice for 1h. The cells were resuspended in 1ml of sterile ice cold 0.1M $CaCl_2$ and incubated on ice for 1h before spinning for 2 minutes at 13000 rpm at 4ºC. They were then resuspended again in 200µl of ice cold 0.1M $CaCl_2$ and stored at 4ºC awaiting transformation.

3.3.1.5. Topo Cloning and *E. coli* transformation

The entry vector PCR8/GW/TOPO TA (Figure 3.1) and the purified *CYSTEINE PROTEASE* PCR product were used in this step. The cloning reaction was set up as shown in table 3.1. The reagents were mixed by gently flicking the tubes and incubated for 5 minutes at room temperature. The mixture was stored at -20ºC before being used to transform E. *coli*.

Table 3. 1 Reaction mix for cloning of the *CYSTEINE PROTEASE* gene into PCR8/GW/TOPO TA

Reagent	Reaction volume (µl)
PCR product	2
Salt solution	0.5
TOPO vector	0.5
Total volume	3

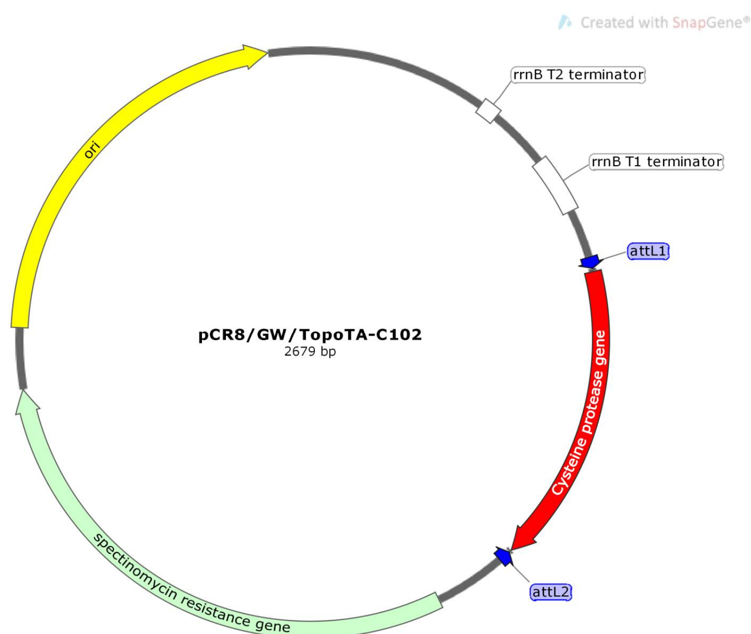

Figure 3.1: Map of PCR8/GW/TOPOTA entry vector. Schematic representation of the entry vector topo with the *CYSTEINE PROTEASE* sequence flanked by attL1 and attL2 recombination sites and Spectinomycin resistance gene for selection of positive colonies.

The PCR8/GW/TOPOTA recombinant vector containing a fragment from the putative gene was used to transform the chemically competent *E. coli* cells. Briefly, 50µl of the chemically competent cells was thawed on ice after which 3µl of the cloning reaction

was added and mixed by finger flicking. This mixture was incubated on ice for 30minutes and heat shocked in a 42℃ water-bath for 90 seconds then immediately transferred to ice. LB broth media (250µl) was added and incubated on a rotator shaker at 37℃ for the cells to grow. Fifty (50) µl of the bacterial culture was spread on pre-warmed LB solid media containing 100mg/L spectinomycin and grown overnight at 37℃. Positive (pTF102 vector with a spectinomycin resistance gene) and negative (non-transformed competent cells) controls were included.

3.3.1.7. Plasmid extraction of the PCR/GW/TOPO/TA recombinant vector

Two colonies of the cloned *CYSTEINE PROTEASE* fragments were selected and grown overnight in 40ml LB broth media containing spectinomycin at 37℃ on a rotary shaker. The overnight culture was centrifuged at 6000 revolutions per minute for 15 minutes. The cells were resuspended in 300µl of buffer P1 (Tris-EDTA and glucose in addition to RNAase A) following the Qiaprep spin miniprep kit (Qiagen, CAT. 27104, Valencia) manual. Then, 300µl of P2 lysis buffer was added and mixed thoroughly by inverting the tube 5 times and incubated at room temperature for 5 minutes. 300µl of neutralizing buffer (N3) was added, mixed by inverting the tube 5 times and centrifuged for 10 minutes at 13000 rpm. The supernatant was applied to a Qiaprep spin column by pipetting and centrifuged for 1 minute at 13000 rpm followed by discarding of the flow through. 500µl of binding buffer (PB) was added, centrifuged for 1 minute and the flow through discarded. Then, 750µl of the wash buffer containing ethanol (PE) was added and centrifuged for 1 minute. The flow through was discarded and the columns centrifuged for 1 minute to remove the residue wash buffer. The QIAprep spin columns were placed in clean 1.5ml eppendorf tubes and 50µl of elution buffer added directly to membrane and let to stand for 1 minute before centrifugation. To confirm the presence of plasmid DNA a gel was run at 110 volts for 45 minutes and then visualized under UV light in a trans-illuminator. The plasmid was then stored at -20ºC.

3.3.1.8. Restriction digestion of PCR8/GW/TOPO TA/*CYSTEINE PROTEASE* and sequencing

To verify successful ligation of the *CYSTEINE PROTEASE* insert in the vector, a restriction digestion was carried out using EcoRV enzyme. A 30µl reaction containing 10µl of the template DNA, 3µl buffer, 16.5µl PCR water and 0.5µl of EcoRV (New England Bio-labs Inc., MA, USA) was set up. This was aliquoted into PCR tubes, incubated at 37℃ for 1h and gel electrophoresis done thereafter to confirm restriction. The positive clones were sent for sequencing at the International Livestock Research Institute (ILRI) using the forward *CYSTEINE PROTEASE* primer.

3.3.1.9. Sub-cloning of *CYSTEINE PROTEASE* into binary vector

To sub-clone *CYSTEINE PROTEASE* from entry into the expression vector pStargate (Figure 3.2), an LR Clonase™ reaction was set up using 3µl of the linearized clean PCR8/TOPOTA/ *CYSTEINE PROTEASE*, 1µl of the binary vector (pStargate) and 1µl of the Clonase enzyme and the mixture incubated for 1 hour at room temperature according to the LR cloning kit user manual (Invitrogen Corp. Carlsbad CA, USA). Then, 0.5µl of proteinase K was added to the reaction followed by 10 minutes of incubation at 37ºC. After the reaction, 2µl of the reaction was used to transform 30µl of *E. coli* chemically competent cells (prepared in section 3.3.1.4).

Confirmation of presence of the *CYSTEINE PROTEASE* insert into the binary vector was carried out using colony PCR targeting the *CYSTEINE PROTEASE* transgene. In summary, a PCR reaction mix (without template DNA) was set up using Taq polymerase reaction mix and *CYSTEINE PROTEASE* primers as earlier described. The reaction mixture was then aliquoted into 200µl tubes. Template DNA was added by touching a bacterial colony, using a sterile pipette tip, and re-suspending the bacterial

cells into the reaction mixture. Finally, the PCR conditions described (section 3.2.1.3) were used for amplification and later confirmed on agarose gel after electrophoresis. To verify orientation of the transgene in the vector, a restriction digestion reaction was set up. A 30µl reaction containing 10µl of the template DNA, 3µl buffer, 16.5µl PCR water and 0.5µl of EcoRV (New England Bio-labs Inc., MA, USA) was set up. This was aliquoted into PCR tubes, incubated for 1h at 37 ºC and gel electrophoresis was done to confirm the positive clones. A positive control from a vector with *CYSTEINE PROTEASE* was also included. EcoRV cuts the vector at 3 restriction sites and it is therefore expected to release 2 fragments and a backbone. One colony of the clone with the expected fragments was picked from which minipreps were prepared and used for *Agrobacerium* transformation.

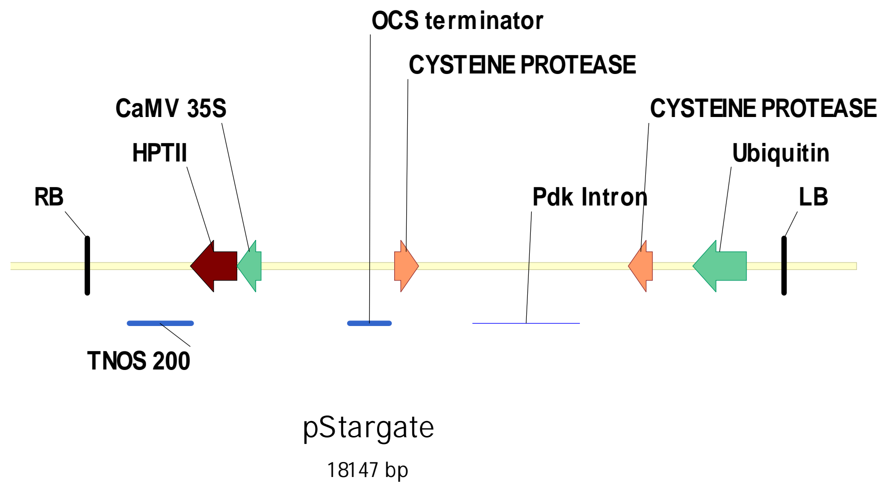

Figure 3.2: pStargate 12 silencing vector with CYSTEINE PROTEASE. Schematic representation of the T-DNA region of binary vector pStargate 12 with the *CYSTEINE PROTEASE* sequences in sense and antisense orientation. The silencing sequences are separated by the pdk intron and driven by maize ubiquitin promoter with an octopine synthase terminator downstream. It has hygromycin (HPTII) resistance gene for plant selection driven by CaMV promoter and Nos terminator.

3.3.1.10. Transformation of *Agrobacterium* with *CYSTEINE PROTEASE*

Disarmed *Agrobacterium tumefaciens,* strain EHA 105, was used to deliver the cloned construct into maize. A loopful of empty *A. tumefaciens* was cultured in 30ml of liquid LB media overnight with shaking at 28ºC. Chemically competent cells were prepared using a protocol according to Tu *et al*. (2005) as described in section 3.3.1.4. To transform EHA105, the competent cells were thawed on ice and 2µl of plasmid DNA containing the recombined binary vector with *CYSTEINE PROTEASE* added with gentle mixing. A control (plasmid DNA of the binary vector without the *CYSTEINE PROTEASE*) was also included. The mixture was frozen in liquid nitrogen for 10 minutes and thawed in a water bath (37□) for 5 minutes. Two (2)ml of LB broth was added and the culture incubated in darkness at 28ºC for 3h on a shaker. Fifty (50)µl of the bacterial culture was spread on LB agar plates containing rifampicin and spectinomycin for selection of the bacteria and construct respectively. The plates were sealed with parafilm and incubated at 28ºC for 3 days in darkness. Emerging colonies were screened for presence of the hairpin *CYSTEINE PROTEASE* through PCR using *CYSTEINE PROTEASE* specific primers. One colony carrying the hairpin *CYSTEINE PROTEASE* selected and used to transform maize.

