ANALYSIS OF EXPRESSION PATTERNS OF ZmRCP1 AND CaMV35S IN PLANTAINS IN ASSOCIATION WITH NEMATODES INFECTION

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156/12260/2009

A thesis submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

April 2013
DECLARATION

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

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DEDICATION

To God Almighty Father for His merciful guidance. Lucy, Antionette and George, you were the impetus behind my success. To my dear parents, your guidance directed my focus.
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TABLE OF CONTENTS

TITLE PAGE ......................................................................................................................... i
DECLARATION ..................................................................................................................... ii
DEDICATION ....................................................................................................................... iii
ACKNOWLEDGEMENT .......................................................................................................... iv
TABLE OF CONTENTS .......................................................................................................... v
LIST OF TABLES ................................................................................................................... vii
LIST OF FIGURES ................................................................................................................ viii
ABBREVIATIONS AND ACRONYMS ................................................................................... ix
ABSTRACT .......................................................................................................................... x

CHAPTER ONE ..................................................................................................................... 1
1.0 INTRODUCTION ............................................................................................................ 1
1.1 Background information .............................................................................................. 1
1.4 Economic importance of plantains .............................................................................. 3
1.5 Plantains production challenges ................................................................................. 5
1.6 Effects of nematodes on bananas and plantains ......................................................... 6
1.7 Problem statement and justification ........................................................................... 7
1.8 Null hypothesis ............................................................................................................ 8
1.9 General objective ........................................................................................................ 8
1.10 Specific objectives ...................................................................................................... 8
1.11 Significance and anticipated output ........................................................................... 9
1.11.1 Significance of the study ...................................................................................... 9
1.11.2 Anticipated output .............................................................................................. 9

CHAPTER TWO ................................................................................................................... 10
2.0 LITERATURE REVIEW .................................................................................................. 10
2.1 Genetic improvement of plantains ............................................................................. 10
2.2 Transgenic technologies for nematode resistance ....................................................... 11
2.3 Promoters used for transgenic control of nematodes ................................................. 12
2.4 Molecular characterization of transgenic plants ......................................................... 13
2.4.1 Reporter genes .................................................................................................... 13
2.4.2 Polymerase chain reaction ................................................................................. 15
2.5 Stability of transgenes in plants ................................................................................ 16
2.6 Detection of transgene stability in plants ................................................................. 18
2.7 Allometric function for estimation of banana leaf area .............................................. 19

CHAPTER THREE .............................................................................................................. 20
3.0 MATERIALS AND METHODS ..................................................................................... 20
3.1 Transgenic plants production .................................................................................... 20
3.2 Maintenance and multiplication of transgenic lines ................................................... 20
3.3 Root induction and establishment of plants in soil ...................................................... 21
3.4 Genomic DNA extraction from transgenic plants ..................................................... 21
3.5 Agarose gel electrophoresis of extracted genomic DNA ......................................... 22
3.6 Estimation of DNA concentration ............................................................................ 22
3.7 Maintenance of A. tumefaciens culture and plasmid DNA isolation ......................... 22
3.8 PCR amplification ................................................................. 23
3.9 Histochemical GUS analysis on young uninfected transgenic plants 24
3.10 Inoculation of transgenic lines with nematodes .......................... 25
3.11 Analysis of gusA gene expression in infected plantain roots ........ 26
3.12 Determination of effects of nematodes infection on growth characteristics ... 26
3.13 Extraction and counting of nematodes from infected plant roots .... 27
3.14 Histochemical GUS assay and nematodes staining ........................ 27

CHAPTER FOUR ........................................................................... 29
4.0 RESULTS .................................................................................... 29
4.1 Maintenance and multiplication of transgenic plants ......................... 29
4.2 Polymerase chain reaction on transgenic plants ............................... 30
4.3 Histochemical GUS assay on young uninfected transgenic plants .... 31
4.4 Weaning and hardening of plants prior to inoculation ..................... 32
4.5 Symptoms on transgenic plants due to R. similis infection ............... 33
4.6 Analysis of reporter gene activity in infected plantain roots .......... 35
4.9 Extraction and enumeration of nematodes in infected roots .......... 37
4.10 Histochemical GUS assay and nematodes staining on infected roots 40

CHAPTER FIVE ........................................................................... 42
5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS ........... 42
5.1 Discussion ................................................................................. 42
5.2 Conclusion .................................................................................. 48
5.3 Recommendations ...................................................................... 49

6.0 REFERENCES ............................................................................ 50
7.0 APPENDICES ........................................................................... 59
7.1 Stock solutions ........................................................................... 60
7.3 Yeast Mannitol Broth (YMB) ........................................................ 60
7.4 Luria Bertani (LB) medium .......................................................... 61
7.5 Dilution scheme for 0.1M sodium phosphate buffer ....................... 61
7.6 Antibiotics .................................................................................. 61
7.7 Mastermix preparation for PCR amplification of desired target DNA 62
7.8 Macronutrients stock .................................................................. 62
7.9 Micronutrients stock .................................................................. 62
7.10 Fe EDTA .................................................................................... 63
7.11 MS vitamins .............................................................................. 63
7.12 Growth regulators .................................................................... 63
7.13 Proliferation media (1L) and rooting media (1L) ......................... 63
7.14 Histochemical GUS assay fixation solution ................................. 64
7.15 Histochemical GUS assay substrate solution .............................. 64
LIST OF TABLES

Table 1. Confirmatory Student’s t-test for determination of significant difference between TLA(I) and TLA(UI) clones. .......................................................... 38

Table 2. Confirmatory Student’s t-test for determination of significant difference between TPB(I) and TPB(UI) clones. ......................................................... 39
LIST OF FIGURES

Figure 1. Morphological differences between bananas and plantains. ..................... 2

Figure 2. Schematic representation of plasmid T-DNA regions. ............................. 21

Figure 3. Transgenic plants (RCP1 lines) in proliferation medium ......................... 29

Figure 4. PCR analysis showing amplification of a 500 bp corresponding to internal fragment of gusA gene. ...................................................... 30

Figure 5. Histochemical GUS assay performed on leaves and roots of young transgenic lines in the tissue culture laboratory ........................................... 32

Figure 6. Transgenic plants in green house .......................................................... 33

Figure 7. Potted infected and uninfected RCP1, CaMV35S lines and non-transgenic plants in the screen house one month after infection with Radophilus similis. .................................................................. 34

Figure 8. Typical symptoms of Radophilus similis on roots: black roots rots on corms and roots attacked by the nematodes .............................................. 34

Figure 9. Histochemical GUS assay performed on roots of infected plants. ............. 35

Figure 10. Histochemical GUS staining performed on the roots of infected CaMV35S promoter lines ................................................................. 36

Figure 11. Comparison between TLA of infected and uninfected plantains ............ 38

Figure 12. Comparison between total plant biomasses of infected and uninfected Gonja manjaya promoter lines at 8 weeks after infection period by R. similis. ... 39

Figure 13. Radophilus similis observed under compound light microscope after nematodes extraction from the infected roots ............................................. 40

Figure 14. Population of Radophilus similis per gram of root weight in different infected plantain promoter lines ......................................................... 40

Figure 15. Nematodes staining performed at 8th week after infection with Radophilus similis .............................................................. 41
## ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BAP</td>
<td>Benzylaminopurine</td>
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<td>BBSRC</td>
<td>Biotechnology and Biological Sciences Research Council</td>
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<tr>
<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>DFID</td>
<td>Department for International Development</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
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<tr>
<td>FAOSTAT</td>
<td>Food and Agriculture Organization Statistics</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GNA</td>
<td><em>Galanthus nivalis</em> agglutinin</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<td>IITA</td>
<td>International Institute of Tropical Agriculture</td>
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<tr>
<td>Kan R</td>
<td>Kanamycin resistance</td>
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<tr>
<td>LB</td>
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<tr>
<td>MES</td>
<td>Morpholino ethanesulfonic acid</td>
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<tr>
<td>NOS</td>
<td>Nopaline synthase</td>
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<td>PBS</td>
<td>Program for Biosafety Systems</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermophilus aquaticus</em></td>
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<tr>
<td>UNCST</td>
<td>Ugandan National Council for Science and Tech.</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
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<tr>
<td>YMB</td>
<td>Yeast mannitol broth</td>
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<tr>
<td>ZmRCP1</td>
<td><em>Zea mays</em> root cap specific promoter-1</td>
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<td>NARL</td>
<td>National Agricultural Research Laboratory</td>
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ABSTRACT

