EXPRESSION OF AMPHIPATHIC PROTEIN (SAP1) FROM SWEET PEPPER FOR INDUCTION OF RESISTANCE TO *Xanthomonas Campestris pv. Musacearum* IN BANANA

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DECLARATION

I, **Namukwaya Betty** declare that this study is original and has not been published or submitted for any other degree award to any other university.

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This thesis has been submitted for examination with our approval as the University appointed supervisors.

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DEDICATION

This thesis is dedicated to my dear son Shafic Muwulya and my beloved father Mr. Ezra Lutakoome, all my friends and whoever contributed to this book, and most of all to the Almighty God who strengthened me all through.

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ABSTRACT

Bananas and plantains constitute the most important staple food crop in sub-Saharan Africa. They are also a source of income for millions of people in this region. The livelihoods of millions of Ugandan farmers have been threatened by the recent outbreak of the Banana Xanthomonas wilt (BXW) disease caused by *Xanthomonas campestris* pv.*musacearum*. BXW is currently the most destructive emergent disease in Uganda, causing up to 100% plantation loss where no control measures are employed. It attacks all banana cultivars, including East African Highland Bananas (EAHBs) and there is no banana cultivar that is resistant to BXW. Due to lack of resistant cultivars in the available germplasm, coupled with the high infertility of the triploid banana cultivars, genetic engineering seems to be the most feasible way of introducing resistance into banana germplasm. This study reports *Agrobacterium*-mediated transformation of embryogenic cell suspensions of two banana cultivars 'Nakinyika' (EA-AAA) and 'Sukali Ndiizi' (ABB), using amphipathic protein gene (*sap*1) that confers resistance against plant Banana Xanthomonas Wilt diseases. Transformed cells were selected on kanamycin supplemented medium and regeneration frequencies of 62% and 38% were observed for cultivars 'Nakinyika' and 'Sukali Ndiizi,' respectively. The presence of *sap*1 gene was confirmed by PCR analysis of transformed lines. The integration of *sap*1 gene into the plant genome was confirmed using Southern blot analysis. The efficacy of *sap*1 gene was tested by evaluating the transgenic lines for resistance against BXW using *in vitro* plantlets under laboratory conditions. The preliminary results obtained suggest that *sap*1 gene could provide significant resistance to BXW in banana.

CHAPTER ONE

INTRODUCTION

1.1 Background

Bananas and plantains constitute the most important staple food crop globally. They are cultivated in over 100 countries covering about 10 million hectares, with annual production of 88 million tones (Sharrock and Frison, 1999). In Africa, bananas and plantains provide more than 25% of food energy requirement for more than 100 million people of whom 20 million are from East Africa alone. Uganda ranks second after India in the world in banana production with an annual output of 10.5 million tones with 90% consumed locally (FAOSTAT 2004). In terms of revenue, banana is one of the most important cash crops contributing up to 22% of national agricultural revenue (Kalyebala *et al.,* 2007). Despite the importance of bananas, the crop is threatened by various production constraints such biotic and abiotic factors (Ortiz *et al*., 2002).

Biotic factors in general significantly reduce yield of banana in comparison to abiotic factors. The major biotic factors include banana weevils (*Cosmopolites sodidus*), nematodes (*Rodopholus similis*), black sigatoka disease (*Mycosphaerella fijiensis*), Fusarium wilt (*Fusarium oxysporum* f.sp *cubense*) and banana Xanthomona*s* wilt disease (*Xanthomonas campenstris* pv.*musacerum*). The abiotic factors include low soil fertility with associated yield loses typically in the range of 30-60%. Overall, low soil fertility and Banana Xanthomonas wilt (BXW) appear to pose the most damaging threat to banana productivity. Banana Xanthomonas wilt has become a new threat and is currently rated the most serious constraint to banana production in Uganda.

The Banana Xanthomonas Wilt (BXW) disease caused by the bacterium *Xanthomonas campestris* pv. *musacearum* endangers the livelihood of millions of farmers in East Africa (Tushemereirwe *et al*., 2004). BXW is threatening the banana production in the Great Lakes region of Eastern Africa including Burundi, Rwanda, Democratic Republic of Congo, Uganda, Kenya, and Tanzania (Kalyebala *et al*., 2007). The disease was first reported about 40 years ago in Ethiopia on Ensete, which is closely related to banana (Yirgou *et al.*, 1968). Outside Ethiopia, BXW was first reported in Uganda in 2001 (Tushemereirwe *et al*., 2004) and has now spread to almost all major banana producing districts of the country (Tripathi *et al*., 2009). The disease has contributed to decreased household and national food security and income (Tushemereirwe *et al* 2004; Tushemereirwe *et al.,* 2003). The disease has also been reported in Democratic Republic of Congo (Ndungo *et al*., 2006), Rwanda (Reeder *et al*., 2007), Tanzania (Mgenzi *et al*., 2006), Kenya (Mbaka *et* a*l*., 2007) and Burundi (Carter *et al*., 2009).

1.2 Banana Xanthomonas wilt and its economic impact

Banana Xanthomonas Wilt (BXW) is an emerging disease of banana in East Africa. It is a vascular disease that results in permanent wilting and eventual death of the plant. All banana cultivars are susceptible to BXW, although field observations suggest that the disease appears to be more prevalent on 'Pisang'Awak' (ABB commonly known as Kayinja). In susceptible banana cultivars yield losses of up to 100% have been reported (Tushemereirwe *et al.,* 2004; 2003). By the end of 2004, the disease had infected an average of 33% mats nationwide (Karamura *et al*., 2006). The total banana yield loss due to BXW infection was estimated at 30-52 % between 2001 and 2004 and it was estimated that Uganda would lose up to 4 billion

US shillings by end 2010 if the epidemic is not arrested (Karamura *et al*., 2006). Most susceptible cultivars have been reported to include 'Bogoya,' 'Pisang Awak' and 'Sukali Ndiizi' (Tushemereirwe *et al.,* 2006).

The impact of BXW is both extreme and rapid, unlike those of other diseases, which cause gradual losses over years. The economic impact of BXW is due to death of the mother plant that would otherwise contribute to the ratoon plant production cycles (Tripathi *et al*., 2007). Fields infested with *X. campestris* pv. *musacearum* cannot be replanted with bananas for at least 6 months due to carryover of soil borne inoculum (Turyagyenda *et al*., 2007). BXW has similarities to other bacterial wilts of banana caused by *Rastonia solanacealum*, including moko, blood, and bugtok diseases (Thwaites *et al*., 2000). Once these pathogens have established, disease control is very difficult (Eden- Green *et al*., 2004).

All banana types are susceptible to the BXW disease, and no resistance gene has been identified in bananas (Tripathi *et al*., 2008; Sekiwoko *et al*., 2006). The disease has spread throughout the major banana producing districts of Uganda, causing losses of up to 100% in poorly managed banana plantations (Tushemereirwe *et al*., 2004). Yield losses are associated with early ripening and rotting of fruits even in the absence of apparent external symptoms of the disease, and wilting and death of the banana plants. As a result of its spread within the eastern, northern and central districts of Uganda, several farmers have abandoned banana cultivation (Tripathi *et al.,* 2009).

1.3 Statement of the Problem

Banana Xanthomonas Wilt (BXW) is the most devastating disease of banana in the entire Great lakes region of Africa. So far, No naturally occurring resistance to BXW has been identified in banana, yet banana breeding is very difficult because of polyploidy, long generation cycles, sterility in most edible cultivars, and small gene pool resulting in lack of resistance in cultivated *Musa* sp (Stover and Simmond, 1987). Therefore, genetic engineering is considered the most feasible approach since genes with potential resistance against most of the banana diseases and pests using genes from other plant species (Tripathi *et al.,* 2008) can be obtained from other plant species.

1.4 Justification

The use of genetic engineering approach has been identified as a potential option that could be utilized to facilitate and/or enhance the process of developing resistant banana cultivars to BXW without significantly changing their original characteristics. Developing transgenic plants with effective resistance to bacterial diseases is generally difficult to accomplish. However, efforts including the expression of pathogenesis related proteins and others with antimicrobial effects have been reported. Antimicrobial proteins so far reported to enhance resistance against bacterial diseases include ferredoxin-like protein (*sap*1) in rice, cana lily, sweet pepper and Oncidium orchid (Liau *et al*., 2003, Yip *et al*., 2006). Sweet pepper amphipathic protein (*sap*1*)*, previously isolated and cloned from sweet pepper (*Capsicum annuum*), is reported to significantly enhance harpin-mediated hypersensitive response (Dayakar *et al*., 2003). Although the *sap*1 gene enhanced plant protection against gram-negative bacteria, there is no information concerning the use of this gene in the management

of bacterial diseases in banana. However, based on the available information, *sap*1 has potential of enhancing banana's resistance against BXW.