3.3.2. Maize transformation

3.3.2.1. Growth of maize for explant production

Immature zygotic embryos of maize inbred line CML 144 were transformed using the above-generated *A. tumefaciens* harboring the construct. Seeds were grown and maintained at Kenyatta University plant transformation laboratory. The maize plants were self-pollinated at silking stage and covered with a cellophane bag to prevent cross-pollination. Maize ears were harvested 11 days after pollination and stored at 4ºC to await transformation.

3.3.2.2. Surface sterilization of maize ears and excision of embryos

Husks covering maize cobs were removed and the cobs surface sterilized with 3.5% sodium hypochlorite (NaOCl). The cobs were then rinsed twice with autoclaved distilled water. A sterile scapel blade was used to chop off the top part of the kernels and a sterile excisor used to remove the immature embryos and suspended in 5ml of filter sterilized infection medium comprising the following components; 4.4g/l MS salts with vitamins, `34.25g/l sucrose, 10g/l glucose, 1.5mg/l 2,4-D, 1g/l Casein hydrolysate and 100µM acetosyringone at pH 5.2.

3.3.2.3. Embryo infection and cocultivation.

Prior to infection, one positive colony selected from section 3.2.1.10 above was grown in liquid LB media supplemented with 100mg/l spectinomycin and 50mg/lrifampicillin. The cells were refreshed in 20ml of infection medium containing 100µM acetosyringone in sterile 50ml centrifuge tubes. The bacterial culture was incubated at 28ºC for 3h in darkness with shaking. To transform maize, embryos were removed from the infection media and placed on a sterile petri plate and 5ml of the *Agrobacterium* suspension added to them. The plates were covered with aluminium foil to provide darkness and left for 5 minutes for efficient infection. The infected embryos were transferred on to cocultivation medium comprising 4.4g/l MS salts with vitamins, 30g/l sucrose, 10g/l glucose, 0.7g/l proline, 1.5mg/l 2,4-D, 100mM $CuSO_4$, 0.5g/l MES monohydrate pH 5.8 and 8g/l agar to initiate callus formation. Excess *Agrobacterium* suspension was pipetted off and the embryos oriented to ensure that the scutella were facing upwards on the medium. The plates were sealed with parafilm and covered with aluminium foil then incubated at 22ºC for 3 days. Embryos were then transferred onto resting medium (pH 5.8) whose components were 4.4g/l MS salts with vitamins, 20g/l sucrose, 0.7g/l proline, 1.5mg/l 2,4-D, 0.5g/l MES monohydrate, 8g/l agar supplemented with 1.6mg/l of filter

sterilized $AgNO_3$ and 250mg/l carbenicillin (for elimination of Agrobacterium). The plates were sealed with parafilm and incubated 26ºC in the dark for 10 days.

3.3.2.4. Selection of putative transgenic maize

Explants forming calli were subjected to a 2-week pre-selection on media that comprised 4.4g/l MS salts with vitamins, 30g/l sucrose, 0.7g/l proline, 1.5mg/l 2,4-D, 0.5g/l MES monohydrate and 3g/l gelrite supplemented with 250mg/l carbenicillin and 15mg/l hygromycin. The cultures were covered with aluminium foil to provide darkness and incubated at 28°C. Surviving calli were transferred to a second selection media similar to the first selection medium except that it contained 30mg/l hygromycin for 2 weeks. Surviving calli with somatic embryos were transferred to embryo maturation medium comprising 4.4g/l MS salts with vitamins, 60g/l sucrose, 0.7g/l proline, 0.5g/l MES monohydrate and 3g/l gelrite, pH 5.8 supplemented with filter sterilized 250mg/l carbenicillin. Calli with somatic embryos were transferred to regeneration medium (4.4g/l MS salts with vitamins, 30g/l sucrose, 0.7g/l proline, 0.5g/l MES monohydrate and 3g/l gelrite, pH 5.8) and incubated in light to induce shoot formation. Regenerated plants with well-developed roots were acclimatized in the glass house according to Ishida *et al*. (2007). According to the protocol, plants were transferred into plastic pots (70cc) containing autoclaved peat moss to acclimatize and harden. The plants were covered with plastic bags to prevent dehydration. The plastic bags were gradually lifted to decrease humidity and allow hardening of the plants. These were maintained in a glass house at 25ºC for 10 days and later transferred to larger pots (90cc) with soil and hardened for 10 days with regular watering. Plants that survived were allowed to grow to maturity.

3.3.3.0. Analysis of transgenic plants

Molecular analysis of putative transgenic plants was carried out using PCR and RT-PCR, while phenotypic analysis was carried out using infection assays with *S. hermonthica* to screen for differences in resistance between *CYSTEINE PROTEASE* transgenic plants, empty vector control transgenic plants and wild type plants.

3.3.3.1. Confirmation of transgenic status of putatively transformed plants

To verify transgenic status of putative transgenic T0 plants, a PCR analysis was done using *CYSTEINE PROTEASE* specific primers and the hygromycin resistance gene specific primers. This was also done on T1 plants to identify positive plants for the transgene from those that were negative due to segregation. Genomic DNA from the putatively transformed T0 as well as T1 plants was isolated as described by Zidani *et al*. (2005). Briefly, leaf samples from each of the putative transgenic plants including the mock and wild type maize was harvested, wrapped in aluminium foil and immediately chilled in liquid nitrogen. Tissues were removed from the liquid nitrogen and crushed to a fine powder using a mortar and pestle under liquid nitrogen. The powder (20mg) was transferred to a 2ml centrifuge tube containing 800µl of CTAB extraction buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA and 1% β- mecarptoethanol) and incubated for 30 minutes in a water bath at 65°C. Then, 800µl of chloroform isoamyl-alcohol (24:1) was added to the samples and inverted several times to mix.

The mixture was centrifuged at 13000rpm for 10 minutes and later 1ml of the supernatant transferred to a new centrifuge tube. DNA was precipitated by addition of 0.7ml ice cold isopropanol to each tube and gently inverted to mix. The tubes were chilled at -20°C for 30 minutes and then centrifuged at 13000rpm for 30 minutes. The supernatant was discarded, 1ml of 70% ethanol added to wash the DNA pellet and centrifuged for 10 minutes. The supernatant was carefully discarded and the DNA pellet

air dried after which 50µl of de-ionized water was added to dissolve the pellet. Five microliters of the DNA was run on agarose gel to confirm that isolation was successful and that the DNA was of good quality. The DNA was stored at 4°C for PCR analysis. For amplification, the Hygromycin primers were Hygro F-CGCGTCTGCTGCTCCATACAAG and Hygro R-TTCGATGTAGGAGGGCGTGGAT. The two sets of primers were used to set up 20ul reaction mixture containing 10ng DNA, µl of X5 Taq reaction mix (New England Bio-labs Inc., MA, USA), and 0.5µl of 0.25µM of each primer (forward and reverse). The PCR profile was set as described in section 3.2.1 but the annealing temperature of hygromycin was 61°C. The PCR outcome was determined by running 5µl of the product on 1% agarose gels stained with SYBR Green at 110 V for 30 minutes.

3.3.3.2. Confirmation of gene expression in the transgenic plants using RT-PCR

Total RNA was isolated from leaf tissues of each of the first generation (T1) plants using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase digestion. In brief, approximately 20mg of ground tissue was used for total RNA extraction according to the instructions of the RNeasy®mini kit manual (Qiagen, Valencia.USA). This followed the procedure described in section 3.2.1 above. Total RNA was quantified using a nanodrop and 5µg used for cDNA synthesis. Complementary DNA (cDNA) synthesis was carried out using the first strand Superscript^{TM}III reverse transcriptase kit using random hexamers (Invitrogen) according to the manufacturer's protocol. The cDNA was then used as a template to run an RT-PCR as described in section 3.2.2 above. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis was done using two primers; one targeting *CYSTEINE PROTEASE* transgene and 18S rRNA (internal amplification control for maize) to confirm the expression of the hp-*CYSTEINE PROTEASE* cassette in putatively transformed maize plants.

3.3.4.1. Bulking of transgenic maize seed

Plants that were positive after PCR were maintained in the glass house in potted soil until the silking stage. Plants were self-pollinated and on reaching maturity they were harvested and sun dried. Cobs were shelled and the grains further dried in a glass house at 28ºC and 24ºC day and night temperatures respectively for 2 weeks. First generation (T0) kernels were put in storage bags and then stored at 4ºC. These seeds were grown and self-pollinated to produce second generation (T1) that were used for *S. hermonthica* resistance screening.

3.3.4.2. Preconditioning Striga seeds

Striga hermonthica seeds (obtained from maize growing fields in Kibos, Western Kenya in 2015) were preconditioned as described by Gurney *et al*. (2003) before germination following the steps described in section 3.1.1 above. The preconditioned seeds were germinated by adding 5ml of 0.1ppm GR24 and incubated overnight at 29ºC. Germination efficiency of the *S. hermonthica* seedlings was determined through observation using a microscope, Leica MZ7F (Leica inc.) and plates showing more than 70% germination used to infect maize roots.

3.3.4.3. Infection of maize roots using the rhizotron system and screening for resistance phenotypes

Wild type CML 144 and transformed maize seeds (mock and Transgenic line, (TL1-6) were first germinated on vermiculate and after 5 days, they were transferred to Rhizotrons prepared as described in section 3.1.2. Maize seedlings with well-developed roots were infected with 25mg pre-germinated *S. hermonthica* seeds after 11 days by aligning the *S. hermonthica* seeds along the maize roots with a soft paint brush. The Rhizotrons were then covered with Aluminium foil and maintained in a glasshouse with controlled conditions with regular watering using Long Ashton nutrient solution. Roots

infected were screened for *S. hermonthica* resistance at 9 and 21DAI. Six events were screened with five maize plants per event and the experiment replicated three times. At 9 DAI, *S. hermonthica* seedlings attached on maize roots were observed and documented using a Stereomicroscope (Leica MZ4 fitted with DFC320FX camera (Leica, Germany).