Plantains are among staple foods in Africa. Worldwide, Musa spp. have an annual production of 88 million tonnes. Nematodes cause significant annual yield losses to these crops. The losses are estimated at about 20% worldwide. The most disastrous nematodes are Radopholus similis and Platylechnus goodeyi. Genetic improvement of the crops by conventional breeding has proved to be laborious and time consuming. Transgenic technology can offer sustainable solutions to the problem of controlling plant parasitic nematodes. To date, only constitutive heterologous promoters such as maize ubiquitin 1, rice actin 1, viral promoter (CaMV35S) have been used for the production of transgenic banana plants. This study defined the potential of Zea mays root cap specific promoter-1 (ZmRCP1) to drive biosynthesis of an anti-nematodes effector in plantain roots. Transgenic plants used in this study were regenerated from embryogenic cell suspension (ECS) of Gonja manjaya transformed by Agrobacterium tumefaciens strain EHA105 harboring the binary vectors pBI121 and pBI-ZmRCP:GUS. The study was designed to establish the difference in expression patterns of gusA gene under Cauliflower Mosaic Virus 35S (CaMV35S) constitutive promoter and ZmRCP1 and to assess the impact of R.similis infection on plantains at various stages of growth. To achieve the objectives, the study involved confirmation of the presence of gusA gene in plantain genomic DNA, inoculation of the plantains with nematodes and analysis of gusA gene expression pattern in the infected plants. Nematodes infection was achieved with R. similis. PCR amplicons were observed in all transgenic lines when DNA amplification was done using gusA specific primers. A histochemical GUS assay showed root cap expression of gusA in RCP1 lines. R.similis infection had no influence on ZmRCP1 expression pattern. The effect of R. similis infection on total leaf area (TLA) and total plant biomass (TPB) during infection period was analyzed. Consequently, a two-tailed t-test performed at 95% confidence level revealed a significant difference (P<0.05) between TLA and TPB of infected and uninfected plants. Infected lines showed lower TLA than uninfected lines. Nematodes infection reduced TPB of infected clones. The decline in TLA and TPB shows minimal or negligible natural resistance from G.manjaya against R. similis. As nematode population increased in roots, gusA expression at the root tip of RCP1 plants intensified. ZmRCP1 restricts gene expression at the root cap hence making it favourable for use in delivering a chemodisruptive peptide that can be released into rhizosphere with the root exudates. A lethal peptide can be used to achieve a complete resistance status in Musa spp.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Plantains (Musa spp.) are cultivated in more than 120 countries throughout the tropics and sub-tropics. They are very important staple crops in Africa and fruit crops in the rest of the world (FAO, 2010). Plantains are predominantly smallholder crops, and most growers cannot afford costly chemicals to control pests and diseases. As diseases and pests continue to spread, there is a growing demand for new improved varieties.

Genetic improvement of plantains and bananas through conventional breeding is hampered due to long generation times, various levels of ploidy, sterility of most edible cultivars and the lack of genetic variability (Tripathi et al., 2005). Genetic transformation has become an important tool for crop improvement. Relative success in genetic engineering of plantains and bananas has been reported to enable the transfer of foreign genes into the cells of the plants.

Genetic engineering can therefore be used as a means to induce resistance in plantains to curb the menace of soil pathogenic nematodes that negatively impact on the production of the plant.

1.2 Differences between plantains and bananas

Banana and Plantain are two fruits that look alike but they have some difference between them. Bananas have thicker skin when compared to the plantains. The main difference between the two plants (Figure 1) is that plantains have taller
pseudostems as compared to bananas.

Figure 1. Morphological differences between bananas and plantains. Bar=0.5 cm

Banana fruits appear green when not fully ripe but yellow when ripe. Plantains resemble green bananas, but may be green, yellow or black when ripe. It is highly recommended to cook plantains very well before consumption because of their high starch content. Bananas are versatile type of fruits because they can be eaten when raw. Bananas can be used to make chips. Both plantain and banana are rich in dietary fiber as well as rich source for vitamin A and vitamin C. Bananas are a good source of potassium and are hot favorites of body builders and people who want to build muscles. The plantain fruits are good anti-oxidants as well. Hence, they are very effective in cleansing the body off the toxic elements and free radicals. Bananas are sweet while plantains are less sugary (Nelson et al., 2006).
1.3 Natural resistance of plantain and banana cultivars to nematodes infections

Nematodes pose challenges to plantain production (Gowen and Queneherve, 1990). The challenges contribute to approximately 20% annual loss worldwide (Sasser and Freckman, 1987). *Radophilus* is considered the most disastrous species (Sarah *et al.*, 1996). Resistance in banana to migratory endoparasites has been reported in some genotypes including both male (AA, AABB genotypes) and female parents (AA, AAB genotypes) and one hybrid (AAAB). The resistance previously demonstrated in one genotype Pisang Jari Buaya (AA) could be transferred to other *Musa* genotypes (Vaiene *et al.*, 2003). Possible sources of resistance/tolerance to *Pratylenchus coffeae* have also been reported in some genotypes of banana. The resistance of dwarf *Musa* cv ‘Kunnan’ to *R. similis* seems to be related to its high levels of condensed tannins in the roots. The tannin level remains unresponsive to infection in dwarf *Musa* cv ‘Kunnan’. The tannins of cv ‘Kunnan’ had a mostly procyanidin character, but also contained propelargonidins (Collingborn *et al.*, 2000). This background research information provides an opportunity that can be exploited to induce resistance using natural resistance genes (R-genes).

1.4 Economic importance of plantains

It is important to clarify that plantains are used widely for various purposes depending on the values of the crop that a community links to its cultural practices. For instance, plantains are basic food-crops supplying up to 25% of the carbohydrates for over 70 million people in Africa (IITA Report, 2009). Leaves
are used for wrapping other food during steaming. Banana leaves are also used as environmentally friendly "disposable plates" in southern India (Singh, 1996).

Brazilians use plantains to treat dehydration in infants, as the tannins in the fruit tend to protect the lining of the intestinal tract against further loss of liquids (Robinson, 1996). Plantains are appropriate for consumption when a low-fat, low-sodium, and/or cholesterol-free diet is required, making it particularly recommendable for people with cardiovascular and kidney problems, arthritis, gout, or gastro-intestinal ulcers (Robinson, 1996).

Alcoholic beverages such as beer, vinegar, and wine can be produced from banana fruits. In French Polynesia, vinegar is produced in small quantities by small-holding farmers using Yangambi km5 (AAA, Ibota subgroup) bananas, introduced initially for livestock but found to be perfectly edible and excellent for fermenting into vinegar (Nelson et al., 2006).

Some Musa species and hybrids with colourful floral bracts and flowers are utilized in ornamental landscapes and tropical flower arrangements as cut flowers. Bananas and plantain fruits and stems are made into silage and used as cattle feed. Underground parts are also used for pig and cattle feed. The sap is used as dye and ashes used for dye, tanning (India), and, in some Pacific islands, for tattooing. Leaves are used to wrap root balls of seedlings or plants before transplanting. (Nelson et al., 2006)
1.5 Plantains production challenges

Plantain is considered as one of the major staple food in Africa. However, its production remains a major challenge for food security and sovereignty which must be part of agricultural policies at the national and global levels. When grown in monoculture, plantains suffer great pests and disease pressure. This is due to low biotic diversity and short trophic chain. Technical assistance and innovation systems in a participatory form are generally weak. Irrigation is also infrequent due to technical and financial reasons (Nelson et al., 2006).

The general trend globally is the increasing prices of plantains. The price increases for the consumers are not being transferred to the farmers. The production constraints are so high and contribute to low yields as 4 to 15 tons/ha. Therefore, farmers are often not able to improve productivity of plantains (Nelson et al., 2006).

The major production constraints of plantains are pests and diseases. Nematodes cause significant yield losses worldwide. Nematodes also reduce the viability of rhizomes and roots, resulting in low yields. Nematodes, weevil borers, viruses, fungi and bacteria are the major limiting factors for the crop sustainability. The nematodes and weevils during production reduce the viability of the rhizome and roots. The black sigatoka disease reduces the plant leaf area. However, the control strategies for theses biotic stresses are limited for technical and financial reasons. The use of pesticides for control of pests and diseases is also an environmental hazard posing danger to human and environmental health (Jones, 2000).
1.6 Effects of nematodes on bananas and plantains

All the important nematodes that feed on *Musa spp.* are root parasites. Five nematode genera have been found to be associated with banana (Karim, 1994). *Pratylenchus goodeyi*, *Radopholus similis* and *Helicotylenchus multicinctus* are migratory endoparasites. Both *Meloidogyne* spp. and *Rotylenchulus* spp. are sedentary endoparasites. *P. goodeyi* and *R. similis* are the most serious on the East African highland bananas (Mbaka and Mwangi, 2008).

*R. similis* and *Pratylenchus* spp. are burrowing and lesion nematodes respectively. Nematodes penetration occurs by preference near the root apex, but *R. similis* can invade any portion of the root length. As the nematode migrates intercellularly and intracellularly, it feeds on the cytoplasm of cortex cells, collapsing cell walls, and causing cavities and tunnels which evolve as a necrosis and may extend to the whole cortex. The stele is not damaged by *R. similis* although it can penetrate young stelar tissues. This mode of feeding promotes root rots and hence these nematodes are associated with banana toppling which is a major cause of yield loss where high winds occur (Jackson *et al*., 2003).

*R. similis* invades and feeds on root and corm tissues of bananas. The nematode activity within the root cortex causes reddish-brown to black, elongated lesions which are readily seen when the roots are split open. Roots eventually blacken and die (Jackson *et al*., 2003). Plantains tend to be more susceptible to nematode damage than bananas (Anon, 1977). *Pratylenchus* enters the host’s root and creates black necrotic lesions. Secondary symptoms then appear above ground. These include stunting, chlorosis in the leaves, wilt like symptoms, and may
eventually lead to death (Barker et al., 1998).

The root-knot nematodes, *M. incognita* and *M. javanica* occur on banana and plantain roots. They induce multinucleate giant cells derived from vascular parenchyma or differentiating vascular cells in the central part of the stele. Multiplication of cortical cells is also induced resulting in the formation of galls. These lead to formation of giant cells that block the surrounding xylem vessels (Waele and Davide, 1998).

*Helicotylenchus spp.* destroy the plant cells on which it feeds before advancing to attack other cells. Such nematodes provide a resource for necrotrophic microorganisms (O’Bannon and Inserra, 1989). *Rotylenchulus reniformis* also induce feeding cells in host plants. It is a major pathogen of some other crops such as pineapple and cotton and not bananas (Caswell et al., 1990, Starr and Page, 1990).