1.5 Objectives

The main objective of this study was to investigate whether the expression of s*ap1* gene from sweet pepper can be used in the control of BXW.

1.5.1 Specific objectives

(i) To transform two banana cultivars 'Nakinyika' and 'Sukali Ndiizi' with amphipathic protein (s*ap*1) gene from sweet pepper (*Capsicum annuum*).

(ii) To evaluate the presence and integration of *sap*1 gene in the genome of transformed banana.

(iii) To evaluate the efficacy of *sap*1 gene in banana transgenic lines for enhanced resistance against BXW.

1.5.2 Hypotheses

(i) Banana cells can be transformed using *sap*1 gene and regenerated into individual plantlets.

(ii) Integration patterns of transgene *sap*1 differ in different transgenic lines of cultivars 'Nakinyika' and 'Sukali Ndiizi'.

(iii) The expression of transgene *sap*1 significantly enhances the resistance of cultivars 'Nakinyika' and 'Sukali Ndiizi' to BXW.

CHAPTER TWO

LITERATURE REVIEW

2.1 Banana and its Importance in East Africa

Bananas belong to genus *Musa*, family *Musaceae* and order *Zingiberales*. Bananas are believed to have originated from South East Asia and Indochina (Simmonds, 1962) where the earliest domestication of bananas is also believed to have happened. From here, they were introduced to all tropical and subtropical regions of the world thus gaining great importance. It is suggested that edible bananas originated from two wild seed forming species, *Musa acuminata* Colla (2n = 2X= 22) and *Musa balbisiana* Colla (2n=22) and provide "A" and "B" genomes of bananas, respectively. Bananas were introduced into East Africa by Arab traders between India and East Africa, or could have reached East Africa via the west coast of Africa. Somatic mutations gave rise to the large variability in the East African highland banana (EAHB) cultivars making East Africa the secondary center of diversity for this group.

Banana has many attributes that makes it an important crop. The progressive conversion of starch into sugars after harvest makes some banana cultivars to be consumed as fruits (for example, Cavendish) (Bagamba *et al*., 2006) while others like plantain are considered to be a carbohydrate staple. Depending on the juice yield, some fruit type cultivars are used to produce wine and gin. East African highland bananas are mainly produced as a starch staple that compete with other crops such as cereals (like maize and millet) and tubers (like sweet potatoes and cassava) (Bagamba *et al*., 2006). In addition to providing a reliable source of food, banana is an important source of income with excess production sold in local markets. Average per capita annual consumption of bananas in Uganda is the largest in the world,

estimated to be about 1kg per person per day. Banana are consumed as fruit, prepared by cooking, roasting or drying, used for production of banana juice and fermented for production of alcoholic beverage (beer, wine and gin) (Edmeades *et al*., 2006). Bananas are also a source of animal feeds (fresh pseudostems, male buds, banana peelings and by-products of fermentation), wrapping material for produce in storage, construction materials (thatch and binding ropes) and handicrafts (mats, baskets, hand bags, necklaces and decorations) (Karugaba and Kimaru, 1999). It also provides soil surface cover, reduces soil erosion on steep slopes and a principal source of mulch for maintaining and improving soil fertility.

2.2 Banana Production Constraints

In Africa, bananas and plantains provide more than 25% of food energy requirement (Robinson, 1996) for more than 100 million people of which 20 million are from East Africa alone. In terms of revenue, banana is one of the most important cash crops contributing up to 22% of national agricultural revenue (Kalyebala *et al.,* 2007). In Uganda, the East African highland banana (*Musa spp*. cv. EA-AAA), popularly known as 'matooke', provide staple food for over 70% (Picq *et al*., 1998) of the population. Banana production is threatened by various biotic constraints such as pests (banana weevil and nematodes) and diseases (Ortiz *et al*., 2002). Of the diseases, Black Sigatoka, Fusarium wilt and Banana Xanthomonas Wilt (BXW) cause significant yield losses in farmers fields with yield losses of up to 90% (Aritua *et al*., 2007). In 2006, the estimated loss if BXW was not controlled was 295 million USD worth of banana output valued at farm gate (Kalyebara *et al*., 2006). This expected loss translates to around 200 USD of food and income per household. As reported previously (Tushemereirwe *et al*., 2006), all banana varieties so far screened are susceptible to BXW. Once the pathogen (*Xanthomonas campestris* pv. *musacearum*) has established, the disease

control is very difficult and eradication impossible (Eden-Green, 2004). Management of BXW in banana is challenging due to lack of resistance in the available banana gene pool and continuous association of host and inoculum over a long period of time (Tripathi *et al*., 2009). Recommended BXW control measures are mainly cultural practices. However, these measures were reported to reduce 10% of the losses to BXW (Tushemereirwe *et al*., 2006). Thus there is a strong need to use non conventional disease control strategies for example genetic engineering with genes that have potential resistance against BXW.

2.2.1 History of Banana Xanthomonas wilt disease

The Banana Xanthomonas wilt (BXW) disease caused by the bacterium *Xanthomonas campestris pv musacearum* (Xcm), has threatened millions of Ugandan farmers due to its severe effect on banana production (Tushemereirwe *et al*., 2004). The disease was first reported in Ethiopia in 1968 on Enset (wild/false banana) and then in banana in 1974 (Yirgou and Bradbury 1968, 1974). It was later described on bananas in the Keffa, Shoa and Sidamo, Harerge and Game-Goffa regions of Ethiopia, on cultivar Casse Hybrid (Yirgou and Bradbury, 1974), with incidence between 70 and 80% (Korobko *et al*., 1987). In Ethiopia where BXW disease is considered important on Enset, banana cultivation is less extensive and therefore its destructive nature on the latter may be difficult to determine. In Uganda it was first reported in 2001(Tushemereirwe *et al*., 2003) and has since spread rapidly with plantation incidence of up to 70% and affecting both EAHB and exotic (dessert/ beer) bananas.

2.3.1 Etiology of Banana Xanthomonas Wilt disease

Little is known about the etiology of BXW. It is reported that the pathogen enters the host through wounds on roots, pseudostems and leaves (Yirgou and Bradbury, 1968, 1974;

Korobko *et al*., 1987). It is suspected that the bacterium also enters the plant through the male buds as reported for the Moko disease (Korobko *et al*., 1987). According to Yigou and Bradbury (1974) long distance transmission of the disease is aided through: contaminated farming tools such as, pangas and pruning knives, which transmit the bacteria through injuries on roots and aerial parts and movement of infected plant materials (suckers, bunches, leaves). The major transmitters of the disease are the insects as they move from one plant to another looking for nectar in flowers (Sekiwoko *et al*., 2006).

Xanthomonas campestris pv. *musacearum (Xcv)* attacks the vascular system of both banana and *Ensete ventricosum* (Enset) causing wilting and death of the plants. Xcm is motile, Gram-negative rod shaped, possesses a single polar flagellum and produces typically yellow, convex, mucoid, slimy colonies on nutrient agar and other media (Yirgou and Bradbury, 1968, 1974; Tripathi *et al*., 2007). Phylogenetic relationships were evaluated for 20 isolates of the bacterium collected within a period of about four decades, between 1968 and 2005, from Ethiopia, Uganda, Democratic Republic of Congo, Tanzania and Rwanda. Sequence analyses of the internally transcribed spacer (ITS) locus (Aritua *et al*., 2008) and the gyrase B (gyrB) gene revealed only limited (<2%) nucleotide divergence among the isolates (Aritua *et al*., 2008).

2.3.1.1 Banana Xanthomonas wilt Disease symptoms

Affected banana plants develop symptoms characterized by a progressive yellowing and wilting of leaves, with fruits ripening prematurely and unevenly with internal brown discoloration (Fig .1). When stems are cut, a pocket of pale yellow bacterial ooze appears within 5-15 min (Yirgou and Bradbury, 1974; Tushemereirwe *et al*., 2004). Yellow or brown

streaks occur in the vascular tissues of infected plants. Other symptoms on the floral parts include wilting of bracts, shriveling and rotting of the male buds, and flower stalks turning yellow-brown (Yirgou and Bradbury, 1968; Tushemereirwe *et al*., 2004). Plant death commonly results from infection.