After 21 days, *S. hermonthica* attached to maize roots were harvested, placed on 90mm petri plates and photographed. Image analysis using ImageJ, v. 1.45 (http://rsb.info.nih.gov/ij/) was then carried out to determine the length and the number of *S. hermonthica* parasitizing each host plant. To determine the total *S. hermonthica* biomass attached on maize roots, harvested *S. hermonthica* seedlings were oven dried for 7 days at 45ºC and weighed. To investigate the interaction between the parasite and host, tissues were collected at 9 DAI for histological analysis. Tissues collected at 9 DAI were taken through the stages of fixation, dehydration, pre-infiltration, infiltration, embedding, mounting, sectioning and staining as described in section 3.1.3.2. The sections were observed and photographed using a Leica microscope mounted with a DFC camera (mm). Five plants per line were used for histological studies from which three tissues were collected per plant.

3.3.5. Data analysis

Data on the total number of embryos infected, number of calli surviving selection, and the number of calli forming shoots was recorded. The Transformation and regeneration frequencies were determined. The biomass, length and the number of *S. hermonthica* attachments were recorded. Analysis of variance (ANOVA) was carried out to determine the means and standard deviations using the Statistical Analysis Software (SAS) version 9.1. Data on the mean *S. hermonthica* biomass, length and number of attachments was presented as boxplots prepared in R software. Significant differences between the means will according to Tukey's HSD test at at 95% confidence level. The callus induction frequency was calculated as the total number of calli in callus induction media as a percentage of the total number of infected embryos. The transformation frequency was obtained from the total number of calli surviving the second selection as a percentage of the total number of embryos co-cultivated with EHA 105 harboring the RNAi construct. The regeneration frequency was expressed as the total number of calli producing at least one shoot as a percentage of the total number of calli surviving second selection.

$$\text{Callus induction frequency} = \frac{\text{total number of calli in callus induction media}}{\text{total number of infected embryos}} \times 100$$

$$\text{Transformation frequency} = \frac{\text{Total number of calli surviving selection}}{\text{Total number of infected embryos}} \times 100$$

$$\text{Frequency of shoot formation} = \frac{\text{Total number of calli forming at least one shoot}}{\text{Total number of callus cultured on PRM}} \times 100$$

CHAPTER FOUR

RESULTS

4.1.1. Comparative analysis of post-attachment S. hermonthica resistance in maize

An effective measure of host resistance to *Striga spp* is achieved by determining the number, size and total parasite biomass infecting a host plant. A resistance response is characterized by fewer, smaller and less biomass relative to a susceptible host. CML144, the susceptible maize inbred line, recorded significantly higher numbers of *S. hermonthica* attachments (Figure 4.1a), longer *S. hermonthica* seedlings (Figure 4.1b), and a higher *S. hermonthica* biomass (Figure 4.1c). From the data, CML144, the susceptible maize inbred line had significantly higher number of *S. hermonthica* attachments compared to the Open pollinated variety, KSTP'94. 1.6-fold more *S. hermonthica* attached on roots of CML144 compared to KSTP'94 ($P<0.05$). (CML144 =72.9333; KSTP '94 =44.8 therefore 73/45=1.6-fold). The mean length of *S. hermonthica* that attached to maize inbred line CML 144 roots was more than 2.5-fold longer than the length of *S. hermonthica* attached to KSTP'94 roots. To examine the level of support by host plants on *S. hermonthica* survival, the biomass of parasite seedlings that had attached on each maize plant was determined. Data revealed that there was more *S. hermonthica* biomass when parasite is attached to CML 144 by more than 2.6-fold higher compared to the biomass of *S. hermonthica* attached to KSTP'94.

The microscopic screening of *Striga*-host plant connection analysis showed that attachment and penetration by *S. hermonthica* haustoria in both maize lines occurred by 9 DAI. In the susceptible line, vascular connections had already been established at this time point with the parasite having a relatively larger volume of vegetative tissue compared to KSTP '94 (plate 4.2ai and bi).

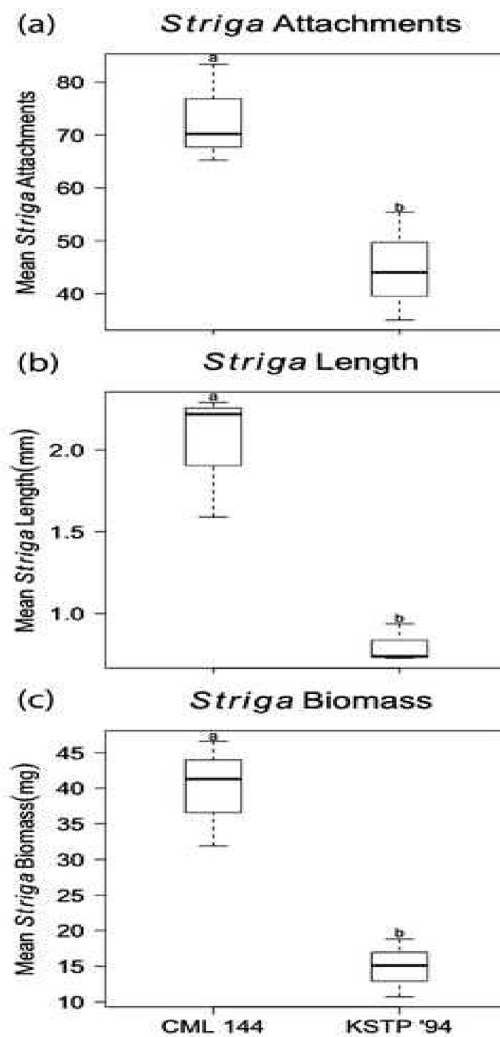

Figure 4.1: Post-attachment resistance evaluation of maize inbred line CML144 and KSTP'94 following infection with *S. hermonthica* seeds. (a). Mean number of *S. hermonthica* seedlings attached to host roots. (b). Mean length of *S. hermonthica* seedlings in millimetres and (c). Mean *S. hermonthica* dry biomass (mg) of parasite seedlings attached to each host roots. Data was collected 21 days after infection and is a mean of 3 replicates. Letters above each bar indicate significant differences ($p \leq 0.05$).

Plate 4.1: ***S. hermonthica*** **seedlings growing on the roots of maize lines screened on rhizotrons 21 days after infection with** a *S. hermonthica* ecotype from Kibos. a. Susceptible maize inbred line CML144 characterised by numerous *S. hermonthica* attachements. b. Resistant open pollinated maize KSTP'94 characterised by fewer and smaller attachements. White arrows indicate attachment points to the host by the parasite. Scale bar is 0.2 mm.

4.1.2. KSTP'94 exhibits mechanical resistance mechanism to *S. hermonthica*

To further elucidate the underlying resistance mechanism of KSTP'94 after infection, histological analyses of *Striga*-host interactions were carried out at the attachment point 9 DAI. *Striga* parasitism is considered to be successful when the vascular connection between host and parasite is established followed by efficient nutrient flow into the parasite. According to the stained sections, CML144 roots (Plate 4.2 ai and aii). It was

further evident that the parasite successfully formed xylem to xylem connections with the host after penetration. However, in KSTP '94, a majority of *S. hermonthica* seedlings penetrated host tissue up to the cortical cells but did not go beyond the endodermis, hence failing to make xylem-xylem connections (Plate 4.2 bi and bii).

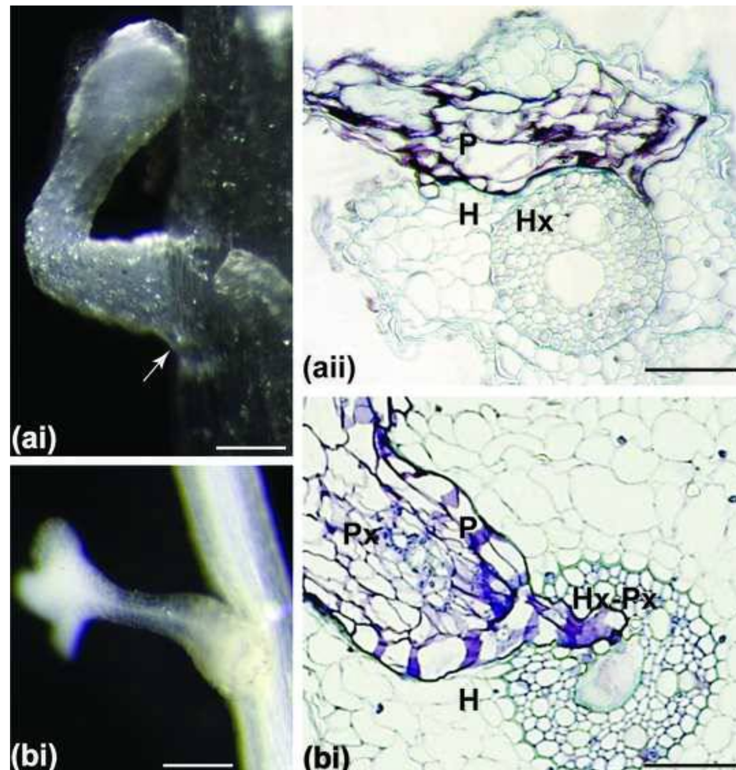

Plate 4.2: Histological analysis of post-attachement resistance mechanisms to *S.hermonthica*. (ai). Colonization of CML144 root by *S. hermonthica* showing successful attachment (scale bar 1 mm). (aii). Transverse section of a stained root tissue of CML144 maize line 9 days after infection showing penetration of the host root cortex and endodermis as well as connections between the host and parasite xylem (Hx-Px). Scale bar = 0.1 mm. P= Parasite, H=host, Px= Parasite xylem. In the susceptible interaction the parasite penetrates the cortex and endodermis and connects to the xylem vessels of the host allowing the haustorium to differentiate. (bi). Resistance interaction in KSTP'94 where the parasite penetrates the host but exits (white arrow). Scale bar is 1 mm. (bii). A transverse section through the haustorium of the resistant maize line KSTP'94. The parasite penetrates the cortex but, it is unable to breach the endodermal barrier and grows around the host vascular cylinder. Scale is 0.1 mm.

4.2.1. *CYSTEINE PROTEASE* mRNAs traffic from parasite to the host

This study employed maize-*S. hermonthica* infection system to study whether mRNAs traffic between a parasite and its host. Semi-quantitative RT-PCR on cDNA from infected maize root tissues collected at the point of infection between maize and *S. hermonthica* revealed that trafficking of *CYSTEINE PROTEASE* mRNAs occurs. RT-PCR analysis revealed the expected band (446bp) of *S. hermonthica CYSTEINE PROTEASE* gene in the infected maize and *S. hermonthica* leaves with the transcript detectable up to 15cm in maize roots. There was no amplification in the cDNA from uninfected maize (CML 144) with the 18S ribosomal RNA used as an internal control producing the expected fragment (324bp) on agarose gel (Plate 4.3A and B).