### 1.7 Problem statement and justification

Bananas and plantains (*Musa* sp.) are a major staple food, supplying up to 25% of the carbohydrates for approximately 70 million people in Africa’s humid forest and mid-altitude regions (IITA, 2009). Many pests and diseases have significantly affected *Musa* cultivation. *R. similis* is the major biotic constraint of plantain and cooking bananas grown in the lowlands of Central and Eastern Africa (Sarah et al., 1996).

Transgenic technology offers sustainable solutions to the problem of controlling plant parasitic nematodes. To date, only constitutive heterologous promoters such
as maize ubiquitin 1 and the rice actin 1 promoters (Ordoñez, 2008) or viral promoter (CaMV35S promoter) (Tripathi et al., 2008) have been used for the production of transgenic banana plants.

The lack of a suitable tissue specific promoter calls for a need to test root specific promoters that can drive biosynthesis of a repellent peptide. The main aim of this study was to define the potential of Zea mays Root Cap specific Promoter-1 and more generally those characteristics required of a root specific promoter to deliver an effective anti-nematodes resistance in plantains’ roots.

1.8 Null hypothesis
Maize derived ZmRCP1 promoter cannot drive root specific gene expression in the roots of transgenic plantains.

1.9 General objective
To compare the transgenic expression patterns of ZmRCP1 and CaMV35S promoters in uninfected and nematodes-challenged transgenic plantains roots.

1.10 Specific objectives
i. To confirm presence or absence of gusA gene in plantain genomic DNA
ii. To analyze the expression patterns of gusA gene under regulation of RCP1 and CaMV35S promoters in transgenic plantains in association with nematodes infection
iii. To assess the impact of Radophilus similis roots infection on the total leaf area and total plant biomass at different stages of growth in plantains
1.11 Significance and anticipated output

1.11.1 Significance of the study

Nematodes are recognized as severe production constraints plantains (Gowen and Queneherve, 1990). There is need to find a long lasting solution to the menace. Genetic transformation has emerged as a complementary tool for nematodes control through a transgenic resistance strategy. However, there are public complains about the safety of genetically modified organisms to human life (Sonal, 2013).

This study established the possibility of genetic engineering of plantains using ZmRCP1 promoter to induce resistance to soil pathogenic nematodes while restricting the biosynthesis of toxic peptides in the roots to avoid intoxication of non-target organisms that feed on plantain fruits and pseudostems.

1.11.2 Anticipated output

The study was expected to demonstrate the effectiveness of Zea mays root cap specific promoter (ZmRCP1) as a potential driver for the biosynthesis of anti-nematodes effectors in the roots of transgenic plantains.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Genetic improvement of plantains

Development of disease resistant *Musa* spp. by conventional breeding remains a difficult endeavor because of the long generation times, various levels of ploidy, sterility of most edible cultivars and limited genetic variability. Therefore, genetic transformation has shown great prospects of becoming an important complementary tool for disease management strategy in *Musa* spp. (Tripathi *et al*., 2005). Genetic transformation requires protocols for integration of transgenes, many of which have been developed.

Microprojectile bombardment of embyogenic cells suspension (ECS) of *Musa* spp. is routine (Sagi *et al*., 1995, Becker *et al*., 2000). *Agrobacterium* - mediated transformation is the most preferred to direct gene transfers due the possibility of transferring one or a few DNA fragments with the gene of interest at high transformation frequencies and low cost. Large DNA fragments can also be transferred with minimal rearrangements (Gheysen *et al*., 1998, Hansen and Wright, 1999, Shibata and Liu, 2000).

*Agrobacterium*-mediated transformation protocols for ECS from various cultivars have been developed (Ganapathi *et al*., 2001, Ghosh *et al*., 2009, Tripathi *et al*., 2010, Khanna *et al*., 2004). Transformation protocols using meristematic tissues from various cultivars of *Musa* are now available (May *et al*., 1995, Tripathi *et al*., 2003, Tripathi *et al*., 2008).
2.2 Transgenic technologies for nematode resistance

The use of proteinase inhibitors (PIs) such as cystatins (a cysteinase inhibitor) as a nematodes anti-feedant has been effective (Ryan, 1990). It also addresses biosafety concerns since cystatins hardly involve in mammalian digestion. This is a natural defense strategy in some plants. Cystatin has been isolated from sunflower, cowpea, maize soybean and rice (Atkinson et al., 1995).

Engineered oryzacystatin-I expression has shown resistance to nematodes in tomatoes (Urwin et al., 1995), Arabidopsis (Urwin et al., 1997), rice (Vain et al., 1998) and pineapple (Urwin et al., 2000). Cavendish banana has been transformed using A. tumefaciens to express a protein engineered rice cystatin and tested in glasshouse for nematode resistance (Atkinson et al., 2004).

Natural resistance genes (R-genes) like Hs1pro-1 from wild species of beet have shown resistance to cyst nematodes Heterodera schachii (Cai et al., 1997). Mi-1.2 gene from tomato has shown resistance to Meloidogyne species (Milligan et al., 1998). No report exists about the use of Mi-1.2 gene in plants other than tomatoes. Lectins such as snowdrop lectins (GNA) have been proved to have biological activity against nematodes (Burrows et al., 1998).

However, toxicity to insects and mammals (Pusztaei et al., 1996) is often a limitation to the use of lectins. Some Bacillus thuringensis (Bt) cryptochrome proteins (Cry proteins) are effective against saprophagous nematodes (Borgonie et al., 1996). The Cry5B protein is toxic to wild type C. elegans whereas some mutants of C. elegans are resistant to it but susceptible to Cry6A toxin.
(Marroquin et al., 2000). The approach using Cry genes has potential for plant nematode control (Wei et al., 2003).

2.3 Promoters used for transgenic control of nematodes

Various gene constructs harboring both root specific and constitutive promoters have been used to develop crops that are resistant to many species of nematodes. CaMV35S provides a strong constitutive expression in dicots but expression in monocots is unfortunately weak (Fromm et al., 1985). CaMV35S has been used to drive cystatin expression in control of Globodera pallida in Potatoes (Urwin et al., 2001). However, cystatin expression is downregulated at syncytial stages of feeding cells of cyst nematodes (Goddijn et al., 1993).

The expression patterns of CaMV35S, tubulin-1(TUB-1) from Arabidopsis and ubiquitin-1 (UBI-1) promoter from Zea mays has been studied in rice plants using gusA gene fusions (Green et al., 2002). TUB-1 showed a higher GUS activity in roots than in tillers. UBI-1 and TUB-1 activity were both high in root tips and zone of elongation. TUB-1 activity declined more in older roots than UBI-1. Nematodes activity did not alter the expression of both promoters. However, giant cells induced by M.incognita retained TUB-1 activity as roots matured. Pratylenchus zeae invaded older roots than M.incognita with no changes in promoter activity realized. Hence TUB-1 proved to be the best promoter for delivery of anti-feedants such as cystatins to M.incognita (Green et al., 2002).

The potential of the AtMDK4-20 promoter of Arabidopsis thaliana to direct effective transgenic expression of a secreted nematode-repellent peptide has been
investigated in *Arabidopsis thaliana* and *Solanum tuberosum*. The peptide under the control of AtMDK4-20 has been shown to be effective against *Heterodera schachtii* in *Arabidopsis* and *Globodera pallida* in potatoes (Lilley *et al.*, 2010).

Transcription profile analyses have shown that up regulation of genes in the maize root cap reveals a link between clusters of genes and major metabolic processes in the root cap. These processes include detection and response to stressful biotic and abiotic factors, transport, processing and integration of hormonal activities (Jiang *et al.*, 2006). Hence, *ZmRCP1* may be an efficient promoter to deliver anti-nematodes effectors in plantains roots challenged by nematodes.

### 2.4 Molecular characterization of transgenic plants

The most significant recent development in genetics has been the introduction of molecular tools for genetic analysis. These techniques are based on the central dogma of molecular genetics.

#### 2.4.1 Reporter genes

A reporter gene is a gene that researchers attach to a regulatory sequence of another gene of interest in cell culture, animals or plants. Certain genes are chosen as reporters because the characteristics they confer on organisms expressing them are easily identified and measured, or because they are selectable markers. Reporter genes are often used as an indication of whether a certain gene has been taken up by or expressed in the cell or organism population (Darbani *et al.*, 2008).
Commonly used reporter genes that induce visually identifiable characteristics usually involve fluorescent and luminescent proteins. Examples include the gene that encodes jellyfish green fluorescent protein (GFP), which causes cells that express it to glow green under blue light, the enzyme luciferase which catalyzes a reaction with luciferin to produce light and the red fluorescent protein from the *dsRed* gene. The *gusA* gene has been commonly used in plants but luciferase and GFP are becoming more common (Koo *et al*., 2007).

The (β-glucuronidase gene (*gusA*)) is a reporter gene particularly useful in plant molecular biology (Jefferson *et al*., 1987). The purpose of this technique is to analyze the activity of a promoter (in terms of expression of a gene under that promoter) either in a quantitative way or through visualization of its activity in different tissues. The technique is based on beta-glucuronidase, an enzyme from the bacterium *Escherichia coli* (Blanco *et al*., 1982). This enzyme when incubated with some specific colorless or non-fluorescent substrates, can transform them into coloured or fluorescent products (Jefferson *et al*., 1986).