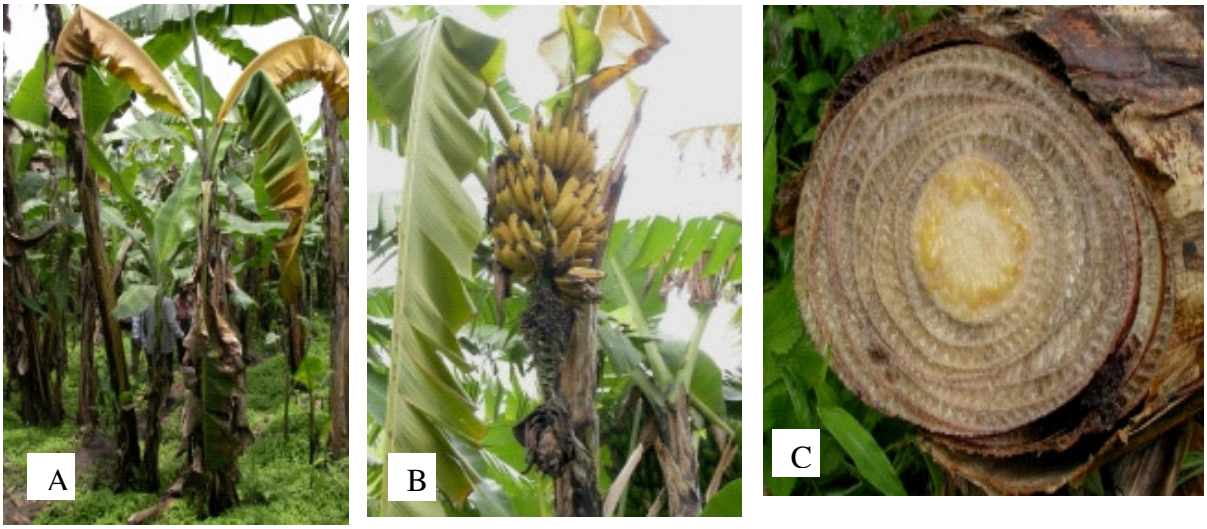

Figure 1. BXW symptoms; wilting of leaves (A), premature ripening of the bunch (B) and(C) cross-section of banana pseudostem showing yellow oozing.

2.4 Resistance to BXW

Banana Xanthomonas Wilt (BXW) attacks banana cultivars of all genome types including East African Highland bananas. The EAHBs are reported to be slightly tolerant than plantain and dessert cultivars (Karamura *et al*., 2006). Some of the EAHB cultivars like 'Mbwazirume' and 'Nakitembe' have been reported to escape the infection due to the persistence male bracts (Tripathi *et al*., 2009). Cultivar 'Bluggoe' was most infected (74%), followed by 'Pisang Awak' (30%), EA Highland bananas (12%) and lastly 'Sukali Ndizi' (3%) (Tushemereirwe *et al.,* 2003). A study done on germplasm screening in Mukono district

on 42 genotypes identified no resistant cultivar among the edible ones except *Musa balbisiana* which is a natural wild type (Ssekiwoko *et al.*, 2006).

2.5 Management of Banana Xanthomonas Wilt diseases

Management of diseases in tropical perennial crops such as banana is a challenge due to continuous association of host and inoculum over a long period of time (Ploetz *et al*., 2007). The recommended measures for BXW management involve a mixture of approaches combining exclusion, eradication, host resistance, and crop protection. Control of BXW and similar bacterial diseases of banana depends on prevention of disease spread (containment), reduction of disease impact in affected farms (management), and rehabilitation of previously affected areas. In Uganda, BXW is mainly controlled by improved cultural practices in well organized banana production areas (Tushemereirwe *et al.,* 2003). Cultural practices that have been used so far include the use of clean planting materials, clean tools which are sterilized in fire or diluted sodium hypochloride, de-budding by breaking the male buds with a forked stick, cutting and burying of diseased plants, and crop rotation (Tushemereirwe *et al*., 2004). These cultural practices have not been used effectively by farmers as they are expensive, labour intensive and time consuming. As a result, low level of adoption has been observed which has led to continuous disease development, outbreak and further spread (Ssekiwoko *et al*., 2006). Therefore, this calls for use of genetically engineered genotypes whose development and management are cheap and less intensive.

2.6 Genetic engineering for resistance to bacterial diseases in plants

Genetic transformation approach has been used to control bacterial wilts in many crops (Huang *et al*., 2004). The path system-specific plant resistance (R) genes that mediate resistance to bacterial, fungal, viral and nematode pathogens have been cloned from several

plant species (Bent, 1996). Many of these R gene products share structural motifs, which indicate that disease resistance to diverse pathogens may operate through similar pathways. For example, the *Bs*2 resistance gene of pepper specifically recognizes and confers resistance to strains of *X. campestris* pv. *vesicatoria (Xcv*) (Wang *et al*., 1996) that contain the corresponding bacterial a virulence gene, avr *Bs2* (Tai *et al.,* 1999). Transgenic tomato plants expressing the pepper *Bs2* gene suppress the growth of *Xcv*. The *Xa1* gene in rice confers resistance to Japanese race 1 of *X. oryzae* pv. *oryzae*, the causal pathogen of bacterial blight (Yoshimura *et al.,* 1998). Transgenic bananas expressing *Hrap* gene conferred resistance to BXW (Tripathi *et al*., 2010). *Sap*1 is an amphipathic protein isolated from the sweet pepper, *Capsicum annum* (Lin *et al*., 1997). The use of S*ap*1 has been shown to delay the hypersensitive response induced by *Pseudomonas syringae* pv. *syringae* in non-host plants through the release of the proteineous elicitor, harpins (Lin *et al.,* 1997). Further analysis also showed that S*ap*1 functioned in a dose-dependent manner by competitively inhibiting the interaction between harpins and its receptor on the plant cells and consequently suppressed bacterial growth. This transgene has showed enhanced hypersensitive response against various pathogens in many dicot and monocot crops (Tripathi *et al*., 2009).

2.7 Plant defense mechanisms

Different steps in plant defense pathways have been evaluated to get a better understanding of defense responses and potentially produce transgenic plants with improved defense systems. One step in the defense-signaling cascade that has been evaluated is the *NPR1* gene from *Arabidopsis*, which regulates salicylic acid signaling (Chern *et al.*, 2001). When *NPR1* was over expressed in *Arabidopsis* as well as in rice, plants with stronger protein induction and

enhanced bacterial and fungal resistance were generated. When bacterial salicylic acid (SA) generating enzymes were expressed in transgenic tobacco, salicylic acid (SA) accumulation was substantially increased and proteins were constitutively expressed conferring enhanced resistance to fungal as well as viral infections (Verberne *et al.*, 2000).

Expression of an amphipathic protein (*sap*1) which was isolated from sweet pepper, *capsicum annuum* has been shown to delay the hypersensitive response induced by *Pseudomonas syringae* pv. *syringae* in non-host plants through the release of the proteineous elicitor harpin (Lin *et al.,* 1997). Therefore, since previous reports in other studies have shown that *Sap*1 inhibited the interaction between *Xanthomonas* and the host plant in other species. It is expected that *sap1* might cause the same interaction between *Xanthomonas campestris pv.musacearum* (Xcm) and the host plant so that transgenic banana plants expressing the *sap1* gene show resistance to BXW. SAP1 is a protein designated as *PFLP* (Plant ferredoxin-like protein) by virtue of its high homology with plant ferredoxin protein containing an N-terminal signal peptide responsible for chloroplast targeting and a putative 2Fe-2S domain responsible for ferredox activity.

2.8 *Sap*1 mediated resistance mechanism

*Sap*1 is a ferredoxin-like protein that is involved in many redox reactions leading to the production of Reaction Oxygen Species (ROS) a characteristic of plant defense response. It intensifies the harpin-mediated HR. Inhibition of the harpin-mediated HR by *sap*1 was shown to be dosage-dependent and revealed a competitive pattern. *Sap*1 may interact with harpin as a putative receptor so as to prevent the binding between receptor and the active fragment of harpin. In this way, harpin may retain its ability to activate an HR via the signal transduction

system (Lin *et al.,* 1997). The production of reactive oxygen species (ROS) is one of the earliest events during HR and considered as a characteristic of plant defense responses (Levine *et al.,* 1994). Moreover, *SAP*1 protein is a ferredoxin that is involved in many redox reactions leading to the production of ROS and it is found that *sap*1 enhances ROS production, so as a result intensifies the harpin-mediated HR (Dayakar *et al.,* 2003).

2.8.1 Transformation studies with *sap1*

The *sap*1 gene enhanced resistance against various pathogens in many plants (Tang *et al*., 2001). Transgenic rice expressing *sap*1 was produced showing enhanced resistance against one of the most devastating diseases of rice in Africa and Asia, bacterial leaf blight, which is caused by the Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Tang *et al.,* 2001). Another study showed that *sap*1 confers resistance against soft rot disease which is caused by *Erwinia carotovora* in *Oncidium (*orchid) even when the entire plant was challenged with the pathogen (Liau *et al.,* 2003; You *et al*., 2003).