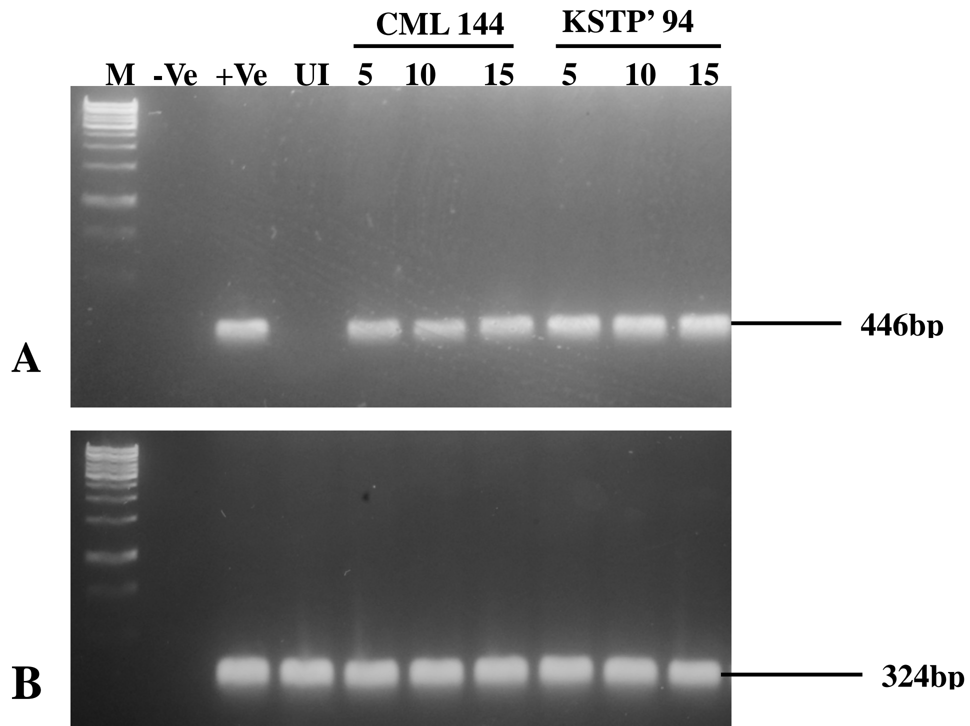

Plate 4.3: (A). Detection of *S. hermonthica CYSTEINE PROTEASE* transcripts by semi-quantitative RT-PCR in maize root tissues of CML 144 and KSTP '94 infected with *S. hermonthica*. Lane M was loaded with 1 kb DNA ladder (1kb plus Gene ruler ladder). -Ve was negative control (PCR reagents and no template cDNA). Total RNA was assayed from *S. hermonthica* leaves (+Ve control), uninfected (UI) maize roots (negative) and infected root tissues at 5cm, 10cm and 15cm from the point of attachment. **(B).** Amplification of 18S ribosomal RNA gene used as the internal amplification control.

4.2.2. Phylogenetic relatedness of *S. hermonthica CYSTEINE PROTEASE* to those of other plant species

Following confirmation of the trafficking above, this study went further to compare how *CYSTEINE PROTEASE* has evolved between *S. hermonthica* and selected plant species through a phylogenetic analysis. Retrieved sequences did not capture any evolutionary relatedness between *S. hermonthica CYSTEINE PROTEASE* and those of host species (Figure 4.2). The *S. hermonthica CYSTEINE PROTEASE* could be segregated alone, with only 16% similarity to a clade comprising that from the common bean (*P. vulgaris*) and other non-host species (Figure 4.2). Maize *CYSTEINE PROTEASE* clustered with that from *Oryza sativa* (another *Striga* host) with 81% similarity. However, their relatedness to *S. hermonthica CYSTEINE PROTEASE* could not be deduced from this phylogeny.

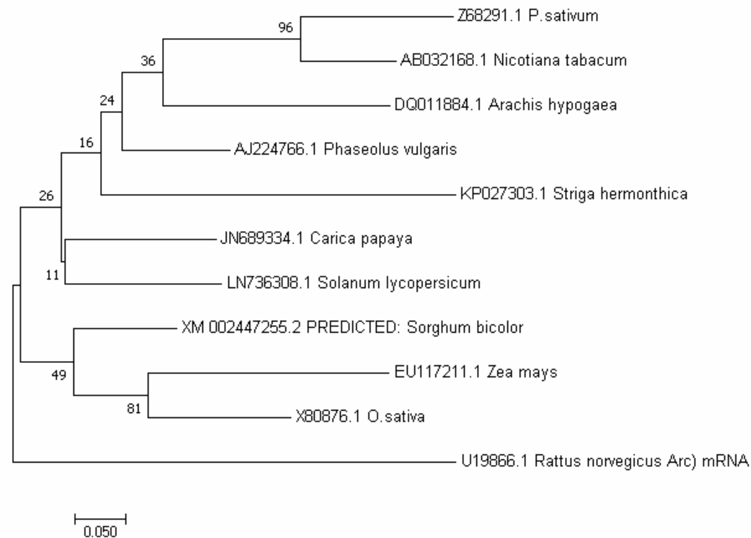

Figure 4.2: A phylogenetic tree of selected plant *CYSTEINE PROTEASEs*. The analysis was done using sequences retrieved from the gene bank after a sequence alignment by MEGA and the Neighbor-joining tree algorithm with 1000 bootsrap. The rat Arc gene sequence was used to root the tree.

4.2. Developing *Striga*-resistant transgenic maize through host induced gene silencing of the *S. hermonthica CYSTEINE PROTEASE*

4.2.1. Construction of RNAi cassette for maize transformation

PCR amplification of *S. hermonthica* cDNA using *CYSTEINE PROTEASE* specific primers generated the expected fragment (446bp of the *CYSTEINE PROTEASE* gene) that was then used for cloning (Plate 4.4 A). Restriction digestion using EcoRV allowed linearization of the TOPO-PCR product for sub-cloning into the binary vector (Plate 4.4 B). PCR analysis on *E. coli* colonies after bacterial transformation revealed the presence of *CYSTEINE PROTEASE* insert in pStargate12 vector (Plate 4.4 C) with a similar result also observed for presence of the construct in *Agrobacterium tumefaciens* (Plate 4.4 D).

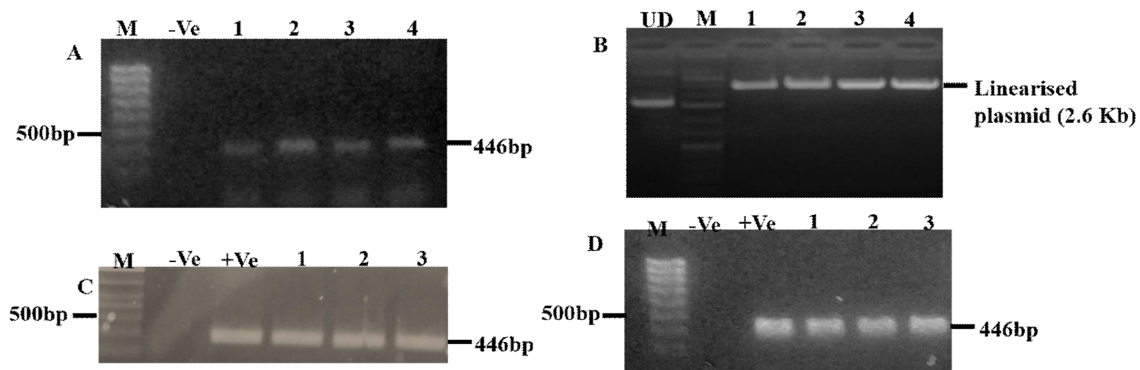

Plate 4.4: PCR cloning of *S. hermonthica CYSTEINE PROTEASE* for maize transformation. (A). Amplification of the *CYSTEINE PROTEASE* (CP) gene using CP primers alongside a 100bp Gene ruler DNA ladder (M). Samples 1-4 were positively amplified revealing the expected fragment A negative control (-Ve) was also included. (B). Restriction digestion of PCR8/TOPOTA/CP vector in *E. coli* using EcoRV. Lane M was loaded with a 1kb DNA ladder, UD-Undigested plasmid DNA. (C). Amplification of CP in *E. coli* through colony PCR using CP primers with a positive control carrying the *CYSTEINE PROTEASE* gene and negative (–Ve) control, Water. Lane M was loaded with 1kb Gene ruler DNA ladder. **(D).** Verification by PCR amplification of the CP gene cloned in pStargate12 in *A. tumefaciens* strain EHA 105 colonies via PCR using CP primers. Lane M was loaded with 100bp DNA ladder, (-ve) negative control, (+ve) positive control, plasmid DNA.

4.2.2. Maize transfromation and regenaration of putative transgenics

Infection of immature zygotic embryos using *Agrobacterium tumefaciens* harboring the above-generated construct, subsequent selection and regeneration steps according to Ishida *et al*. (2007) resulted in putative transgenic maize. Callus induction was initiated on co-cultivation media and showed swelling of the embryo by the 3rd day of culture (Plate 4.5 A). On resting media, compact creamy white calli were formed by the 10th day with some of the calli forming root-like structures (Plate 4.5 B). On selection media, non-transformed tissues appeared necrotic, turned brown and eventually died while those that survived formed somatic embryos (Plate 4.5 C and D). Calli with somatic embryos on regeneration media turned green by the 4th day of exposure to light (Plate4.5E). Calli surviving second selection with double hygromycin concentration

(Plate 4.5 D) were later regenerated into whole plants. Seven days post embryonic calli turning green shoots were formed (Plate 4.5 Gand H). The maize plantlets were successfully hardened and acclimatized in peat moss (Plate 4.5 I).

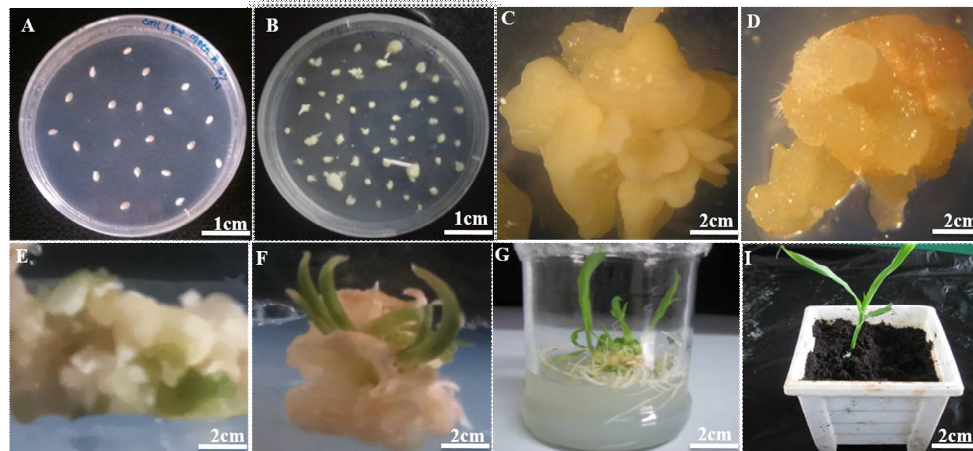

Plate 4.5: Transformation and regeneration of maize (CML 144) using a hairpin construct with *CYSTEINE PROTEASE.* (A and B). Maize embryos on resting media **(C). Pu**tatively transformed maize calli maintained on MS medium with 15 mg/l hygromycin for selection. **(D).** Maize callus obtained after the second selection (30mg/l hygromycin) showing the presence of somatic embryos. **(E).** Callus with somatic embryos **(F).** Multiple maize shoots on regeneration medium. **(G).** Maize plantlets with established roots on rooting medium. **(I).** Acclimatization of a maize plantlet in peat moss.