There are actually different possible glucuronides that can be used as substrates for the beta-glucuronidase, depending on the type of detection needed (histochemical, spectrophotometrical, fluorimetrical). The most common substrate for GUS histochemical staining is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc): the product of the reaction is in this case a clear blue color. Other common substrates are p-nitrophenyl β-D-glucuronide for the spectrophotometrical assay and 4-methylumbelliferyl-beta-D-glucuronide (MUG) for the fluorimetrical assay (Keith *et al*., 1995).
The GUS assay, and other reporter gene systems, can be used for other kinds of studies other than the classical promoter activity assay. Reporter systems have been used for the determination of the efficiency of gene delivery systems, the intracellular localization of a gene product, the detection of protein-protein or protein-DNA interactions, the efficiency of translation initiation signals and the success of molecular cloning efforts (Koo et al., 2007).

The use of gene reporter system for the analysis of promoter activity (GUS, luciferase, GFP, β-galactosidase, chloramphenicol acetyltransferase (CAT), alkaline phosphatase) is mainly dependent on the organism of interest (Keith, 1995).

2.4.2 Polymerase chain reaction
Polymerase chain reaction (PCR) was developed in 1983 by Kary Mullis (Bartlett and Stirling, 2003). PCR is now a common and often indispensable technique used in medical and biological research laboratories for a variety of applications (Saiki et al., 1988). PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to approximately 10 kilo base pairs (kb) Some techniques allow for amplification of fragments up to 40 kb in size (Cheng et al., 1994).

A basic PCR set up requires several components and reagents. These components include: DNA template that contains the DNA region (target) to be amplified, two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target, Taq polymerase or another DNA
polymerase with a temperature optimum at around 70°C. Deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand, buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, divalent cations, magnesium or manganese ions; generally Mg$^{2+}$ is used, but Mn$^{2+}$ can be utilized for PCR-mediated DNA mutagenesis (Sambrook et al., 2001), as higher Mn$^{2+}$ concentration increases the error rate during DNA synthesis (Pavlov et al., 2004).

Currently, a master mix that contains nearly all PCR components is used. An example is GoTaq® Green Master Mix (Promega) which is a premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, MgCl$_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. Reactions assembled with GoTaq® Green Master Mix have sufficient density for direct loading onto agarose gels. GoTaq® Green Master Mix is recommended for any amplification reaction that will be visualized by agarose gel electrophoresis and ethidium bromide staining (Glebs et al., 2003).

2.5 Stability of transgenes in plants

Transgenes are widely known to be susceptible to loss of expression as a result of gene loss and transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) (Stam et al., 1997, Kooter et al., 1999, Fagard, 2000 and
Vaucheret, 2001). Unstable expression of transgenes is of particular concern for trees as a result of their long life cycles (Han et al., 1997, Bradshaw et al., 2000).

Meiosis is widely known to promote gene silencing (Scheid et al., 1991, Iglesias et al., 1997). For some applications of transgenic trees, biosafety traits such as reproductive sterility, may be required by regulatory agencies, marketplace forces, or stewardship programs (Strauss et al., 1995).

Expression of a transferred gene can be initially silenced or inactivated over time or generations by either TGS or PTGS triggered by diverse host–defense responses (Matzke et al., 2000). Several factors that might serve as a trigger for silencing mechanisms include transgene copy number, T-DNA structure and integration sites. The presence of multiple copies of transcripts that are coded by transgenes might cause homology-dependent transgene silencing due to TGS or PTGS (Scheid et al., 1991, Gallo-Meagher and Irvine, 1996).

Formation of transgene duplications and its association with stability have been reported in both annual plants and trees (Stam et al., 1997, Kumar, 2000). Transgenes that are organized as inverted repeats can show low expression or complete silencing (Morino et al., 1999), but this is not always the case (Meza et al., 2002, Lechtenberg et al., 2003).

Stress-induced transgene inactivation has been reported in plants (Broer, 1996). For instance, a heat treatment (37°C) lasting for 10 days resulted in an almost complete (95%) loss of the phosphinothricin resistance in suspension culture cells derived from a single Medicago sativa transgenic line (Walter et al., 1992).
Transgenic instability has been frequently observed in annual plants undergoing sexual propagation (Brunner et al., 2007). Several studies have been conducted in perennial plants, including poplar trees, over multiple seasons and after vegetative propagation (Gallo-Meagher and Irvine, 1996). Most of these studies reported that transgene expression following vegetative propagation was highly stable and predictable.

2.6 Detection of transgene stability in plants

There is a correlation between multiple gene copies with transgene silencing. Thus, an early detection of transgene copies can be useful in sorting out the transgenic plants with higher tendency of transgene silencing. A number of methods are available for analyzing transgene copy number. These include Southern blotting, comparative genomic hybridization, multiplex probe amplification and hybridization (Armour et al., 2000) and chip hybridization (Lucito et al. 2000). There are a number of shortcomings, for example laborious protocol, requirement of large amounts of DNA and inaccurate estimation of the foreign gene copy number when tandem integration or rearrangement of the foreign gene occurs associated with these methods (Mason et al., 2002).

A powerful real-time fluorescence quantitative real-time PCR (qRT-PCR) method has been used to analyze gene copy number in transgenic corn, rapeseed rice, and cotton plants (Song et al., 2002, Weng et al., 2004, Yang et al., 2005a, Yang et al., 2005b). This method does require certain application conditions, such as the selection and copy number identification of the endogenous gene, accurate determination of DNA concentration.
2.7 Allometric function for estimation of banana leaf area

A method established by Nyombi et al., (2009) has helped in the study of various parameters of growth in bananas. This method can be applied in the analysis of growth pattern of plantains too. Indeed, researchers have been able to evaluate different stages of banana growth for use in growth assessment, understand banana crop physiology and also to predict yield. In the allometric function, total leaf area (TLA) is estimated as the product of the measured middle leaf area (MLA) and the number of functional leaves. MLA is estimated as:

\[ \text{MLA (m}^2\text{)} = -0.404 + 0.381 \text{ height (m)} + 0.411 \text{ girth (m)} \]

A leaf is considered as functional if over 50% of the leaf is green and is still fully attached to the pseudostem. The heights of the plants are measured from the base of the pseudostem to the axil of the top most pair of the fully expanded leaves.
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Transgenic plants production

Transgenic plantain lines of *Gonja manjaya* cultivar used in this study were provided by IITA. The CaMV35S lines were generated though transformation of embryogenic cell suspension of *Gonja manjaya* with *A. tumefaciens* strain EHA105 (Hood et al., 1993) harboring the binary vector pBI121 containing β-glucuronidase (*gusA*) gene driven by the CaMV35S promoter and the neomycin phosphotransferase (*nptII*) gene as selection marker (Chen et al., 2003). The RCP1 lines were generated through transformation using plasmid construct pBI-RCP1:GUS containing β-glucuronidase (*gusA*) gene driven by the RCP1 promoter and the neomycin phosphotransferase (*nptII*) gene as selection marker.

Schematic representation of the pBI121 and pBI-RCP1:GUS vectors’s T-DNA regions are shown in Figure 2.

3.2 Maintenance and multiplication of transgenic lines

The transgenic plants and non transgenic control plants were micropropagated on proliferation medium (Tripathi et al., 2003). Regenerated shoots were aseptically removed from baby food jars in the laminar hood. The shoots were cut to approximately 2cm from the base of the plant’s pseudostem. The roots were trimmed off and a small longitudinal cut was made on the shoot meristem. The explants were cultured on the proliferation media and incubated at 26 ± 2°C with 16h photoperiod furnished with fluorescent tube providing light of 94μmolm⁻²s⁻¹
for 4 weeks. The plants were maintained by subculturing on fresh culture medium every 4 weeks.

**Figure 2.** Schematic representation of plasmid T-DNA regions. **A:** The T-DNA of pBI-RCP1:GUS construct. **B:** The T-DNA region of pBI121 construct. The nptII gene driven by nopaline synthase promoter (NOS) was inserted near the right border of the T-DNA. The gusA gene under the CaMV35S or RCP1 promoter and with a CaMV35S terminator sequence was sited near the left border of the T-DNA region.

3.3 Root induction and establishment of plants in soil

The individual plantain shoots were transferred to root induction media (Tripathi *et al.*, 2003) and incubated in the same conditions as above for 4 weeks. The rooted plantlets were transferred to sterile soil in plastic pots for acclimatization. The pots were kept in a humid and shady place for 12-15 days and then transferred to normal greenhouse conditions. After hardening, the plants were transferred into big pots (25cm in diameter) for further growth and development in the screen house.

3.4 Genomic DNA extraction from transgenic plants

Genomic DNA extraction was done from young fully expanded leaves of transgenic plants and non transgenic control plants following the protocol outlined
in Qiagen DNeasy Plant Mini kit (Qiagen, GmbH, Germany). Leaves of each plantain cell line weighing 200mg were lyophilized in a microcentrifuge tube and crushed using sterilized micropestles. The extracted and pelleted DNA was resuspended in 100 ml of DNase free elution buffer and stored at -20°C.

3.5 Agarose gel electrophoresis of extracted genomic DNA

The quality of DNA extracted was checked by electrophoresis on a 0.8% (w/v) agarose. Two microlitres of the extracted genomic DNA was mixed with 5µl of the loading dye (New England Biolabs, Massachusetts, USA) and run on 0.8% (w/v) agarose gel against 5µl of 1kb ladder (Invitrogen, California, USA). The gel was run at 70 volts for 45 min and the gel visualized using a Syngene transiluminator, Cambridge, United Kingdom.