2.9 Genetic transformation of banana

Biotechnological techniques that are required for genetic transformation of banana have been reported (Sagi *et al*., 2000). Different embryogenic cell suspensions (ECSs) technologies have been reported (Cote *et al*., 1996; Strosse *et al*., 2006). Genetic transformation using direct gene transfer methods (*Sagi et al*., 1995) and *Agrobacterium* based gene transfer system (May *et al*., 1995; Hernández *et al*., 1999; Ganapathi *et al*., 2001; Khanna *et al*., 2004) are used routinely in different laboratories. Biotechnology, therefore, offers the most feasible and precise tools to introduce useful genes such as those for pest and disease resistance into locally available varieties without changing their preferred characteristic.

Genetic transformation of banana, which started with the use of micro projectile bombardment, is now routinely used (Becker *et al*., 2000; Sagi *et al*., 1995). Later, *Agrobacterium* mediated transformation was reported (May *et al*., 1995; Sagi *et al*., 1995; Hernández *et al*., 1999; Ganapathi *et al*., 2001; Khanna *et al*., 2004). *Agrobacterium* mediated transformation system is more efficient than particle bombardment system in bananas. This is because high transformation frequencies are obtained (Khanna *et al*., 2004) with higher frequencies of transgenic lines containing single transgene copy numbers (Tzafira and Citovsky, 2005). Many banana cultivars of variable genome types have been transformed so far (Sagi *et al*., 1995; May *et al*., 1995; Becker *et al*., 2000; Ganapathi *et al*., 2001; Tripathi *et al*., 2008; Arinaitwe *et al*., 2004). Recently, ECSs of highland banana were developed at the National Agricultural Research Laboratories (NARL) Kawanda. The transformation protocol of ECSs obtained was also developed, opening several avenues of genetic improvement of EAHB cultivars. Therefore, this study considers transformation of EAHB cultivars with *sap*1 gene, so as to increase their resistance to Xanthomonas Wilt.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study 1. Genetic transformation of banana cultivars `Sukali Ndiizi' and `Nakinyika'

3.1.1 Plant material

The study was carried out at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda.

Embryogenic cell suspensions (ECS) of the banana cultivars 'Sukali Ndizi' (AAB) and 'Nakinyika' (AAA-EA) were used in the study. ECS were sub-cultured and maintained as previously reported Cote *et al.* (1996).

3.1.2 *Agrobacterium* strains and binary vectors

Agrobacterium tumefaciens strain EHA105 (Hood *et al*., 1986), harboring binary vector pBISAPI, was used in all transformation experiments with pCAMBIA as a positive control. The construct pBSAP1 was provided by Academia sinica Taiwan through IITA. The T-DNA of binary vector pBISAPI contains a Sweet pepper amphipathic gene (*sap*1) gene driven by Cauliflower Mosaic Virus promoter (CaMV35S) and neomycin phosphotransferase II *(nptII*) as a plant cell selectable marker gene (Figure.2). The T-DNA of binary vector pCAMBIA1305.1, used for control transformation, contains an improved *gusA* gene version (*GusPlus*, www.Cambia.org) isolated from *Staphylococcus* sp. and hygromycin phosphotranferase (*hpt*) gene for plant cell selection (Figure 2). The size of the Plasmid used was 11729bp (Figure 3).

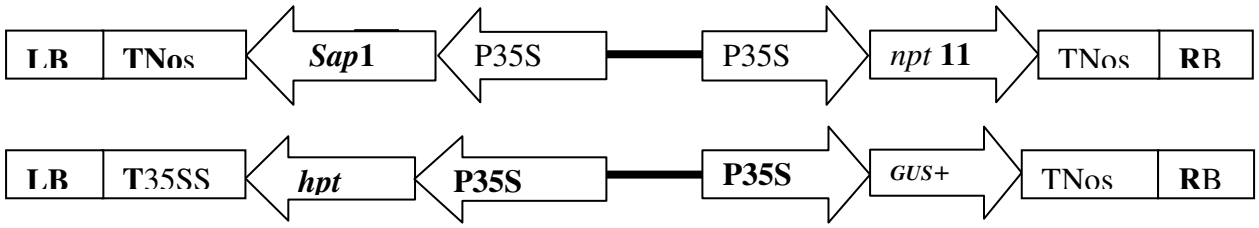

Figure 2. T-DNA regions of the two binary vectors used in the transformation of ECS line 299 and 274-2 from cultivars 'Sukali ndiizi' and 'Nakinyika', EHB (AAA). A refers to T-DNA of binary plasmid pBISAPI; B, pCAMIBIA1305.1; Tnos, terminator of the nopaline synthase gene, *Sap*I, Sweet pepper amphipathic protein; P35S, CaMV 35S promoter; T35S, CaMV 35S terminator;*nptII*, neomycin phosphotransferase gene; *hptII* ,hygromycin phosphotransferase RB-right border and LB- left T-DNA border.

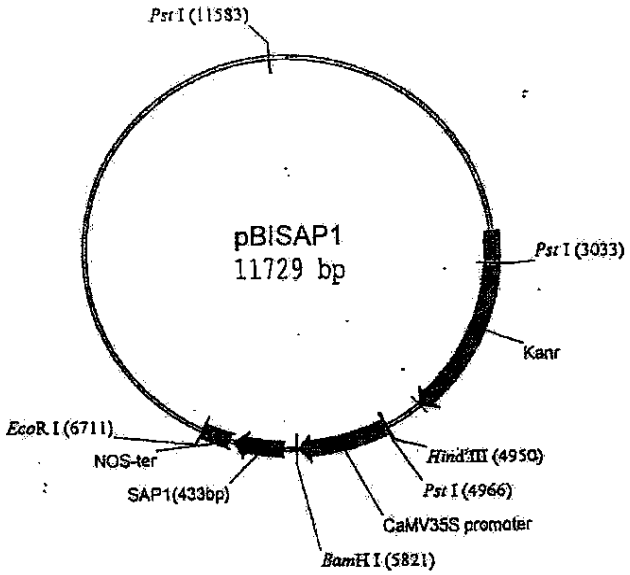

Figure 3. Binary vector map (pBISAP1, 11729bp) containing sap1 gene**.** Restriction sites *Eco*RI (6711), *BamH*I (5821) and *Hind*III (4950) were used in the vector integrity analysis

3.1.3 Bacterial growth and plasmid isolation

Plasmid was isolated using the QIAGEN plasmid mini prep kit (QIAGEN, 2005). Single bacteria colonies were picked and cultured in 5 ml of selective (Kanamycin 50) LB medium. Cultures were incubated at 37°C with shaking at 200 rpm overnight. Plasmid purification was done with the QIAprep Spin mini prep kit as per accompanying manual. Five milliliters of cultures were centrifuged at 6000 rpm for 5min and the supernatant discarded. The pelleted bacterial cells were resuspended in 250 µl of PI buffer and transferred to a microfuge tube. The lysis reaction was initiated by the addition of 250 µl of P2 solution. After gently inverting the tube several times, proteins and polysaccharides were precipitated by the addition of 350 µl of P3 buffer. This was followed centrifugation at 13,000 rpm for 10min after, plasmid DNA in supernatant was loaded onto QUAprep spin column by centrifugation for 2 min at 13,000 rpm. The column was washed with buffers PB (500 µl) and PE (750 µl) by centrifugation at 13,000 rpm for 1 min, in each case. Plasmid DNA was eluted by adding 50 µl buffer EB (10Mm Tris-Cl, pH 8.0) at 70°c. The column was placed into a 1.5 ml microfuge tube, left to stand for 5 min, and centrifuged at 13,000 rpm for 1 min. To check plasmid DNA, electrophoresis using 1% agarose gel was done and later, the isolated DNA was stored at -20°C for further use.

3.1.4 Transformation of *Agrobacterium*

A 50 µl aliquot of competence *E.coli* was thawed on ice for 10 min prior to heat shock procedure. Briefly, 1-5 µl, containing about 100 ng of plasmid DNA, was added to the competent bacterial cells and the tube was gently swirled and tapped for thorough mixing. After 30 min of incubation on ice, the tubes were placed in 42^0C water bath for exactly 30 sec without mixing or shaking. Immediately after incubation, the tubes were placed on ice for 1 to

2 min. Then, 250µl of Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0) were added to the transformation mix and the bacterial cells were incubated for 2 h at 37^0C with shaking at 210 rpm to allow recovery from the heat shock and start expression of the selectable marker gene. After 2 h of incubation, 100 µl of the culture was plated on selective (Rifampicin 50, streptomycin 200 and Kanamycin 50) LB medium pre-warmed to 37^0C and incubated overnight at 37^0C. Single colonies were then picked to initiate cultures for plasmid purification and making glycerol stocks for long-term storage.