A callus induction frequency of 74.93% was obtained as the total number of calli in callus induction media as a percentage of the total number of infected embryos. A transfomation frequency of 28.18% calculated from the total number of calli surviving second selection with hygromycin as a percentage of the sum of all infected embryos was recorded. A regeneration frequency of 38.71%, calculated as the total number of calli producing atleast one shoot as a percentage of the total number of calli surviving second selection was further obtained. Twelve (12) maize plants were regenerated from 3 independent transgenic events (Table 4.1). Both transgenic and wild type maize exhibited normal growth to maturity (Plate 4. 6A and C) except four plants that showed

multiple cobs at the same node, formation of tassel seeds, tillering and folding during flowering; phenomena that are characteristic of somaclonal variation (Plate 4.6 B and C).

Table 4.1: Number of plants regenerated from CML 144 embryos transformed with *S. hermonthica CYSTEINE PROTEASE*.

Transformation experiment	**No of infected embryos**	**CIF (%)**	**No of calli on 2nd selection media**	**TF (%)**	**No of regenerants**	**RF (%)**
1	40	77.5	12	30	3	40
2	45	75.6	13	28.89	6	46.15
3	39	71.7	10	25.64	3	30
Total	**124**	**74.93**	**35**	**28.18**	**12**	**38.71**

CIF- callus induction frequency **TF**- Transformation frequency **RF**- Regeneration frequency

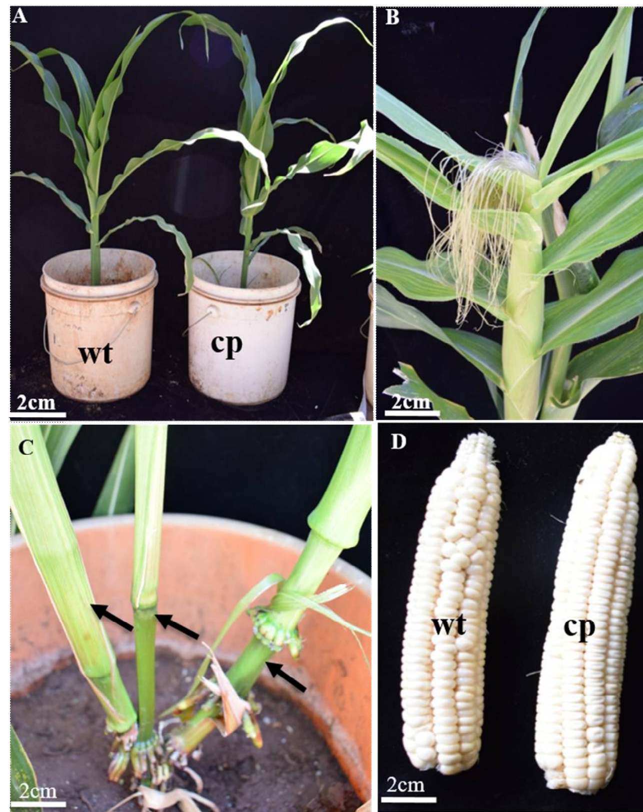

Plate 4.6: Growth of putative transgenic maize transformed with hairpin-*CYSTEINE PROTEASE* in the glasshouse. (A). Appearance of regenerated putative transgenic plant (cp) and non-transformed (wt) potted soil. **(B).** A maturing transformed ear cob before pollination. **(C).** Tillers on a maize plant with soma clonal variation. **(D).** Placement of maize kernels in non-transformed (wt) and transformed (cp) plants.

4.2.3. Determination of transgenic status of putatively transformed maize

To confirm presence of the construct with *CYSTEINE PROTEASE* insert in transgenics, PCR set up targeting the *CYSTEINE PROTEASE* transgene revealed that plants had been successfully transformed. The expected fragment of 446bp was amplified in the transgenic plants but was not detected in non-transformed plants. Of the 12 regenerated plants, 8 were positive for the transgene (Plate 4.7)

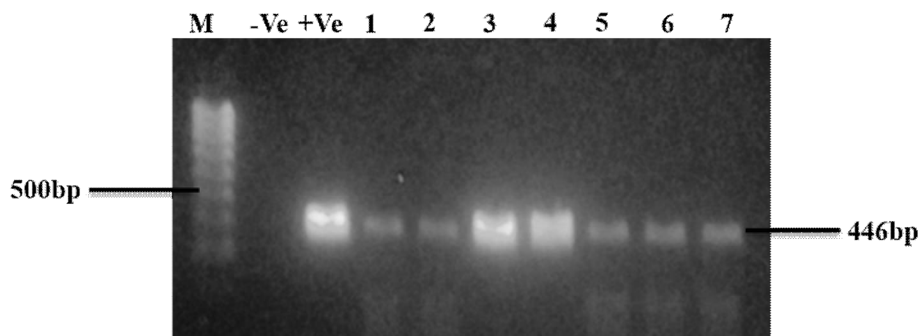

Plate 4.7: PCR amplification of 446 bp size *CYSTEINE PROTEASE* fragment of the leaf genomic DNA extracted from T1 maize plants. M, GeneRuler DNA ladder 100 bp, (-Ve) Negative control, mock plants (+Ve) Positive control (plasmid DNA) 1-7 (Transgenic plants).

Analysis of T_1 maize plants by RT-PCR for *CYSTEINE PROTEASE* revealed that all the 8 positive plants were expressing the construct (Plate 4.8). The band only amplified in the transgenic lines (1-8) but not in the mock plants (-ve). The internal control (18S) was also amplified in all plants with a fragment size of 324bp. Taken together these results confirm the expression of the transgene.

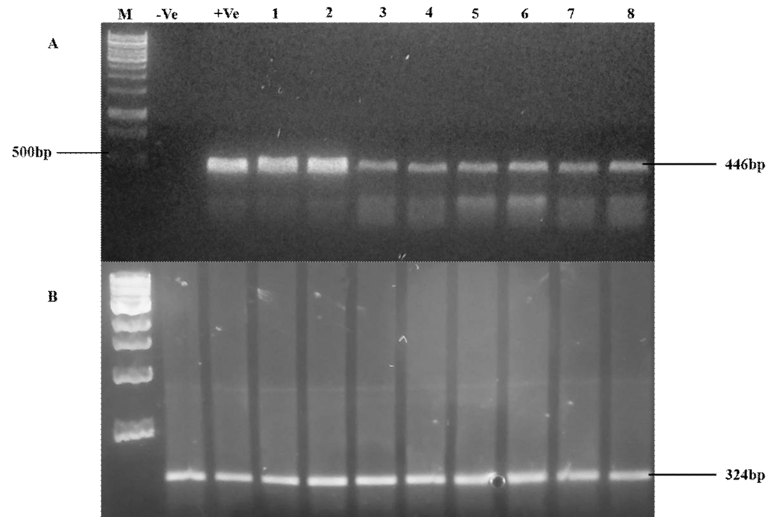

Plate 4.8: Molecular analysis of transgenic maize expressing *CYSTEINE PROTEASE.* Lane M was loaded with Gene ruler 1kb DNA ladder. (-ve) negative control for cDNA from mock maize, (+ve) positive control, plasmid DNA. Lane 1-8 was amplification of cDNA from maize transformed with *CYSTEINE PROTEASE.*

4.2.4. Transgenic plants show no resistance to S. hermonthica

An effective measure of plant resistance to this parasite is achieved by determining the number, size, and total parasite biomass parasitizing the host. *S. hermonthica* resistance was tested on individual transgenic plants obtained from the six events of *S. hermonthica CYSTEINE PROTEASE* and compared to wild type maize (CML 144). Upon infection of plant roots with 25mg of pre-germinated *S. hermonthica* seeds, photographs taken from infected roots at 21 DAI showed no differences in *Striga* parameters (mainly numbers and size of attachments) between wild types and plants transformed with *S. hermonthica CYSTEINE PROTEASE* (Plate 4.9AandB).

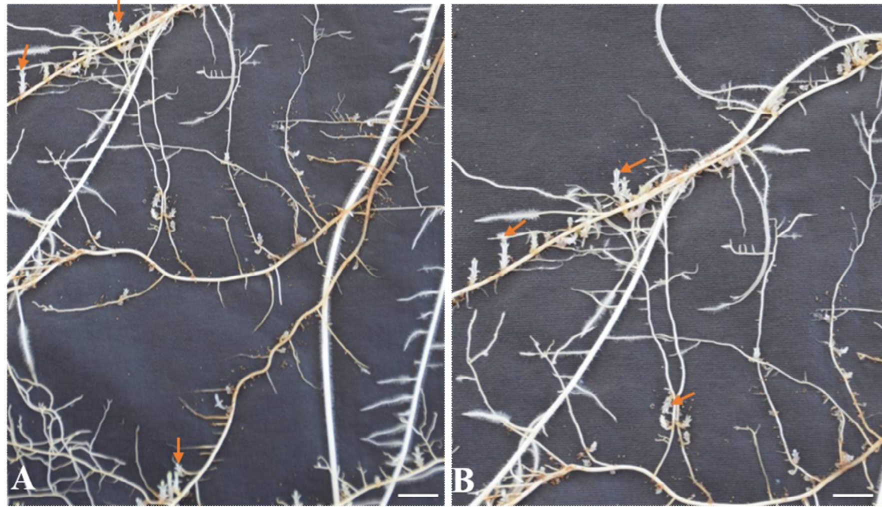

Plate 4.9: Phenotype of resistance against *S. hermonthica* in T1 transgenic plants. (A). Infection on CML 144 maize plant. **(B).** infection on *CYSTEINE PROTEASE* positive. Red arrows indicate sampled points of *S. hermonthica* attachment on maize roots (Bar=0.2mm)

Data collected showed no statistically significant differences in mean number, mean length and mean biomass of *S. hermonthica* seedlings attaching on each plant between transgenic and wild type maize (Appendix II). The highest mean number of *S. hermonthica* attachment was 88.67 ±6.83 recorded in transgenic event five (TE5) and this represented no significant difference when compared to 72.93±9.40 attachments on CML 144 maize (Figure 4.3A). Transgenic event one (TE1) had the highest mean length of *S. hermonthica* attachments of 2.70 ± 0.01 mm compared to CML 144 2.03 ± 0.04cm (Figure 4.3B). The mean biomass of *parasite* attachments was highest in TE2, (43.54± 13.48 mg) which was not significantly different ($p<0.05$) from CML 144 wild type maize that recorded 39.93±7.46mg (Figure 4.3C).