3.6 Estimation of DNA concentration

The DNA concentration was determined using a Thermo Scientific NanoDrop 2000c (Wilmington, USA). The concentrations were determined by directly pipetting 1µl of the DNA samples onto the measurement surface and recording the determined concentrations in nanogram. The purity of DNA was determined at A260/A280 and A260/A230 ratios. Following the NanoDrop concentration estimation, the samples were diluted to stock concentrations from which 200 ng of DNA for PCR analysis could be obtained.

3.7 Maintenance of A. tumefaciens culture and plasmid DNA isolation

Transformed Agrobacterium tumefaciens EHA105 strain cultures were provided by IITA. The EHA105 cells harboring pBI121 and pBI-ZmRCP1:GUS plasmids
were separately and aseptically streaked on LB solid media (Appendix 7.4) supplemented with kanamycin and rifampicin (Appendix 7.6) to final concentrations of 50µg/ml and 20µg/ml respectively. The plates were incubated at 28°C for 32h and then stored at 4°C. The bacterial cultures were subcultured on a fresh plate after every 2 weeks to retain the plasmids.

The plasmid DNA extraction from Agrobacterium tumefaciens EHA105 was done from pelleted cells as outlined in the procedures of Qiagen’s QIArep Spin Miniprep kit bench protocol (GmbH, Germany). A colony of EHA105 cells with the pBI121 plasmid was cultured in 25ml of LB broth supplemented with kanamycin and rifampicin to final concentrations of 50µg/ml and 20µg/ml respectively. The cultures were incubated at 28°C for 24h in an incubator shaker (New Brunswick, Enfield, USA).

The cells were harvested by centrifugation using sterile 2ml eppendorf tubes in a microcentrifuge at 12,000 rpm for 1 min at room temperature. The supernatants were discarded and plasmid extraction was afterwards done according to the procedures outlined in the Qiagen’s miniprep kit. The plasmid DNA extracted was resuspended in 50µl of the elution buffer.

3.8 PCR amplification

PCR analysis was done with gusA gene specific primers to confirm the presence or absence of the transgene in the plant genome. A total of 25 RCP1 lines and 15 CaMV35S lines were analyzed by PCR on genomic DNA. The forward primer 5'TTTAACTATGCGGATCCATCGC3' and reverse primer 5'CCAGTCGA-
GCATCTCTTCAGCGTA3' were used in the analysis. PCR was performed in 200µl Eppendorf PCR tubes. The PCR mastermix (Appendix 7.6) was prepared using GoTaq Green Master Mix (Promega, Madison, USA) which is a premixed ready-to-use solution containing Taq DNA polymerase, dNTPs, magnesium chloride and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

The PCR conditions used were 95°C for 5 min for initial denaturation followed by 35 cycles of amplification with each cycle consisting of the following steps: 94°C for 50 sec, 55°C for 40 sec and 72°C for 50 sec with a final extension of 72°C for 10 min. The amplified products were fractionated on 0.8% agarose gel. The pBI121 plasmid DNA was used as positive control and genomic DNA of non-transgenic plants was treated as a negative control. PCR grade water served as a negative control to verify absence of a DNA contaminant in the PCR master mix. The fractionated DNA was stained in ethidium bromide (0.5µg/ml) for 15 min and visualized using transilluminator (Syngene, Cambridge, United Kingdom).

3.9 Histochemical GUS analysis on young uninfected transgenic plants

All the PCR positive plants were tested for expression of gusA gene by histochemical assay according to a modified protocol of Jefferson et al., (1987) as described by Tripathi et al., (2005) for banana. The analysis was done using the leaves and roots of the RCP1 and CaMV35S lines. The explants were washed in 70% v/v ethanol for 2 min and then incubated in a fixation solution containing 0.3% v/v formaldehyde, 10mM MES pH 5.6, 0.3M mannitol (Appendix 7.14) for 45 min at room temperature. For proper fixation, the explants were vacuum-
infiltrated for 4 min and then washed at least thrice in 50mM sodium phosphate, pH 7.0 (Appendix 7.5).

The fixed explants were incubated with substrate solution containing 1mM X-gluc, 50mM sodium-phosphate pH 7.0, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 10mM EDTA and 50mM ascorbic acid (Appendix 7.15). The explants soaked in substrate solution were vacuum-infiltrated for 4 mins and then incubated at 37°C for 48h.

After incubation period, chlorophyll was removed by soaking the explants in 1% v/v NaOCl (stock 3.85%) for 3h. The explants were dehydrated in 50%, 70% and 95% v/v ethanol. The tissues were observed for blue staining due to β-glucuronidase activity and photographed by Sony DSC-F828 8Mega pixels camera, NewYork city, USA.

3.10 Inoculation of transgenic lines with nematodes

The 8 weeks old RCP1 and CaMV35S transgenic lines were transferred to the screen house for inoculation with *R. similis*. The *R. similis* produced by culturing on carrot discs were provided by IITA nematology laboratory at Namulonge in Uganda. Nematodes were extracted from the carrot discs by maceration in a blender and sieving (Speijer and De Waele, 1997). The average number of nematodes was determined by counting the number in 200μl aliquot. Each plant was inoculated with 500 nematodes. To deliver the inoculum to the roots, 2.9ml of the nematodes suspension was spread on a whatman filter paper and placed beneath the root mass of the infected plants. Three replicates of each transgenic
promoter line (RCP1 and CaMV35S) along with control non-transgenic plants were inoculated with nematodes. The uninfected plants were used as negative controls.

3.11 Analysis of gusA gene expression in infected plantain roots

A histochemical GUS assay on infected plants was used as qualitative method to evaluate the effect of nematode infection on promoter expression. The assay was done on all the CaMV35S, RCP1 lines and non-transgenic plants at the time of infection and afterwards at 4 weeks interval. A piece of the root was harvested, cleaned, untangled and stained for GUS activity. The change in intensity and locality of GUS activity along the roots was observed in all the plants.

3.12 Determination of effects of nematodes infection on growth characteristics

The height, girth and number of functional leaves were recorded at 2 weeks interval from the time of infection. An allometric function for determination of total leaf area (TLA) was used (Nyombi et al., 2009). The TLA was calculated as,

\[ TLA = n (0.411G + 0.381H - 0.404) \]

where ‘G’ is girth (m) at the base of the pseudostem, ‘H’ is the plant height (m) measured from the base to the axil of the topmost pair of fully expanded leaves and ‘n’ is the number of functional leaves (more than 50% green and fully attached to the pseudostem).

At the end of the infection period, the total plant biomass which includes root weight and above ground weight was determined by weighing. The roots were washed and photographed using 12M pixels Kodak C143 camera to show the effect of nematodes of infection on the corm and plantain fibrous roots.
3.13 Extraction and counting of nematodes from infected plant roots

The extraction of nematodes was done according to the modified procedure of Spaull and Braithwaite, (1979). The activity was carried out after every four weeks of infection period. The plant variety, plant line, and date of infection were recorded on incubation polythene bags. The roots were weighed and the masses recorded on the bags. The roots were washed to remove soil around them. They were then chopped into 1cm pieces. The chopped roots were ground into fine macerates. The macerated root samples were placed in the incubation polythene bags and mixed with 10 ml of 1% hydrogen peroxide (H$_2$O$_2$). The samples were incubated in the hydrogen peroxide for seven days. After the 7 days of incubation, H$_2$O$_2$ was transferred into 15ml falcon tubes and topped up with sterile distilled water. Using a microscope, the nematodes in H$_2$O$_2$ were counted in 200µl aliquots and the nematodes population per gram of the root weights recorded.

3.14 Histochemical GUS assay and nematodes staining

The procedure was carried out according to Green et al., (2002). GUS stained roots from both the infected and non-infected plants of RCP1, CaMV35S lines and non-transgenic control were placed in 1% sodium hypochlorite for 4 min to improve their transparency. The roots were transferred to a beaker of boiling 10% w/v aqueous solution of stock acid fuchsin solution (1.75g acid fuchsin in 125ml of acetic acid and 375ml of distilled water) for 3 min. The roots were rinsed in water and cleared in 10ml of acidified glycerol for 16h at 65°C. Roots and nematodes were observed under a compound light microscope (10X objective
lens) and photographed using a 12 Mega pixels Kodak C143 camera. The absence or presence of GUS stain around the nematode was recorded.
CHAPTER FOUR

4.0 RESULTS

4.1 Maintenance and multiplication of transgenic plants

Transgenic plantain lines of *Gonja manjaya* cultivar used in this study were provided by IITA. The regenerated transgenic plantain lines (RCP1 and CaMV35S lines) and the non-transgenic control plants were maintained and multiplied on proliferation medium to obtain a sufficient number of plants for various experiments. At the end of 4 weeks, fully developed shoots and roots had emerged increasing the population of plants from three plantlets subcultured in every jar to approximately twelve plants. Figure 3 illustrates how RCP1 lines were proliferated in jars containing proliferation medium. The CaMV35S, RCP1 and non-transgenic lines were proliferated according to Tripathi *et al.*, (2008).

![Transgenic plants (RCP1 lines) in proliferation medium. Bar =1.5cm](image)

*Figure 3.* Transgenic plants (RCP1 lines) in proliferation medium. Bar =1.5cm
4.2 Polymerase chain reaction on transgenic plants

PCR was used to detect *gusA* gene in the transgenic plants. PCR was performed using *gusA* gene specific primers and the amplified fragment of approximately 500 bp corresponding to the amplified internal fragment of *gusA* gene was detected (Figure 4). The amplicons were observed in all the transgenic plants tested. This confirmed the presence of the *gusA* gene in all transgenic plants and no plant escapes. No amplified product was observed in the control non-transgenic plants.