For enhanced growth, *Agrobacterium tumefaciens* strain EHA105 was inoculated on the nutrient-rich semi-solid LB medium rather than Yeast-Mannitol (YM) (10 g/l mannitol, 0.4 g/l yeast extract, 0.1 g/l K_2HPO_4, 0.4 g/l KH_2PO_4, 0.1 g/l NaCl, 0.2 g/l $MgSO.7H_2O$, pH 6.8)YM. Selective LB plates, previously prepared and stored at 4°C in the fridge, were warmed at 28°C for 2 hrs prior to inoculation. For inoculation, 4 µl of non-transformed and disarmed *Agrobacterium* cells (from the glycerol stocks at -80°C) was used as an inoculum. After inoculation, plates were incubated at 28°C for 3 days. A single colony was picked and inoculated in 50 ml YM medium (0.4 gL-1 yeast extract, 10 gL-1 mannitol, 0.5 gL-1 K2HPO4, 0.2 gL-1 MgSO4, 0.1gL-1 NaCl, pH 7.0) medium for 3 days at 28°C till OD_{600} =0.5-0.7. A 50 mL cultures of OD_{600} =0.6 was spun at 6000 rpm for 5min and the supernatant was discarded. The pellet was re-suspended in10ml of ice-cold distilled water; centrifuged for 5 min at 6000 rpm/4°C and supernatant discarded. The pellet was re-suspended in 10ml 0.15M $CaCl_2$, cells spun at 500 rpm for 5min and supernatant discarded as above. This was followed by re-suspending the pellet in 1ml ice-cold 20 mM $CaCl_2$ and was sub-divided into 100µl aliquots. For transformation, an aliquot (100 µl) of cells contained in 1.5ml eppendorf

tube was kept on ice for 5min, prior to addition of 10 µl of plasmid DNA (pBISAPI). The mixture was gently tapped, incubated on ice for 30min, frozen in liquid nitrogen for 1 min and later flashed in water at 37°C followed by addition of 1ml of warm (28°C) LB medium. The culture was incubated at 28°C for 4 hrs, with gentle shaking, in order to allow the expression of selectable marker genes prior to transfer onto selective medium. Finally, aliquots of 200µl each were plated on selective semi-solid LB medium (Rifampicin 50, streptomycin 200 and Kanamycin 50) and incubated at 28°C for 3 days. Single colonies of transformed *Agrobacterium tumefaciens* strain EHA105 were picked, cultured in YM in preparation for transformation of banana cell or preparation of glycerol stocks.

3.1.5 Growth and isolation of Plasmid DNA from EHA 105

A trace of EHA105 containing a plasmid pBISAP1 from the glycerol stock was removed with a sterile inoculating loop and streaked on semi-solid LB agar supplemented with Rifampicin (50 ug/ml), Streptomycin (200 µg/ml) and Kanamycin (100 ug/ml). The plates were incubated for three days at 28^{o}C. A single colony from the putative antibiotic resistant colonies, appearing on the agar plates, was isolated with the help of a sterile loop and inoculated into 10ml of LB broth containing Kanamycin (50 µg/ml), Rifampicin (50 µg/ml), and **S**treptomycin (200 µg/ml) and grown for three days. Plasmid was purified using the QIAGEN plasmid mini prep kit. Confirmation of plasmid DNA presence was done by gel electrophoresis using 1% agarose gel.

3.1.6 Restriction digestion

Double restriction digestion of the plasmid DNA using restriction enzymes (BamH1/Hind III and Hind III /EcoRI) was then done to check the integrity of pBISAPI binary vector (11729Kb). The restriction reaction (20 µl) `contained 1x NEBB2 (New England BioLabs

Buffer2) and 10 Units of each of the restriction enzymes used in double digestion, and 0.8 µg of plasmid DNA. The reaction mixture was incubated at 37ºC over night. After incubation the reaction mixture was checked on 1% agarose gel for presence of the expected fragment.

3.1.7 Preparation of bacterial media and *Agrobacterium* culture for transformation

The method used for plant transformation was the Centrifugation Assisted *Agrobacterium*-mediated Transformation system reported by Khanna *et al*. (2004). Yeast-Mannitol (YM) (10 g/l mannitol, 0.4 g/l yeast extract, 0.1 g/l K_2HPO_4, 0.4 g/l KH_2PO_4, 0.1 g/l NaCl, 0.2 g/l $MgSO.7H_2O$, pH 6.8) and Luria-Bertani (LB) (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0), liquid and solid media were used for culturing and selecting *A. tumefaciens* strains, EHA105 (pBISAP1) and EHA 105 (pCAMBIA 1305.1). Solid and liquid media was prepared using de-ionised water autoclaved at 121^{o}C for 15mins. EHA 105 from pBISAPI glycerol stocks at -80 ºC was streaked on selective YM solid plates with 50 µg/ml kanamycin, 50 µg/ml rifampicin and 200 µg/ml Streptomycin while EHA 105 from (pCAMBIA 1305.1) glycerol stock at -80 ºC was streaked on selective YM solid plates with 100 µg/ml kanamycin and grown at 28ºC for 3 days. Single colonies of EHA (pCAMBIA 1305.1) and EHA105 (pBISAP1) bacteria were inoculated in 25 mls of selective liquid YM Rifampicin 50, streptomycin 200 and Kanamycin 50) and grown on a shaker at 200 rpm and temperature of 28º C for 3days. To increase bacterial cell growth, fresh culture was started by adding 5mls of *Agrobacterium* inoculum from the seed culture into 20ml liquid LB medium. The culture was incubated over night at 28º C and 200 rpm. Prior to transformation, bacterial cells were pelleted by centrifugation at 5000 rpm for 10 min at room temperature. The bacterial pellet was re-suspended in the bacteria re-suspension medium (T-MA1) comprised of standard MS salts and vitamins (Murashige and Skoog, 1962), supplemented with (1 mg/l biotin, 100 mg/l

malt extract, 100 mg/l glutamine, 230 mg/l proline, 20 mg/l ascorbic acid, 5 g/l polyvinyl pyrrolidone 10 (PVP 10), 400 mg/l L-cysteine, 1 mg/l indole acetic acid (IAA), 1 mg/l naphthalene acetic acid (NAA), 4 mg/l 2, 4- dichlorophenoxyacetic acid (2,4-D), 85.5 g/l sucrose, pH 5.3) and 300µM Acetosyringone (AS). The bacteria were then activated by gentle shaking at 25ºC and 70 rpm for 2 hrs until an optical density (OD) of 0.6 (OD_{600nm}) was reached.

3.1.7.1 Transformation of banana embryogenic cells

Embryogenic cell suspension line ECS-ND 299 of 'Sukali Ndizi' and ECS -NAK 274-2 of 'Nakinyika' were transformed using T-MA1 containing Acetosyringon as reported by (Arinaitwe *et al*., 2008). Banana ECS (0.5 settled cell volume) were re-suspended in 10ml of pre-warmed liquid MA2 medium consisted of standard MS salts and vitamins (Murashige and Skoog, 1962), 4.1 µM biotin, 4.5 µM 2, 4-D, 680 µM glutamine, 100 mg/l malt extract, 20 mg/l ascorbic acid, 45 g/l sucrose and the pH of 5.3. Later incubated for 5 min at 45°C (heat shock) as described by Khanna *et al.* (2004). The medium was later decanted leaving settled cells at the bottom of the falcon tubes. The cells were re-suspended in 10ml of *Agrobacterium* suspension, adjusted to 0.6 OD_{600}. ECS were allowed to settle and later maintained at 25°C with shaking at 25 rpm. The infected ECS, drained on 50 µm nylon mesh, were transferred onto semi-solid T-MA1 medium1 -containing 300 µM AS in 15cm Petri dishes. The ECS were co- cultivated for 5 days, prior to their transfer onto selective MA3 medium (containing kanamycin100µg/ml and Timentin 200 µg/ml) in the dark at 22^{o}C. for embryo formation media (MA3) containing (3.2 g/l SH salts, standard MS vitamins (Murashige and Skoog, 1962), 4.1µM biotin, 100 mg/l malt extract, 680 µM glutamine, 230 mg/l proline, 100 mg/l myo-inositol, 60 mg/l citric acid, 40 mg/l ascorbic acid, 10 g/l PVP 10, 400 mg/l L-cysteine,

1.1 µM NAA, 0.2 µM zeatin, 0.5 µM kinetin, 0.7 µM 2-ip, 45 g/l sucrose, 10 g/l lactose and 2.3 g/l phytagel).