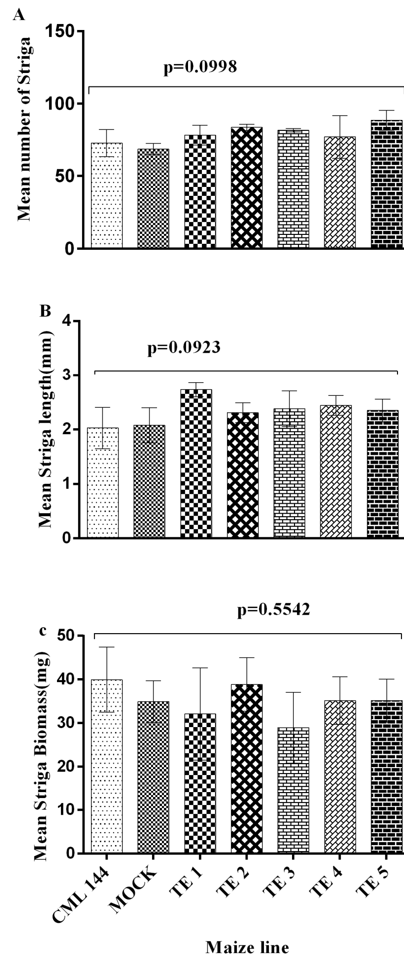

Figure 4.3: Evaluation of resistance to *S. hermonthica* in transgenic and wild type maize plants. (A). Mean number of attachments, **(B).** mean length and **(C).** mean biomass of infecting *S. hermonthica* among transgenic and wild type plants. There were no significant differences according to Tukey's HSD test ($p \leq 0.05$).

To investigate extend of *S. hermonthica* penetration in the transgenics, microscopic screening of Striga-host plant connection point haustorium at 9 DAI was done. The analysis showed that attachment and penetration by *S. hermonthica* haustoria occurred by 9 DAI. The parasite attached to the host revealed rapid growth and were relatively large in volume. It penetrated the host cortex, endodermis and by the 9th day it had

formed connections with the xylem resulting to differentiation of the haustorium and shoot emergence in all the tissues collected. Absorption of nutrients and water from the host caused the parasite enlargement. (Plate 4.10).

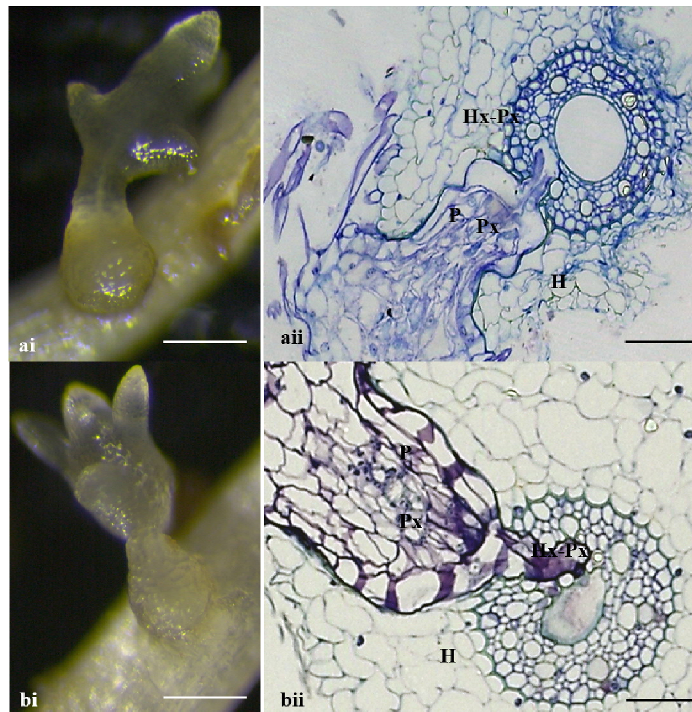

Plate 4.10: Susceptible interaction between transgenic maize and *S. hermonthica*. Colonization of Transgenic **(ai)** and CML 144 **(bi)** maize root by *S. hermonthica* showing successful attachment (scale bar 1 mm). Transverse section of a stained root tissue of transgenic **(aii)** and CML 144 **(bii)** maize line 9 days after infection showing penetration of the host root cortex and endodermis as well as connections between the host and parasite xylem (Hx-Px). Scale bar = 0.1 mm. P= Parasite, H=host, Px= Parasite xylem. Both cases show susceptible interaction in which the parasite penetrates the cortex and endodermis and connects to the xylem vessels of the host allowing the haustorium to differentiate.

CHAPTER FIVE

DISCUSSION

5.1. KSTP'94 exhibits post-attachment resistance to *S. hermonthica*

Striga hermonthica resistance mechanisms act either before (pre-attachment) or after physical contact with the host (post-attachment). Pre-attachment resistance occurs when a host produces low amounts of strigolactones or when *Striga* receptors that perceive germination stimulants are insensitive to the strigolactone (Lumba *et al.*, 2017). This mechanism has previously been shown in KSTP'94 which produced low amounts of sorgomol, a strigolactone that does not efficiently induce *S. hermonthica* germination (Kanampiu, 2012). In contrast, post-attachment *Striga* resistance mechanisms act after *Striga* has attached and attempted to penetrate the host. These mechanisms result in physiological or biochemical barriers, which prevent *Striga* haustorium from connecting to the host xylem (van Dam and Bouwmeester, 2016). *Striga hermonthica* post-attachment resistance in maize has mainly come from its wild grass relatives like *Zea diploperennis* (Lane *et al.*, 1997; Amusan *et al.*, 2008) and *Tripsacum dactyloides* (Gutierezz-Marcos *et al.,* 2003).

The ability of *S. hermothica* to attach to a host and make vascular connections is a critical step in the lifecycle and survival of the obligate hemiparasite. Results obtained in the current study suggest that *S. hermonthica* was able to successfully parasitize the susceptible genotype (CML144) 2.6-fold more frequently compared to KSTP'94. The significantly larger size of *S. hermonthica* seedlings in the susceptible genotype further resulted in higher parasite biomass. *Striga's* ability to penetrate host and complete its lifecycle, as observed in this study, is in line with previous work on a resistant maize inbred line ZD05 (derived from wild maize) (Amusan *et al.*, 2008). Here, the frequency of formation of xylem-xylem connections between *S. hermonthica* and ZD05 was 12%

resulting in 88% less infection. This translated to significantly less *Striga* attachments. Earlier studies on host-parasite interaction have reported host and *Striga* incompatibility. For example, Gurney *et al*. (2006) described the resistance mechanism between rice variety Nipponbare and *S. hermonthica*. Similarly, Amusan *et al*. (2008) showed incompatibility of inbred line ZD05 with *S. hermonthica.* In all these cases, the parasite penetrated the host cortex but was prevented from getting into the endodermis. The exact mechanism for this parasite's inability to penetrate the endodermis is unknown but it seems plausible that molecules that mediate interactions between *Striga spp* and their hosts play an important role in resistance. Particularly, the resistance can be attributed to biochemical or physiological barriers such as a tough sclerenchyma exhibited by the host (Amusan *et al*., 2008; Yoshida and Shirasu, 2009).

KSTP'94 is an open pollinated maize variety that was developed by KALRO for *S. hermonthica* management (Woomer and Savala, 2008). Most farmers in SSA prefer Open pollinated varieties of maize over hybrids due to their ability to thrive and produce more in *S. hermonthica*-infested soils, are affordable, easy to multiply and readily available (Midega *et al*., 2016). In addition, though hybrids are desirable for their high productivity and quality, they show reduced pathogen resistance compared to the open pollinated varieties which have innate defense traits (Schroeder *et al*., 2013). It is therefore vital to understand the genetic make-up of the parents used to develop this variety as this would provide more knowledge on the genes responsible for this mechanism of resistance. Such insight would be useful in crop development resulting in maize and other germplasm with enhanced resistance to *S. hermonthica*.

These findings emphasize the need to continuously screen germplasm for pre and post *Striga* attachment resistance for identification of additional mechanisms of resistance that protect against *S. hermonthica* in maize. Previous studies have employed the use of molecular markers in identification of *Striga* resistance in sorghum (Haussmann, 2004) and rice (Swarbrick *et al*., 2009). The post-attachment Striga resistance shown in

KSTP'94; a first maize line not introgresed with wild germplasm, demonstrate the importance of this line as a potential source of genetic material for *S. hermonthica* management in Eastern Africa. Particularly, the molecular mechanisms underlying the resistance phenotype observed need to be investigated.

5.2. Transgene-specific (*CYSTEINE PROTEASE*) mRNA traffic from parasite to the host.

Most parasitic plants form phloem-phloem connections with their hosts but the case is different for *Striga spp* where the parasite connects to a host by forming xylem-xylem connections (Yoshida *et al.*, 2016). For instance, Cuscuta spp. haustoria make both xylem and phloem connections but *Striga spp* make xylem connections only (Yoshida *et al.*, 2016). In the current study, it was evident that successful trafficking of parasite mRNAs from *S. hermonthica* into maize occurs and this is through a host-parasite interface. In parasitic plants, the haustorium is used as a channel for communication between host and parasite. It not only serves as a conduit through which water, mineral salts and food materials are passed to the host but also as an avenue for transfer of macro molecules such as proteins and mRNA (Westwood *et al*., 2015). Data obtained in this study demonstrated movement of *CYSTEINE PROTEASE* mRNA molecules from *S. hermonthica* to maize and that the molecules move over a long distance. In various parasitic plants, genetic material exchange has been shown to take different paths. For instance, Roney *et al*. (2007) demonstrated direct movement of the pumpkin mRNA to *Cuscuta pentagona* via phloem connections. Tomato RUBISCO SMALL SUBUNIT (LeRbcS) was detected in dodder suggesting mRNA translocation from host to the parasite through the parenchyma cells and the phloem (David-Schwartz *et al*., 2008).