![Figure 4. PCR analysis showing amplification of a 500 bp corresponding to internal fragment of *gusA* gene. M is 1kb plus DNA ladder (Invitrogen, New York, USA); P is pBI121 plasmid used as positive control; RCP1-2, RCP1-3 and RCP1-5 are RCP1 lines; CaMV35S-2, is a CaMV35S lines.](image-url)
4.3 Histochemical GUS assay on young uninfected transgenic plants

The expression pattern of $gusA$ gene under the different promoters was analyzed by a histochemical GUS staining on the roots and leaves of the transgenic plants. Transient $gusA$ expression assay was done on the leaves and roots of transgenic plants transformed with the gene construct containing CaMV35S promoter, showed a uniform blue coloration in all the explants tested (Figure 5A). A blue coloration was noted to be intense at the root tips and declined further away from root tips of RCP1 lines. However, the leaves of the RCP1 lines showed no blue coloration (Figure 5B). Absence of blue coloration was observed in roots and leaves of non-transgenic plants (Figure 5A,B).
Figure 5. Histochemical GUS assay performed on leaves and roots of young transgenic lines in NARL tissue culture laboratory. The experiments were set up at the same time. A: GUS assay on CaMV35S lines. C5, C12, C9, C6 and C14 are CaMV35S lines. B: GUS assay on RCP1 lines. R2, R3, R5, R7 and R16 are RCP1 lines; P is a GUS positive plant; N is a non-transgenic plant. Scale bars=0.5cm.

4.4 Weaning and hardening of plants prior to inoculation

The RCP1, CaMV35S lines and the non-transgenic plants were weaned and transferred to pots. About 80% transgenic plantlets survived when transferred to the soil in pots in containment facility. There were no apparent phenotypic alterations observed during vegetative growth of hardened plants (Figure 6).
Figure 6. Transgenic plants in green house. A: RCP1, CaMV35S lines and non-transgenic control plants undergoing hardening in NARL green house. Bar=3cm. B: Potted RCP1 in the green house. C: Potted non-transgenic plants in the green house. Bar=4cm

4.5 Symptoms on transgenic plants due to R. similis infection

Phenotypic distinction was observed between the infected and uninfected plants. Nematodes infection induced leaf yellowing, stunted growth and a reduced number of functional leaves as observed on infected plants (Figure 7). The migratory endoparasites caused very conspicuous roots and corm damage in plants. On roots, the damage was observed as small dark watery lesions on the outer parts of the roots. Primary, secondary and tertiary roots were all affected. On the corm, large black necrotic lesions were observed (Figure 8).
Figure 7. Potted infected and uninfected RCP1, CaMV35S lines and non-transgenic plants in the screen house one month after infection with *Radophilus similis*. For infected and uninfected RCP1 lines, bar = 5cm and bar = 0.5cm respectively; infected and uninfected CaMV35S lines, bar=10cm and bar=6.7cm respectively; infected and uninfected WT, bar=6.7cm and bar=8cm respectively.

![Figure 7](image1.png)

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Figure 8. Typical symptoms of *Radophilus similis* on roots: black roots rots on corms and roots attacked by the nematodes. Bar=2cm.

![Figure 8](image11.png)
4.6 Analysis of reporter gene activity in infected plantain roots

A histochemical GUS assay done on the RCP1 lines at the time of infection showed intense blue coloration at the root tips of all the tested promoter lines. The blue coloration reduced progressively away from the root tip (Figure 9A). A similar experiment done on the roots at week 4 after infection also showed blue coloration but was confined to the root tips of the tested lines (Figure 9B). No blue color was observed on the roots from non-transgenic controls tested.

<table>
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<th>CONTROLS</th>
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<td>R5</td>
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Figure 9. Histochemical GUS assay performed on roots of infected plants. A: GUS staining performed at the time of infection. Scale bars =0.5 cm. B: GUS staining performed at 4 weeks after infection. Scale bars =0.5 cm. R2, R5, R7 and R11 are RCP1 lines, N is non-transgenic plant, C is non-infected RCP1 control line.

Histochemical GUS assay performed on the roots of CaMV35S lines showed blue coloration on whole section of root (Figure 10A,B). The intensity of the blue
coloration at the time of infection did not vary from the results of the experiment performed at 4 weeks from the time of infection.

<table>
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<tr>
<th>CaMV35S lines</th>
<th>Controls</th>
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<tr>
<td>C5</td>
<td>C12</td>
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**Figure 10.** Histochemical GUS staining performed on the roots of infected CaMV35S promoter lines. **A:** GUS staining performed at the time of infection. Scale bars=0.5cm. **B:** GUS staining performed at 4 weeks from the time of infection. Scale bars=0.5cm. C5, C12 are CaMV35S lines, N is non-transgenic plant, C is uninfected CaMV35S control line.

4.7 Analysis of growth pattern of infected plantains

A graphical representation of the growth pattern (Figures 11) of infected plantains revealed that the total leaf area (TLA) of uninfected clones (UI) were higher than those of infected clones (I). There was largely a decline in TLA of infected plants whereas an increase in TLA was observed for uninfected plants during the eight weeks of infection. The graphical representation (Figure 11) gives a hypothetical difference between the infected and uninfected lines. To conclusively suggest that
there is a significant difference, a Student t-test was performed at 95% confidence level (Table 1). P-values<0.05 showed significant differences between the treatment groups and vice versa.

4.8 Assessment of effects of *R. similis* infection on total plant biomass.
The total plant biomass (TPB) was calculated as a sum of root weight (RW) and above ground biomass (AGB). The TPB of infected and uninfected plants are different. The uninfected plants showed a higher TPB at the end of the four weeks infection period.

4.9 Extraction and enumeration of nematodes in infected roots
Nematodes extraction and enumeration was done to estimate the reproduction of the migratory endoparasites (*R. similis*) in the plantain roots. The nematodes were afterwards observed using a compound light microscope. The nematodes were observed as spherically shaped worms with tapered ends (Figure 13). The sizes of observed worms varied indicating varied stages of development of infecting nematodes. The results of enumeration of nematodes extracted from the roots showed an increase in population of *R. similis* in all plant lines from week 4 to week 8. (Figure 14).
Figure 11. Comparison between TLA of infected and uninfected plantain clones of the same transgenic cell lines. C12, C5 are CaMV35S lines; R2, R5, R7, R11, R16 are RCP1 lines; N is non-transgenic plant.

Table 1. Confirmatory Student’s t-test for determination of significant difference between TLA (I) and TLA (UI) clones. A 2-tailed t-test statistics used at confidence level of 95.0%. The mean TLA of (I) and (UI) promoter lines are significantly different where \( P < 0.05\). The mean TLA was calculated from three replicates.

<table>
<thead>
<tr>
<th>Category</th>
<th>Mean TLA ± SE</th>
<th>t Critical 2-tail</th>
<th>( P(T&lt;\text{t}) ) 2-tail</th>
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<tr>
<td>R2-I</td>
<td>0.004±0.0001</td>
<td></td>
<td></td>
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<tr>
<td>R2-UI</td>
<td>0.012±0.0017</td>
<td>3.182446305</td>
<td>0.017476524</td>
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<tr>
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<td>0.017495917</td>
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<tr>
<td>R7-I</td>
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<td></td>
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<td>0.000233682</td>
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<tr>
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<td></td>
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<tr>
<td>R11-UI</td>
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<td>0.003638093</td>
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<tr>
<td>R16-I</td>
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<td></td>
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<tr>
<td>R16-UI</td>
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<td>0.022602353</td>
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<tr>
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Figure 12. Comparisson between total plant biomasses of infected and uninfected ZmRCP1 and CaMV35S transgenic lines at 8 weeks after infection period by R. similis. I:infected plantain clones. UI:uninfected plantain clones. The averages of TPB were calculated from 3 replicates of each treatment category.

Table 2. Confirmatory Student’s t-test for determination of significant difference between TPB (I) and TPB (UI) clones. A 2-tailed t-test statistics used at confidence level of 95.0%. The mean TPB of (I) and (UI) promoter lines are significantly different where P< 0.05. The mean TPB was calculated from three replicates.

<table>
<thead>
<tr>
<th>Category</th>
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<th>t Critical 2-tail</th>
<th>P(T&lt;=t) 2-tail</th>
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<tr>
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<tr>
<td>C5-UI</td>
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<td>0.024301551</td>
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<tr>
<td>R11-I</td>
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<td>0.024301551</td>
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<tr>
<td>R11-UI</td>
<td>134.67±77.75</td>
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<tr>
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<td>2.776445105</td>
<td>0.012301077</td>
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<td>0.012301077</td>
</tr>
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<td>110.01±63.52</td>
<td>2.776445105</td>
<td>0.011937254</td>
</tr>
<tr>
<td>R5-I</td>
<td>73.23±42.28</td>
<td>2.776445105</td>
<td>0.011937254</td>
</tr>
<tr>
<td>R5-UI</td>
<td>46.56±26.87</td>
<td>2.776445105</td>
<td>0.011937254</td>
</tr>
<tr>
<td>R7-I</td>
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<td>0.011937254</td>
</tr>
<tr>
<td>R7-UI</td>
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<td>0.040662472</td>
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<tr>
<td>N-I</td>
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<td>0.040662472</td>
</tr>
<tr>
<td>N-UI</td>
<td>203.59±117.54</td>
<td>2.776445105</td>
<td>0.040662472</td>
</tr>
</tbody>
</table>
Figure 13. *R. similis* observed under compound light microscope at a magnification of 100x after nematodes extraction from the infected roots. Scale bar=200µm.