3.1.7.2 Histochemical Gus assay of banana cells

To monitor the transformation conditions and later on transformation efficiency, ECS transformed with PCAMBIA 1305.1, 0.2 ml SCV, containing *gusA* gene were assayed for β-glucurodinase activity (GUS assay). Three samples per cultivar were incubated in a subsrate solution containing 100 Mm sodium phosphate (pH 7.0), 50 mM ascorbate, and 0.1% Triton X-100, 0.4 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 1mM 5-bromo-4-chloro-3-indolyl-β-Dglucuronic acid (X-Gluc) (Jefferson, 1987). A sterile sheet of filter paper was placed at the bottom of each clean and transparent 15-cm diameter Petri dish. A volume of 1ml of X-Gluc staining solution was added at the centre of each sterile filter paper. Transformed cells plated on 50 µM nylon mesh, were transferred onto wet filter papers. An additional 300-700 µl of X-Gluc staining solution was added in each sample, and then Petri dishes covered, sealed with plastic film, and incubated at 37°C overnight. Blue foci (blue stained cells or cell clusters) in each sample were observed and counted under a stereo microscope and their photograph taken using a digital camera.

3.1.7.3 Selection and regeneration of transgenic banana plants

After 5 days of co-cultivation in the dark at 22^{o}C, infected ECS were washed with liquid MA2 medium, supplemented with Timentin at 200 µg/ml, and drained. Infected ECS were then transferred onto selective semi-solid MA3 media supplemented with Timentin (200 µg/ml) to kill off *Agrobacterium* and Kanamycin (100 µg/ml) to kill non transformed banana cells. ECS were sub- cultured onto selective MA3 medium on 50µM nylon mesh every two weeks until embryogenic cell clones (EC) were observed. After 3 months of selection in the dark at 26^{o}C,

grayish-white masses of cell clusters appeared. These cell clusters were individually transferred onto selective semi-solid embryo initiating RD1 containing (standard MS salts and vitamins (Murashige and Skoog, 1962), 20 mg/l ascorbic acid, 100 mg/l myo-inositol, 30 g/l sucrose, pH 5.3 and 2.3 g/l phytagel) supplemented with 300 µg/ml Timentin and 100 µg/ml kanamycin for further clone forming. After one month the clones started forming shoot-like structure, these were picked and transferred onto selective semisolid MA4, constituting standard MS salts supplemented with Morel vitamins (Morel and Wetmore, 1951), 0.22 µM 6-benzylaminopurine (6-BAP), 1.14 µM IAA, 30 g sucrose, pH 5.8 and 2.3 g phytagel, Timentin (200 µg/ml) and Kanamycin (100 µg/ml), for shoot development and growth. Developing shoots were transferred onto proliferation medium supplemented with 5 mg/l of Benzylamunopurine (BAP). The shoots that developed were multiplied so as to get enough materials for Molecular analyses and *in vitro* screening.

3.2. Study 2. Molecular characterization of transgenic lines

3.2.1 DNA extraction

Plant genomic DNA was isolated as describe by Stewart and via, (1993). About 1g of young leaf tissue from eight putative transformants was homogenized in liquid nitrogen and mixed in 5ml of preheated (65°C) DNA extraction buffer (0.1M Tris-Cl, 20mM Sodium ethylenediamietetra acetic acid (EDTA), 1.4MNaCl and 20%[w/v] hexadecyltrimethyl ammonium bromide [CTAB] and 0.2% [v/v] ß-mercaptoethanol [pH 0.8] in sterile 15ml falcon centrifuge tubes and incubated at 65°C in a water bath for 90min with occasional gentle swirling. The samples were mixed with chloroform-isoamylalcohol (24:1). The contents were centrifuged at 6000 rpm for 10min at room temperature, and the aqueous phase was transferred to fresh sterile centrifuge tubes, 10µl of RNase was added to each tube and

incubated at 37ºC for 30 min. DNA precipitation was done by the addition of equal volume of isopropanol to the supernatant and tubes were inverted for about 20 times to mix. The contents were left at -20 overnight. The mixture was centrifuged at 6000 rpm for 10min to pellet the DNA. The pellet was washed with 70% [v/v] ethanol together with 200µl of sodium acetate and air-dried. The DNA pellet was dissolved in 200µl of Tris-EDTA (TE) buffer (pH 8.0), followed by centrifugation at 13,000 rpm for 15min. Genomic DNA was checked by running it on 1% agarose gel. The extracted DNA was ready to use for PCR and Southern blot analyses. DNA was quantified, by using a spectrophotometer, to determine the required amount of DNA for PCR and Southern blot analyses.

3.2.2 PCR Analysis

To detect the presence of *sap*1 gene in the plant genome of the randomly selected putatively transgenic lines, PCR analysis was performed. Plasmid DNA of pB1SAP1 was used as positive control. PCR analysis was carried out for the randomly selected lines of 'Nakinyika' and'Sukali Ndiizi' with s*ap*1gene primers (5' CCCTCAATAATGGCTAGTGTCT-3'), as forward and (5' TCAGACTGTGGATAAGCAGCAACAC-3') as reverse, were used in the PCR amplification process. The PCR reactions, each 20 µl, contained 2 µl of plant DNA template and 18 µl of master mix. The master mix consisted of 1x Buffer PCR buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 µm of each primer, 0.5 U/reaction of Taq DNA polymerase (Promega, USA). Reactions were started with initial (94^oC for 2min) and subjected to 35 cycles as 1 min at 94^o C denaturing, 1 min at 55^o C annealing, and 1 min of extension at 72^oC. The final extension phase was prolonged to 7 min at 72^o C. For controls, the plasmid vector was used as a positive control and non-transformed plant DNA as negative control. The PCR

products were separated by electrophoresis in a 1.0% agarose gel and DNA fragments were visualized under UV transluminator after ethidium bromide staining.

3.2.3 Southern blot analysis

To determine the integration profiles of these transgenic lines, Southern blot analyses were performed on selected lines. Restriction digestion of individual DNA samples was carried out under the suitable conditions depending on restriction enzyme. The choice of enzyme was based on the vector map and an enzyme that cuts once within the T-DNA was selected (Hind III). To perform Southern analysis of *sap*1 gene, 10 µg of total banana genomic DNA were digested with 6µl of the restriction enzyme overnight at 37° C. Southern hybridization analysis was done following the standard protocols which include, electrophoresis on 0.8% agarose gel for 5 hrs at 50 V to separate the digested DNA fragments, transfer of separated fragments to a positively charged nylon membrane by upward capillary blotting (Southern, 1975). DIG-labeled probe, specific for the transgene was prepared using a PCR DIG probe synthesis kit (Boehringer Mannheim, Germany). Membranes were probed using a DIG luminescence detection kit (Boehringer Mannheim) as per manufacturer's instructions. The prehybridization, hybridization and high stringency washing conditions of (2x SSC, 0.1% SDS) were developed using the DIG luminescent detection kit (Boehringer Mannheim, Germany). Detection of the hybridized fragments on the nylon membrane was done with CPD according to the manufacturer's instructions.

3.3. Study 3: Evaluation of transgenic lines for resistance to BXW

3.3.1 *In vitro* screening

Thirty transgenic lines containing *sap*1gene were randomly selected. These lines were artificially inoculated with *Xanthomonas campestris* p*v. musacearum* (*Xcm*) as previously

reported (Tripathi *et al.,* 2008*)*. Prior to screening, preparation of bacterial suspension was performed as described below.

3.3.2 Preparation of bacterial suspensions

A single colony of *Xanthomonas campestris* pv. *musacearum* bacterial isolate was inoculated into 25 ml of YTS medium and cultured at 28°C with shaking at 150 rpm for 48 hrs. The bacterial culture was centrifuged at 5000 rpm for 5 min and the pellet was re-suspended in sterile double distilled water. The optical density (OD_{600}) of the bacterial suspension was checked and bacterial concentration was adjusted to 10^8 cfu / ml with sterile water. Fresh inoculum was used for all the experiments in order to increase virulence of the pathogen.

3.3.3 Inoculation of *in- vitro* plantlets

Thirty (30) transgenic lines of banana cultivars 'Nakinyika' and 'Sukali Ndiizi' (fifteen lines each cultivar) were randomly selected and tested with artificial inoculation. The inoculum was injected into the pseudostem of *in vitro* plantlets as described by (Tripathi *et al*., 2008). Two shoots per line were inoculated with the fresh culture of *Xanthomonas campestris* pv. *musacearum* bacteria. For comparison purposes, two non-transformed shoots were inoculated with water and the other two with the *Xanthomonas campestris* pv. *musacearum* bacteria. The plants were inoculated by drawing 100 µl of bacterial suspension into a micro syringe and later injected into the pseudostem of the plantlets, using a hypodermic needle. The inoculated plantlets were put into the baby jars fitted with lid and incubated at room temperature for eight weeks.