Currently, the path through which *S. hermonthica CYSTEINE PROTEASE* trafficked into maize is not fully understood. One of the possible ways through which this could have happened is by horizontal gene transfer. Recent studies have identified massive

movement of mRNA transcripts encoding functional genes from parasitic plants to their hosts. For instance, Yoshida *et al*. (2010) revealed translocation of a gene with an unknown role to *S. hermonthica* from sorghum indicating that nuclear protein coding sequences, obtained from their host species, could be integrated into the genomes of parasitic plants by horizontal gene transfer. In another study a defense related gene with 87% identity to *Sorghum bicolor* trafficked into *S. hermonthica* by horizontal gene transfer (Yang *et al.*, 2016). Such genes are highly expressed in the haustoria suggesting their involvement in parasite-host interactions. In *Cuscuta reflexa,* host RNA was shown to move into the parasite supporting RNA-based mechanism for horizontal gene transfer in parasitic plants (Kim *et al*., 2014). Despite evidence of movement of functional genes from hosts to *Striga spp*, there exists no evidence of movement of *Striga spp* genes to host plants. The current study, however, clearly revealed movement of a *S. hermonthica* gene; *CYSTEINE PROTEASE* mRNA into maize.

In maize, the *CYSTEINE PROTEASE* mRNA was detectable up to 15cm from the maize-*Striga* junction which suggests that it moves over long distances. Proteases are likely play a key role in aiding parasitic plant penetration into their hosts and these transcripts possibly act as long-distance signals of development. The *Striga CYSTEINE PROTEASE* is hypothesized to have a function in weakening of the host tissues for successful parasitism.

In a related study, David-Schwartz *et al*. (2008) described movement of tomato PYROPHOSPHATE (PPi)-DEPENDENT PHOS- PHOFRUCTOKINASE (LePFP) β subunit transcripts in dodder up to 20cm. The trafficking of parasite-mobile transcripts into maize from *S. hermonthica* suggests that this could be useful for delineating the mechanisms and functions of mobile RNA within plants.

Studies on *Cuscuta reflexa* and *Phelipanche aegyptiaca* have shown expression of *CYSTEINE PROTEASE*-encoding genes in the stem and haustoria of these parasites

respectively (Rehker *et al*., 2012). Furthermore, this suggests that Proteases play key roles in host tissues because the expression of inhibitor peptidases relatively reduced parasite infection. A transcriptome analysis of *Cuscuta reflexa* revealed many mRNA transcripts encoding proteins with hydrolytic activity and also associated with plant cell wall structure and function at prehaustorial stage. In maize, a natural defense maize insect resistance 1 *CYSTEINE PROTEASE* (Mir1-CP) has been shown to accumulate at the wounded site indicating that it plays a role in pathogenesis (Mohan *et al.*, 2008). In the current study, phylogenetic analysis revealed that *S. hermonthica* and maize *CYSTEINE PROTEASE* genes are unrelated. This therefore, validated targeting of the parasitic *CYSTEINE PROTEASE* without possibility of having off-target effects in generated transgenic maize. Maize *CYSTEINE PROTEASE* is involved in defense while that of *S. hermonthica* is thought to play a role in haustorial growth and development. This justifies the targeting of *S. hermonthica CYSTEINE PROTEASE.*

5.3. Development and validation of transgenic maize expressing silencing molecules against *S. hermonthica CYSTEINE PROTEASE*

The effect of silencing of *Striga CYSTEINE PROTEASE* in parasite growth and development was investigated through RNAi. The RNAi strategy involves identification of a suitable target gene involved in stress factor and production of dsRNAs through construction of hairpin RNA construct which are then expressed in host plants. The steps in making an RNAi construct include isolation of the gene of interest from the source, amplification of the target gene through PCR and cloning the sense and antisense sequences into suitable vectors for expression. The sequences are separated by an intron to make it effective in silencing (Ergunay, 2004). The activity of the hairpin construct is evaluated by analyzing the phenotype of plants in which the construct has been expressed (Koch and Kogel, 2014). This biotechnological tool has been successfully used to control various plant stresses. For instance, Nowara *et al*. (2010) expressed a hairpin construct in barley against powdery mildew. In the current study, the Gateway

cloning technology (Karimi *et al*., 2013) was used in generation of the silencing construct with the *CYSTEINE PROTEASE* gene in sense and antisense orientation. This technology was previously used by Alakonya *et al*. (2012) to successfully develop transgenic tobacco with reduced efficacy of dodder infection. It is widely used due to its advantages over conventional cloning because as it is efficient, less tedious, the entry clone can be subcloned in a number of destination vectors and is accurate utilizing site specific recombination (Chee *et al.,* 2015).

For successful generation of transgenic plants, it is vital for one to combine a number of factors including ideal size and age of explants, transformation and regeneration media as well as selection agents (Ishida *et al*., 2007). In the current study, the right size of immature embryos (1-1.5mm), was used as explants and this is in line with other studies in transformation of monocotyledonous crops like barley (Kartha *et al*., 1989) and maize (Koziel *et al*., 1993). These tissues have a greater ability to induce and maintain high embryogenic calli as compared to older and bigger sized explants (Ishida *et al*., 2003). The use of *Agrobacterium* for delivery of the construct into maize explants was vital in the current study owing to its advantages such as a wide range of hosts and high transformation efficiency (Komari, 1989). Other strains such as EHA 101, LBA 4404, AGL1 and GV have also been used in maize transformation with different efficiencies (Ombori *et al.,* 2013).

Selection of transformed from non-transformed tissues is key to an efficient transformation system. A selection agent that allows for preferential proliferation of transformed cells and at the same time suppresses or kills untransformed cells is preferred (Que *et al*., 2014). Antibiotics and herbicides are commonly used as selection agents and these are usually determined by the selectable marker gene inserted in the T-DNA region of the vector. In this study, hygromycin was used as a selection agent, with plants obtained after the second selection considered putative transformants. The frequency of transformation (28.18%) obtained in the current study was comparable to

that obtained by Negrotto *et al.* (2000) although the latter study used temperate inbred line A188. A regeneration frequency of 38.71% was further obtained and this was slightly similar to 43.11% obtained in the same line of maize (CML 144) by Masanga *et al.* (2015).

Transgenic plants were obtained from calli induced from immature embryos of maize after the tissues were co-cultivated with *A. tumefasciens*. Regenerated plants appeared normal but, in some instances, phenotypic variations were observed. In these cases, the plants had abnormalities that are commonly known as somaclonal variations and which are typical of plants regenerated through callus phase in tissue-culture. These have further been described as either epigenetic or genetic in occurrence (Larkin and Scowcroft, 1981). Epigenetic changes cannot be passed on from one generation to another and in this study, these were not observed in the second generation. The observation of phenotypic aberrations in regenerated plants has been reported in other local maize genotypes (Oduor *et al.*, 2006). PCR amplification of the transgene is often taken as an indication of the transfer of transgene into the regenerants. PCR analysis revealed the presence of *CYSTEINE PROTEASE* indicating that the maize plants had been successfully transformed. Similarly, RT-PCR analysis result revealed expression of the transgene indicating that not only were the plants positive for the construct but they were also expressing it.

Although molecular analysis revealed transgenic status as well as expression of the construct in transgenic maize, it was noted that transgenics showed no resistance to *S. hermonthica*. Resistance to parasitic weeds can be expressed before or after formation of a host-parasite vascular bridge (Rispail *et al.*, 2007). In the case of the current study, successful parasitism of wild type and transgenic maize was evident by the high number of *Striga* attachments and rapid development of the parasite's vegetative tissues, an indication that they were getting enough nutrient support from the host. This strongly indicated their susceptibility. *CYSTEINE PROTEASE* gene has been shown to play a role

in weakening of host structures in *Cuscuta reflexa* through protein degradation (Bleischwitz *et al*., 2010). The current study hypothesized that its ortholog in *Striga* would have a similar function and therefore silencing it would work to thwart parasite infection. Particularly, a reduction of parasite-derived proteins would be expected to weaken the parasite's infection efficiency and thereby strengthen host defense. However, this was not the case in this study.

The lack of resistance to *Striga* by transgenic maize under the current study could have been due to the following; first, the RNAi mechanism could have been compromised probably due to signals not crossing from host to the parasite and/or lack of enough signal transmission across the host-parasite interface. For RNAi to be successful, there must be production and translocation of enough dsRNAs, formation of siRNAs and degradation of the target mRNA transcripts in the pathogen (Runo *et al.*, 2011). While most studies have reported success in silencing of target genes using this technique, others have not been successful. For instance, production of dsRNA in *Arabidopsis thaliana* against *Phytophathora parasitica* showed ectopic expression of the dsRNAs in the host but was not effective in silencing of parasite's transcripts due to lack of production of enough and translocation of the silencing signals into the pathogen (Zhang *et al.*, 2011). To add strength to this, there is a correlation between presence and abundance of siRNAs and resistance to pathogens as is the case in viruses (Chen *et al.*, 2004). In the current study, it is hypothesized that this could have been one of the reasons for the lack of resistance although it remains to be validated in *Striga*.

Secondly, it is likely that silencing molecules in the host plant failed to confer resistance to *Striga* due to possible suppression of silencing by the pathogen. *Striga* could have counteracted the effect of silencing molecules by expressing its own silencing-suppressor compounds although this remains to be investigated. This process is a common phenomenon exhibited by plant viruses in overcoming host immune responses. For instance, previous reports have shown that suppressors of silencing bind to dsDNA

or siRNAs and prevent assembly of the RISC complex which is an important step in silencing (Csorba *et al.,* 2007). So far, no RNAi suppressor molecules have been identified in *Striga* and therefore this calls for additional work in order to screen for such molecules.

Finally, the lack of a resistance phenotype could have been due to the choice of the gene to be silenced, which might not have been a suitable target. Although *Striga CYSTEINE PROTEASE* gene is involved in haustoriagenesis, it has been shown to be expressed alongside many other genes including *EXPANSIN genes, MANNOSE-6-PHOSPHATE, TV PIRIN* gene *and QUINONE OXIDOREDUCTASE* (Kirigia, 2015). It is therefore possible that haustoriagenesis and effective parasitism by *Striga* is a coordinated multistep and multi-gene process probably involving several pathways. This means that each one of these genes plays a vital role in parasitism and therefore silencing of a single one of them may not result in a desirable phenotype.

The RNAi strategy has also been unsuccessfully used in research seeking to develop maize resistant to *Striga asiatica.* Here, targeting metabolic genes and generation of transgenic plants expressing silencing molecules against the target sequences did not result in the desired phenotype probably due to low transgene expression or even lack of translocation of the RNAi construct signals through the haustorium (de framond, 2007). In another study, Agrobacterium rhizogenes carrying a reporter gene, GFP, was used to transform maize for *S. hermonthica* resistance. However the generated transgenic maize roots did not confer the desired phenotype (Runo *et al.*, 2012). In the wake of the current result, exploring a stacked RNAi constructs with key genes that control haustoriagenesis could be more promising for effective resistance in a susceptible host.