Figure 14. Population of *Radophilus similis* per gram of root weight in different infected plantain promoter lines. C12, C5 are CaMV35S lines; R16, R2, R5, R7 are RCP1 lines, N is non-trasgenic plant. Nematodes were extracted from three replicates of each plant line.

4.10 Histochemical GUS assay and nematodes staining on infected roots

*R. similis* were seen around the zones of GUS activity in both RCP1 and CaMV35S lines (Figure 15).
Figure 15. Nematodes staining performed at 8th week after infection with *R. similis*. a: *R. similis* found around GUS stained area in the RCP1 plant’s root. b: GUS stained root tip of a root hair in RCP1 root. c: *R. similis* in GUS stained area in CaMV35S root. d and e: *R. similis* in unstained root of non-transgenic control. f: a deeply GUS stained root tip of RCP1 plant’s root hair. g: a uniformly GUS stained CaMV35S plant’s root hair. All the photographs represent the root tips of both RCP1 and CaMV35S lines. Scale bars =200µm.
CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The prime objective of this project was to define the potential use of ZmRCP1 promoter isolated from maize in delivering nematode repelling peptides. CaMV35S promoter used in this experiment serves as a control to show the contrast between root specificity of ZmRCP1 (Jiang et al., 2006) and the constitutive nature of CaMV35S promoter (Horsch et al. 1985). Among ZmRCP1 and CaMV35S lines tested by PCR analysis, all the tested plants showed presence of the _gusA_ transgene. There was no amplicon observed when DNA sample from a non-transgenic plant was tested (Figure 4). The absence of plant escape shows that there was optimum selection during regeneration of transgenic lines.

The uniform blue coloration in the leaves and roots of the young tissue culture CaMV35S lines (Figure 5A) confirms stable _gusA_ expression in all the tissues of the transgenic plants. CaMV35S promoter provides a strong constitutive expression in dicots. It is weakly expressed in monocots (Fromm et al., 1985). Besides, the constitutive expression is an undesirable trait in transgenic plant where particular tissues are targeted. The expression of the proteins associated with defense should be achieved in non-aerial parts of the plant thereby restricting the exposure of non-target organisms. However, RCP1 lines showed no blue coloration on the leaves. The blue coloration was noticed only in the roots of the young plants (Figure 5B). The _gusA_ expression was very intense at the root tips and some faint leaky GUS activity away from the root tips of the young RCP1 plant roots. The
GUS expression was more confined to root tips in mature plants. This indicate that RCP1 promoter is very much root cap specific in adult plants. The use of ZmRCP1 promoter would therefore address biosafety concerns in an attempt to develop nematode resistant plantains (Lilley and Atkinson, 1997) as the genes will be expressed only in the plant section where there is nematode infection. This will ensure that the peptide is not present in fruits.

RCP1 and CaMV35S transgenic lines showed phenotypic similarities (Figure 6). This verifies that these promoters when used in genetic transformation of plantains do not induce any difference in the phenotype. The gusA gene in the transgenic plants served as a reporter gene. The expression of the reporter gene is used as a marker for successful uptake of the gene of interest (Jefferson et al., 1987). Any change in the morphology of the plant after infection was primarily due to the parasitic effect of *R. similis*. A lower total plant biomass of uninfected R16 lines as compared to infected lines (Figure 12) was due to a noticeable less attack of *R. similis* on the roots of the plants.

The morphological distinction between the infected and non-infected plants observed is a proof that the disease caused by *R. similis*, also called toppling or blackhead disease, has an impact on the plant growth (Luc et al., 1990). Black patches on leaves, yellowing, stunted growth and wilting of leaves are characteristic of the infected plants (Figure 7). As the nematodes feed, they destroy anchor roots and make banana plants susceptible to toppling. Furthermore, roots damaged by the nematodes cannot supply plants with needed water and nutrients, resulting in reduction of plant growth and development (Sarah et al., 1996). A
further deterioration on the plant growth was noted as time progressed from the
date of infection with *R. similis* (Figure 7). The impact confirms lack of resistance
from the plants transformed with *gusA* which is basically a reporter gene.

The shift of β-glucuronidase gene expression from the entire root structure of
RCP1 plant (Figure 9A) to the root tips (Figure 9B) is a show of root cap
specificity of ZmRCP1 (Jiang *et al.*, 2006). The rest of the root quarters that were
infected with nematodes showed no gene expression after four weeks of infection.
ZmRCP1 promoter expression became localized at the root tips in plantains as the
roots matured. This implies that gene expression in RCP1 lines became localized at
the root tips as the root matured. Due to increased root tissue specificity and
localization of promoter expression observed, it can be inferred that *R. similis*
infection had no negative impact on *gusA* gene expression. Age of the plant roots
was the significant determinant of the pattern of gene expression in the roots. This
property of ZmRCP1 promoter may be so pertinent in delivering anti-nematodes
resistance particularly with a gene that directs effective transgenic expression of a
secreted nematode-repellent peptide.

Root-targeted transgene expression provides biosafety benefits by restricting novel
proteins to nonfood parts of most crops and by reducing the exposure of non-target
organisms to the effector protein (Lilley *et al.*, 2010). Targeted expression of an
effector designed to disrupt nematode invasion requires a promoter that is active in
the outer cell layers of the root to ensure efficient secretion to the rhizoplane and
rhizosphere. Such promoters exist; for instance, phytase has been secreted into the
rhizosphere of potato plants using the promoter of the tomato *LeExt1.1* gene that
directs expression in root hair cells (Zimmermann et al., 2003). ZmRCP1 directs expression at the lateral root cap cells (Matsuyama et al., 1999).

The expression of CaMV35S promoter remained unchanged in both young (Figure 10A) and older roots (Figure 10B) of CaMV35S lines. This confirms that the constitutive pattern of expression of the CaMV35S promoter in roots of CaMV35S promoter lines is not altered by the age of the plant. It is arguable that gusA gene expression was neither determined by the age of the roots nor the root invasion by R. similis.

Nematodes infection accounted for the decline in TPB. A lower TPB of uninfected RCP1-16 promoter lines as compared to the infected lines was due to observed less attack on the roots and corms of the plants (Figure 12). The TLA of the infected lines was evidently lower than TLA of uninfected lines (Figures 11). The results obtained show the magnitude of the effect of R.similis on the TLA of nematodes infected plantains. This justifies the efforts in an attempt to develop transgenic nematode resistant plantains. The TLA of uninfected lines largely show an increase in plant biomass from week 2 to week 8. In case of a decline in the TLA, it is likely to be due to the effect of a decline in the plant height, a variable used in computing the TLA (Nyombi et al., 2009). When an outermost leaf sheath that determines the uppermost axil dries up, the plant height recorded became slightly lower than the previously recorded value.

It is important to note that this study established a foundation for development of gene construct that will deliver anti-nematodes resistance to soil feeding nematodes
attacking bananas and plantains. Hence, a correlation between change in nematodes population in the roots and the pattern of RCP1 promoter expression was established. The population of nematodes increased drastically from week 4 to week 8 (Figure 14).

This drastic increase in the population of nematodes presents the need for use of a promoter that highly directs a root specific gene expression in both young and maturing roots. From the results (Figure 9B), gusA expression at week 4 was already intense and localized at the root tips of the plantain roots. Therefore, the increase in the nematodes population would be void if a nematodes resistance gene is used to develop transgenic plantains.

Some cultivars of plantains and bananas have been found to be resistant, moderately resistant or susceptible to *R. similis* (Viaene *et al.*, 2002). In most susceptible cultivars, on examination of nematodes reproduction and corm damage, a positive correlation between nematodes population densities and damage magnitude has been observed (Viaene *et al.*, 2002). The observed shoot symptoms of nematodes infection (Figure 7) is an indication of nematodes reproduction in the root corms of the plantains. This inference is an additional verification of an increasing population of nematodes over time as is graphically represented (Figure 14). *Gonja manjaya* has been reported as a susceptible cultivar which is consistent with the results (Figure 14). This is verified by the increase in the number of nematodes in all the plant tested from the inoculation time to the end of infection period.
Nematodes were observed around GUS stains in the root tips (Figure 15). Hence, there is no down regulation of the gusA gene expression around the regions of *R. similis* infection. This is contrary to the fear attributed to the previous findings which revealed that cystatins expression under CaMV35S promoter is down-regulated at the feeding cells of cyst nematodes *Globodera pallida* (Urwin *et al*., 1997) and *Meloidogyne spp.* (Goddijn *et al*., 1993). Moreover, Plant defensin gene 2.1 (*Pdf2.1*) is strongly upregulated in syncytia induced by the beet cyst nematode *H. schachtii* in Arabidopsis roots. In addition, *Pdf2.2* and *Pdf2.3* is also strongly expressed in syncytia but contrary to the *Pdf2.1* gene, both genes have a strong expression in uninfected roots (Szakasits *et al*., 2009).
5.2 Conclusion

The integration of the transgene *gusA* gene was confirmed in this study by PCR and a histochemical GUS assay. However, there will be need for a southern blot analysis to establish the stable integration of transgene in nematode resistant transgenic plantains. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of gene sequences in a genome. The presence of multiple copies of transcripts that are coded by transgene might cause homology-dependent transgene silencing due to transcriptional gene silencing and or post-translational gene silencing (Scheid et al., 1991, Gallo-Meagher and Irvine, 1996). A southern blot analysis will therefore be a guide to a proper choice of nematodes resistant transgenic plants to be subjected to a confined field trial to assess stability of gene expression in different generations.