3.3.4 Disease assessment due to BXW

Plantlets incubated under *invitro* conditions were monitored and assessed everyday for eight weeks for disease symptoms. The disease symptoms included chlorosis, necrosis and finally

complete wilting and death of plants. Wilt incidence was measured as number of wilted plants over total number of plants inoculated. Observations were made regularly and the data was recorded on each plantlet. The relative resistance of transgenic lines to BXW was evaluated eight weeks after inoculation. Plantlets were categorized based on disease severity as: resistant if a plant did not show any disease symptoms; moderately resistant, if a plant showed delayed symptoms; and susceptible, if a plant got symptoms within 12-20 days after inoculation similar to control non-transgenic plants.

3.3.5 Data collection

Data on regenerated shoots was collected by counting shoots per medium type and the data was presented in form of pictures and graphs. Data on disease incubation and complete wilting was collected basing on chlorosis and necrosis of leaves and complete wilting of plants, and entered into a table. Average incubation and average wilting was computed basing on number of days for appearing of the symptom and death of the plant. The means presented were for two replicates. Analysis of variance (ANOVA) was conducted using SAS, and interaction between means was by least significant difference (LSD) at P= 0.05 (SAS, 2003).

CHAPTER FOUR

RESULTS

4.1 Transformation and regeneration

4.1.1 Restriction Analysis of Plasmid DNA

Double restriction digest to confirm presence of the binary vector pBISAP1, using restriction enzyme combinations, gave variable fragment sizes (Fig 4). These were; 10,858bp and 871bp for combinations HindIII/BamHI; 9968bp and 1761bp, HindIII/EcoRI; and 890bp and 10839bp fragments for combination BamHI/EcoRI.

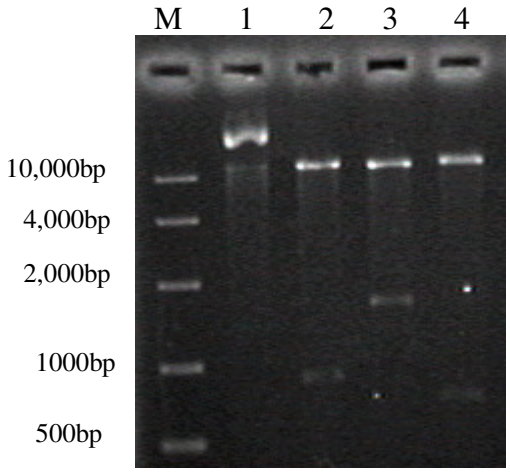

Figure 4. Restriction digests of binary vector pBISAP1 on a 1% agarose gel, M- DNA-Marker; 1,undigested DNA; and lanes 2-4 represent results of restriction analyses of pBISAP1 digested with HindIII/BamHI, HindIII/EcoRI, and BamHI/EcoRI respectively.

4.1.2 Transformation efficiency

Six days after infection, randomly selected samples of, embryogenic cell lines ECS-274 NAK and ECS-299 ND, lines transformed with PCAMBIA 1305.1 were assayed histochemically

for *gusA* expression. For each sample, approximately 80% of the ECSs turned blue after incubation. Corresponding ECS in the non transformed samples remained creamish (Fig 5). Results indicated successful transformation and high transfer efficiency.

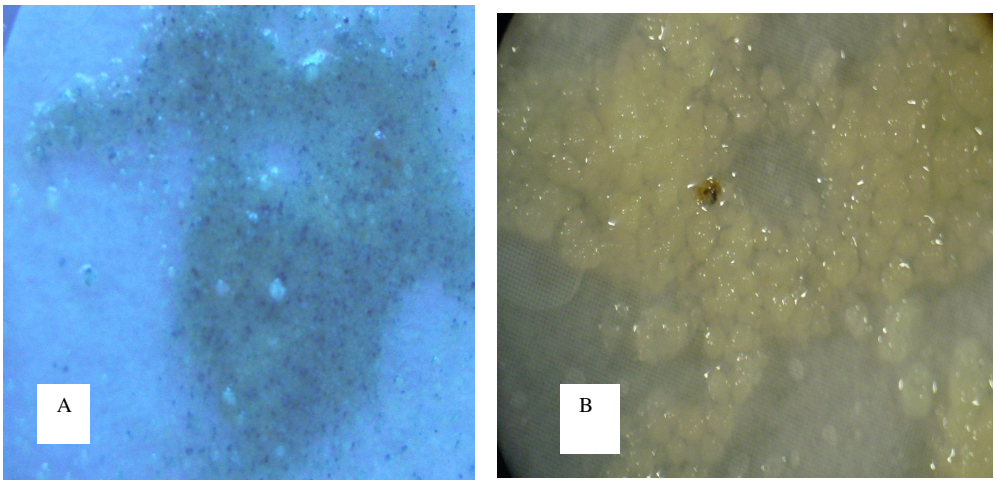

Figure 5. Schematic presentation of histochemical GUS assays, A) Blue foci represent the transformed ECSs of cultivar 'Nakinyika' following *Agrobacterium*-mediated transformation. B) Stained non transformed banana cell.

Table 1. Transformation efficiency of 'Nakinyika' and 'Sukali ndiizi' after Gus assay

Cell line code	Total amount of cells used	No. of blue focil	Transformation efficiency
ND 299	24,000	18960	79%
NAK 274-2	24000	19200	80%

4.1.3 Selection and regeneration of Putatively transformed banana lines

After 3 weeks of culturing on MA3, infected cells turned brown (Fig 6A). Three months later, whitish-grey masses of cell clusters (Cell clones) appeared and continued growing. The cell clusters continued developing and formed embryo-like structures and the remaining cells turned black and died. The non- transformed control cells that had been transferred to non selective medium (MA3 without selection) developed embryos and later into plantlets. (Fig 6B).

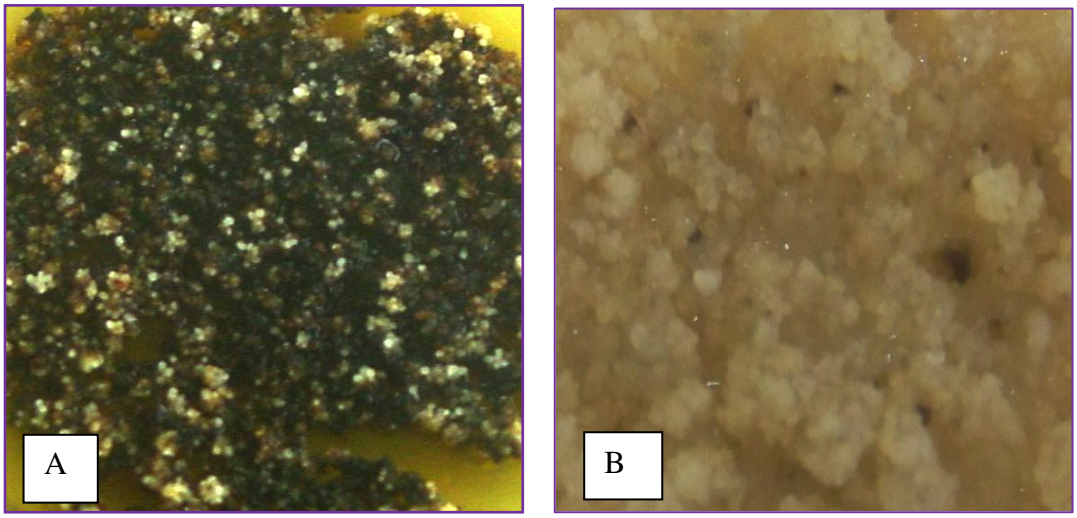

Figure 6. Transformed (A) and non transformed (B) embryogenic cells of banana on selective and non selective medium respectively. The pictures were taken three months of culturing.

After three months on MA3, individual putatively transformed cell clones that survived were picked and transferred onto selective medium RD1 supplemented with appropriate antibiotics. For cultivar 'Nakinyika' 600 cell clones were singly transferred onto fresh selective medium whereas a total of 400 cell clones from cultivar 'Sukali Ndiizi' were transferred. Of the initial total number of cell clones, of both cultivars, only 460 and 320 cell clones of 'Nakinyika' and 'Sukali Ndiizi' respectively, survived and were transferred onto selective embryo initiation

medium (RD1 fig.7). The rest of the cell clones turned brown blackened and finally died (Fig 8A). On selective embryo initiation medium RD1, 260 shoot-like structures of cultivar 'Nakinyika' and 160 of cultivar 'Sukali Ndiizi' survived. These surviving individual shoot-like structures were transferred onto shoot growth and development medium (MA4) from which 135 and 83 shoots of 'Nakinyika' and 'Sukali Ndiizi,' respectively, developed. (Figure 8 B). Although more shoot-like structures were observed on RD1, some never developed into shoots (Figure 8 B). Some started germinating after two weeks and others took longer.