5.4. Conclusion

The study identified post-attachment mechanism of resistance against *S. hermonthica* in KSTP'94.

There was successful trafficking of *Striga CYSTEINE PROTEASE* m-RNA into maize plants.

CYSTEINE PROTEASE gene was successfully transformed in CML 144 inbred line and seeds obtained for further analysis of transgenic plants. However, transgenic maize developed showed no resistance against *S. hermonthica.*

5.5. Recommendations

KSTP'94 has been shown previously to exhibit pre-germination resistance by production of low germination stimulant, this could be combined with the strong post-attachment resistance shown in this study to mitigate *Striga* problem in areas infested by this parasite. There is also need to determine the genetic mechanisms underlying the mechanical resistance exhibited by KSTP'94 maize to *Striga*.

Trafficking of *S.hermonthica CYSTEINE PROTEASE* was shown in this study suggesting that RNAi in *Striga* is viable therefore this control strategy could be exploited for the weed management.

Transgenic maize plants generated by targeting *CYSTEINE PROTEASE* exhibited a phenotype that did not confer resistance to *S.hermonthica* therefore gene stacking could be utilized because the gene is expressed alongside many other genes including *EXPANSIN genes, MANNOSE-6-PHOSPHATE, TV PIRIN* gene *and QUINONE OXIDOREDUCTASE.*

It is vital to screen for suppressors of signal silencing in *Striga* because it could have in one way contributed to lack of effectiveness of the RNAi construct.

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APPENDICES

Appendix I: Prediction of efficient siRNAs and off-targets estimation

SUMMARY OF QUERY

Number of sequences submitted = 1
siRNA length = 21 nt
The 5' end of antisense strand must be A or U
The first 7 bases of antisense strand (5' - 3') have at least 5 A or U bases
The 5' end of sense strand must be G or C
Percentage GC content: 30% to 70%
Mismatch allowed = 0
Target dataset = Zea mays (maize) DFCI Gene Index (ZMGI) version 19 release on 8/12/2010

Query sequence fragments do not have hits in target dataset

Predicted efficient siRNAs

Number	Fragment	siRNA AS (3' - 5')	Hits
1	query_seq (11 - 31)	CCAAAUGUGGAUAUUUUCUCU	0
2	query_seq (30 - 50)	CUCUCGGCAAUAGCAGACAAA	0
3	query_seq (32 - 52)	CUCGGCAAUAGCAGACAAACA	0
4	query_seq (51 - 71)	CACCUACAUGUCUCACUAUAA	0
5	query_seq (53 - 73)	CCUACAUGUCUCACUAUAAUA	0
6	query_seq (112 - 132)	CACGUGCAGACGACAGUCUAU	0
7	query_seq (114 - 134)	CGUGCAGACGACAGUCUAUUU	0
8	query_seq (118 - 138)	CAGACGACAGUCUAUUUCAGA	0
9	query_seq (129 - 149)	CUAUUUCAGAGGGCACUUGAA	0
10	query_seq (218 - 238)	GAGGCAAUAGCCCCUUCUUCA	0
11	query_seq (243 - 263)	CUAGAGUCGGUGGCGCUUUUA	0
12	query_seq (248 - 268)	GUCGGUGGCGCUUUUACUGUA	0
13	query_seq (251 - 271)	GGUGGCGCUUUUACUGUAGUU	0
14	query_seq (252 - 272)	GUGGCGCUUUUACUGUAGUUU	0
15	query_seq (254 - 274)	GGCGCUUUUACUGUAGUUUUU	0
16	query_seq (266 - 286)	GUAGUUUUUGCGGUAAUUGGA	0
17	query_seq (284 - 304)	GGACUACAAGACCCCUUAUAU	0
18	query_seq (285 - 305)	GACUACAAGACCCCUUAUAUU	0
19	query_seq (287 - 307)	CUACAAGACCCCUUAUAUUUA	0
20	query_seq (330 - 350)	GUCAGACGUCCAGCUAUGUCA	0
21	query_seq (334 - 354)	GACGUCCAGCUAUGUCAUCUU	0
22	query_seq (336 - 356)	CGUCCAGCUAUGUCAUCUUUA	0
23	query_seq (337 - 357)	GUCCAGCUAUGUCAUCUUUAA	0
24	query_seq (340 - 360)	CAGCUAUGUCAUCUUUAAUGU	0
25	query_seq (342 - 362)	GCUAUGUCAUCUUUAAUGUCU	0
26	query_seq (379 - 399)	CAUUCAUAUCUCCGACUUUUA	0
27	query_seq (388 - 408)	CUCCGACUUUUAGGUCUACUU	0

http://bioinfo2.noble.org/cgi-bin/RNAiScan/RNAiScan.pl 1

28	query_seq (403 - 423)	CUACUUCGGCUUGCUGAACAU	0
29	query_seq (406 - 426)	CUUCGGCUUGCUGAACAUUCU	0
30	query_seq (409 - 429)	CGGCUUGCUGAACAUUCUCUU	0
31	query_seq (410 - 430)	GGCUUGCUGAACAUUCUCUUU	0
32	query_seq (411 - 431)	GCUUGCUGAACAUUCUCUUUU	0
33	query_seq (415 - 435)	GCUGAACAUUCUCUUUUCAUA	0

Appendix II: The length, biomass and number of *Striga hermonthica* attachments on transgenic maize.

```
                              The ANOVA Procedure

                            Class Level Information

          Class          Levels    Values

          Maize_line          7    CML 144 MOCK TE 1 TE 2 TE 3 TE 4 TE 5

                      Number of Observations Read          21
                      Number of Observations Used          21
                                              17:50 Thursday, February 15, 2018   2

                              The ANOVA Procedure

Dependent Variable: No_of_Striga_attachments_   No of Striga attachments_

                                        Sum of
      Source                   DF      Squares     Mean Square    F Value    Pr > F

      Model                     6     804.049524     134.008254       2.24    0.0998

      Error                    14     835.920000      59.708571

      Corrected Total          20    1639.969524

           R-Square     Coeff Var      Root MSE    No_of_Striga_attachments_ Mean

           0.490283      9.801257      7.727132                          78.83810

      Source                   DF       Anova SS     Mean Square    F Value    Pr > F

      Maize_line                6    804.0495238     134.0082540       2.24    0.0998
                                              17:50 Thursday, February 15, 2018   3

                              The ANOVA Procedure

Dependent Variable: length_of_Striga_attachments_   length of Striga attachments_

                                        Sum of
      Source                   DF      Squares     Mean Square    F Value    Pr > F

      Model                     6     0.00966109      0.00161018       2.31    0.0923

      Error                    14     0.00975753      0.00069697

      Corrected Total          20     0.01941862

         R-Square     Coeff Var      Root MSE    length_of_Striga_attachments_ Mean

         0.497517      11.31641      0.026400                              0.233290

      Source                   DF       Anova SS     Mean Square    F Value    Pr > F

      Maize_line                6     0.00966109      0.00161018       2.31    0.0923
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                              The ANOVA Procedure

Dependent Variable: biomass_of_Striga_attachments_   biomass of Striga attachments_

                                        Sum of
```

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	6	0.00025228	0.00004205	0.85	0.5542
Error	14	0.00069391	0.00004956		
Corrected Total	20	0.00094618			

R-Square	Coeff Var	Root MSE	biomass_of_Striga_attachments_ Mean
0.266626	20.10399	0.007040	0.035019

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Maize_line	6	0.00025228	0.00004205	0.85	0.5542

The ANOVA Procedure

Welch's ANOVA for No_of_Striga_attachments_

Source	DF	F Value	Pr > F
Maize_line	6.0000	4.65	0.0442
Error	5.8049		

Welch's ANOVA for length_of_Striga_attachments_

Source	DF	F Value	Pr > F
Maize_line	6.0000	2.44	0.1481
Error	6.1417		

Welch's ANOVA for biomass_of_Striga_attachments_

Source	DF	F Value	Pr > F
Maize_line	6.0000	0.47	0.8132
Error	6.1848		

The ANOVA Procedure

Level of		--No_of_Striga_attachments_--		-length_of_Striga_attachments_-	
Maize_line	N	Mean	Std Dev	Mean	Std Dev
CML 144	3	72.9333333	9.4028365	0.20333333	0.03865312
MOCK	3	68.8666667	3.7753587	0.20833333	0.03237365
TE 1	3	78.4000000	6.7734777	0.27260000	0.01333717
TE 2	3	83.8666667	2.2120880	0.23130000	0.01845020
TE 3	3	81.8666667	1.1718931	0.23823333	0.03250513
TE 4	3	77.2666667	14.7137124	0.24443333	0.01795838
TE 5	3	88.6666667	6.8303245	0.23480000	0.02095877

Level of		-biomass_of_Striga_attachments_-	
Maize_line	N	Mean	Std Dev
CML 144	3	0.03993000	0.00745722
MOCK	3	0.03492667	0.00474426
TE 1	3	0.03211000	0.01057397
TE 2	3	0.03887000	0.00609948
TE 3	3	0.02896667	0.00810694

TE 4	3	0.03516333	0.00548825
TE 5	3	0.03516667	0.00489688

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for No_of_Striga_attachments_

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	59.70857
Critical Value of Studentized Range	4.82895
Minimum Significant Difference	21.543

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Maize_ line
A	88.667	3	TE 5
A			
A	83.867	3	TE 2
A			
A	81.867	3	TE 3
A			
A	78.400	3	TE 1
A			
A	77.267	3	TE 4
A			
A	72.933	3	CML 144
A			
A	68.867	3	MOCK

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for length_of_Striga_attachments_

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.000697
Critical Value of Studentized Range	4.82895
Minimum Significant Difference	0.0736

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Maize_ line
A	0.27260	3	TE 1
A			
A	0.24443	3	TE 4
A			
A	0.23823	3	TE 3
A			
A	0.23480	3	TE 5

```
A
A       0.23130      3    TE 2
A
A       0.20833      3    MOCK
A
A       0.20333      3    CML 144
```

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The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for biomass_of_Striga_attachments_

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.00005
Critical Value of Studentized Range	4.82895
Minimum Significant Difference	0.0196

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Maize_ line
A	0.039930	3	CML 144
A			
A	0.038870	3	TE 2
A			
A	0.035167	3	TE 5
A			
A	0.035163	3	TE 4
A			
A	0.034927	3	MOCK
A			
A	0.032110	3	TE 1
A			
A	0.028967	3	TE 3

Appendix III: The sequence of Cloned *S. hermonthica CYSTEINE PROTEASE*

NCBI accession Number (KP027303)

aatcgccctttacactctcaaagctggtggacttatgcgagagaaggactatccttacaccggcactgatcgtggggcttgcaa
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tcaactgttgctgcta