The results of this study demonstrated the potential of ZmRCP1 to limit expression of gene to the root cap while maintaining the efficacy of both sedentary and migratory nematodes defense. ZmRCP1 confers spatially restricted expression at the root cap and hence is not suitable for directing anti-feeding resistance. An anti-feeding resistance would not be effective since only nematodes residing and feeding at the root cap would be intoxicated by the peptides in the root. Instead, the promoter can be used to direct biosynthesis of a peptide that is released into the rhizosphere and rhizoplane with root exudates. The peptide disrupts chemoreception of the nematodes in a nonlethal manner (Liu et al., 2005). Consequently, the peptide hinders localization and invasion of roots by nematodes. Targetted expression of an effector designed to disrupt nematodes invasion
requires a promoter that is active in the outer cell layers of the root to ensure efficient secretion to the rhizosphere and rhizoplane. ZmRCP1 directs strong gene expression at lateral root cap cells in maize (Matsuyama et al., 1999) and suits best a chemodisruptive transgenic resistance strategy. However, a complete resistance can be achieved by using a lethal peptide released with the root exudates. Production of transgenic plantain resistant to nematodes using cystatin and repellent peptide has been reported (Roderick et al., 2012).

5.3 Recommendations

Future attempts to develop nematodes resistant transgenic plantains by introducing nematode repellent gene must be coupled with a procedure that enumerates nematodes at uniform segments of sampled root systems. This procedure will help to reveal how responsive each section of the root synthesizing toxic peptides is to the invasive migratory parasites.

Further characterization of the ZmRCP1 promoter is necessary in a confined field trial of the transgenic plants. The transgenic plants used in this study were mainly tissue culture plants in the laboratory and a containment facility. Therefore, there is need to verify further if the age of the plants in their natural setup will influence the pattern of gusA expression in the roots.
6.0 REFERENCES


7.0 APPENDICES

7.1 Stock solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>concentration</th>
<th>FW (g/mol.)</th>
<th>Solvent</th>
<th>Sterilization</th>
<th>Storage</th>
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7.2 Tris-Acetate EDTA buffer (TAE)

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<td>1000 ml (1L)</td>
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<td>0.5M EDTA</td>
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7.3 Yeast Mannitol Broth (YMB)

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<td>Dipotassium hydrogen phosphate</td>
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<td>sodium chloride</td>
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<td>Magnesium sulphate</td>
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7.4 Luria Bertani (LB) medium

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<td>Agar (for solid media)</td>
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7.5 Dilution scheme for 0.1M sodium phosphate buffer (Ellis and Morrison, 1982)

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<th>Volume of 0.5M Na\textsubscript{2}HPO\textsubscript{4} (ml)</th>
<th>Volume of 1M NaH\textsubscript{2}PO\textsubscript{4} (ml)</th>
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<td>7.2</td>
<td>136.8</td>
<td>31.6</td>
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<tr>
<td>7.4</td>
<td>154.8</td>
<td>22.6</td>
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<tr>
<td>7.6</td>
<td>169</td>
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<td>7.8</td>
<td>179.2</td>
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<td>8</td>
<td>186.4</td>
<td>6.8</td>
</tr>
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</table>

Dilute the combined stock solutions to 1000 ml final volume with distilled water. The pH is calculated according to Henderson-Hesselbalch equation:

\[
pH = pK_a + \log_{10} \left( \frac{[\text{conjugate base}]}{[\text{conjugate acid}]} \right) = pK_a + \log_{10} \left( \frac{[\text{proton acceptor}]}{[\text{proton donor}]} \right)\]

\[pK_a=6.8\] at 25°C

7.6 Antibiotics

<table>
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<th>Concentration</th>
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<td>water</td>
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<tr>
<td>Rifampicin</td>
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<td>Cefotaxime</td>
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<td>water</td>
<td>300µl/ml</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
7.7 Mastermix preparation for PCR amplification of desired target DNA. 
\( n = \text{number of reactions required, } V = \text{volume/20µl} \)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock conc</th>
<th>Final conc</th>
<th>V (µl)</th>
<th>V*n(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq X2</td>
<td>X1</td>
<td>10</td>
<td>10*n</td>
<td></td>
</tr>
<tr>
<td>FW primer 10µM</td>
<td>0.2µM</td>
<td>0.5</td>
<td>0.5*n</td>
<td></td>
</tr>
<tr>
<td>RV primer 10µM</td>
<td>0.2µM</td>
<td>0.5</td>
<td>0.5*n</td>
<td></td>
</tr>
<tr>
<td>Template DNA</td>
<td>200 ng</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR grade water</td>
<td>5</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>20</td>
<td>( \Sigma V*n )</td>
<td></td>
</tr>
</tbody>
</table>

The master mix was distributed into the PCR tubes at the rate of \( \Sigma V*n/\text{n µl} \) and then spun before thermo-cycling in the PCR machine.

7.8 Macronutrients stock

<table>
<thead>
<tr>
<th>Medium components</th>
<th>Conc. 1X (mg/L)</th>
<th>Conc.10X (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>1650</td>
<td>16.5</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>1900</td>
<td>19</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>440</td>
<td>4.4</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>370</td>
<td>3.7</td>
</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>170</td>
<td>1.7</td>
</tr>
</tbody>
</table>

7.9 Micronutrients stock

<table>
<thead>
<tr>
<th>Components</th>
<th>Conc.1X (mg/L)</th>
<th>Conc.100X(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>6.2</td>
<td>0.62</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>22.3</td>
<td>2.23</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>8.6</td>
<td>0.86</td>
</tr>
<tr>
<td>Molybdic acid</td>
<td>0.25</td>
<td>0.025</td>
</tr>
<tr>
<td>Coppersulphate</td>
<td>0.025</td>
<td>0.0025</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>0.025</td>
<td>0.0025</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.83</td>
<td>0.083</td>
</tr>
</tbody>
</table>
### 7.10 Fe EDTA

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration.1X (mg/L)</th>
<th>Concentration.100X(g/500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA.₂H₂O</td>
<td>37.3</td>
<td>1.87</td>
</tr>
<tr>
<td>FeSO₄.₇H₂O</td>
<td>27.8</td>
<td>1.37</td>
</tr>
</tbody>
</table>

### 7.11 MS vitamins

<table>
<thead>
<tr>
<th>Components</th>
<th>1X (mg/l)</th>
<th>1000X (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Thiamine.HCl</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

### 7.12 Growth regulators

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Type</th>
<th>Solvent</th>
<th>Dilutant</th>
<th>Storage</th>
<th>Sterilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>Cytokinin</td>
<td>1N NaOH</td>
<td>H₂O</td>
<td>0-5°C</td>
<td>CA</td>
</tr>
<tr>
<td>NAA</td>
<td>Auxins</td>
<td>1N NaOH</td>
<td>H₂O</td>
<td>0-5°C</td>
<td>CA</td>
</tr>
<tr>
<td>IBA</td>
<td>Auxins</td>
<td>1N NaOH</td>
<td>H₂O</td>
<td>0°C</td>
<td>F</td>
</tr>
<tr>
<td>IAA</td>
<td>Auxins</td>
<td>1N NaOH</td>
<td>H₂O</td>
<td>0°C</td>
<td>F</td>
</tr>
</tbody>
</table>

Key: CA-Coautoclavable with other media components; F-Filter sterilization (0.22µl)

### 7.13 Proliferation media (1L) and rooting media (1L)

<table>
<thead>
<tr>
<th>Media components</th>
<th>Final conc.</th>
<th>Vol.in prolif. Medium(ml)</th>
<th>Vol.in rooting medium (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients 10X</td>
<td>1X</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Micronutrients 100X</td>
<td>1X</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Fe EDTA 100X</td>
<td>1X</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Vitamins 1000X</td>
<td>1X</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ascorbic acid 4mg/ml</td>
<td>4 mg/L</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>IBA 1mg/ml</td>
<td>1 mg/L</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>BAP 1mg/ml</td>
<td>5 mg/L</td>
<td>5</td>
<td>....</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3% w/v</td>
<td>30 g</td>
<td>30g</td>
</tr>
<tr>
<td>Gelrite</td>
<td>2.4 g/L</td>
<td>2.4 g</td>
<td>2.4 g</td>
</tr>
</tbody>
</table>

pH=5.8 and autoclaved
### 7.14 Histochemical GUS assay fixation solution

<table>
<thead>
<tr>
<th>Components</th>
<th>Working conc.</th>
<th>Amount in 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>0.30%</td>
<td>300µl</td>
</tr>
<tr>
<td>MES(0.5M)</td>
<td>10mM</td>
<td>2 ml</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.3M</td>
<td>5.47g</td>
</tr>
</tbody>
</table>

### 7.15 Histochemical GUS assay substrate solution

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock’s conc</th>
<th>Working conc.</th>
<th>Amount in 100ml solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-Gluc</td>
<td>0.1M</td>
<td>1mM</td>
<td>1 ml</td>
</tr>
<tr>
<td>Sodium phosphate buffer</td>
<td>0.5M, pH=7.0</td>
<td>5mM</td>
<td>50 ml</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>50mM</td>
<td>5mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>Potassium ferrocyanide</td>
<td>50mM</td>
<td>5mM</td>
<td>10 ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>10mM</td>
<td>10mM</td>
<td>2 ml</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>50mM</td>
<td>50mM</td>
<td>0.88g</td>
</tr>
</tbody>
</table>