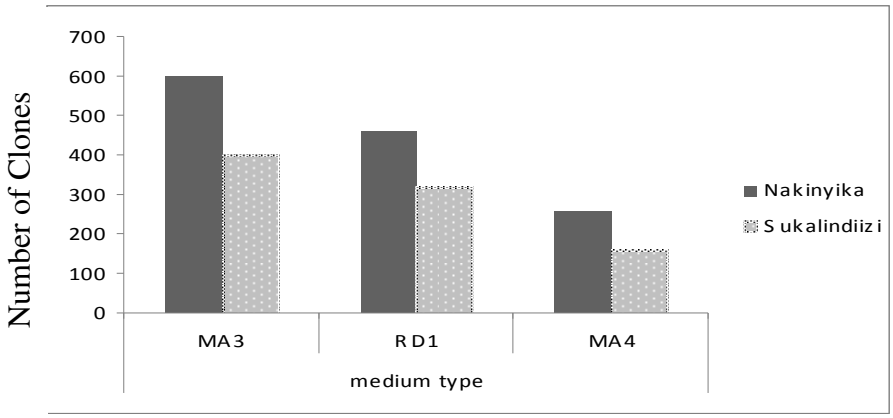

Figure 7. Number of embryogenic cell clones (ECs), in cultivar 'Nakinyika' and 'Sukali Ndiizi' at different stages of selection and shoot regeneration.

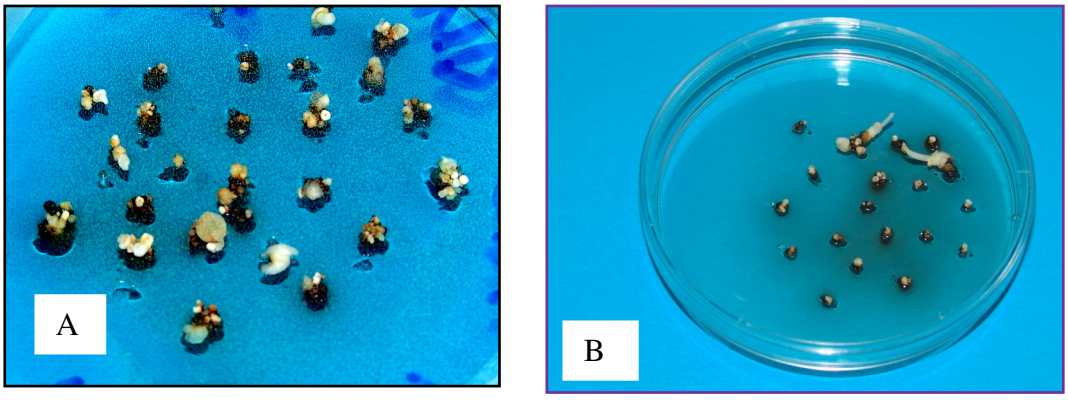

Figure 8. Embryo initiation (A) and shoot development (B) of transformed ECS of cultivar 'Nakinyika' and 'Sukali ndizi' after 2-3 months of selection on kanamycin (50µg/ml) supplemented MA3 and MA4 media, respectively.

Regeneration of putatively transgenic lines was done using (Murashige and Skoog, 1962), medium. The numbers of shoots that were regenerated from shoot-like structures, for both cultivars, are shown in (Fig 7). For the cell lines used (ECS-274 NAK and ECS-299 ND), transformed cell clones of cultivar 'Nakinyika' had the highest regenerability (62%) compared to 38% in cultivar 'Sukali Ndiizi' (Figs 9).

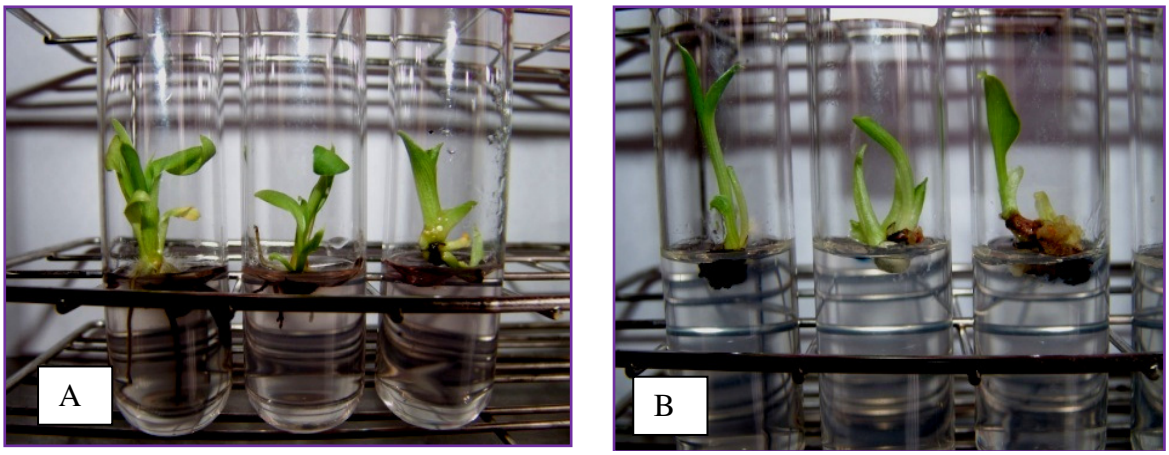

Figure 9. Shoot regeneration in cultivars 'Nakinyika' (A) and 'Sukali Ndiizi' (B) following the 2-3 months of selection process.

4 .2 Mocular Characterization of transgenic lines

4.2.1 PCR Analysis

Molecular characterization of putatively transgenic lines was done using PCR and Southern blot analyses. PCR analysis, using gene specific primers, gave a 420 bp and 600-bp fragments for coding regions of *sap*1 and *npt*II genes respectively. Twenty nine (29) putatively transformed plants that were randomly selected and tested, 26lines were PCR positive (Figure 10 and 11). These lines included ND 39, ND 49, and ND 44 for 'Sukali Ndiizi' and for 'Nakinyika' lines were NAK 9, NAK 14, and NAK 12. However, three lines did not show any

amplification. This could be due to the presence of contaminants within the DNA samples or these could have been escapes.

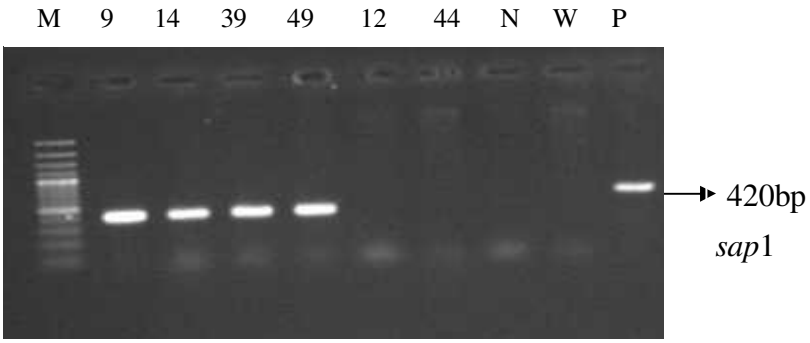

Figure 10. PCR products for *sap*1 primers; M- DNA marker, Lines 9, 14, 39, 49, 12 and 44 are transgenic samples, N-control (non-transformed), W-water p-positive control (plasmid). The arrows indicate the expected PCR products of *sap*1.

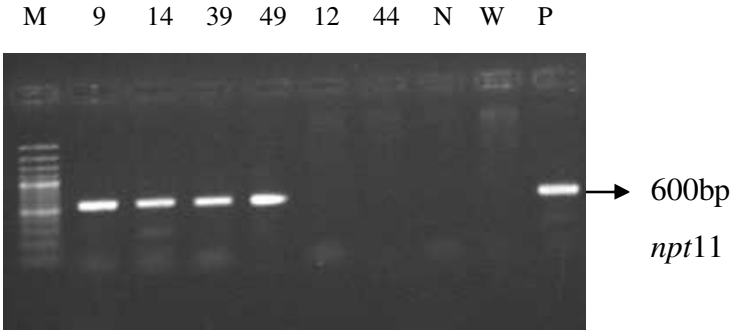

Figure 11. PCR products for *npt*II primers; M- DNA marker, Lines 9, 14, 39, 49, 12 and 44 are transgenic samples, N-control (non-transformed), W-water P-positive control (plasmid). The arrows indicate the expected PCR products of *ntp*II.

4.2.2 Southern blot analysis

Southern blot analysis was done to confirm the integration patterns of *sap*1 in cultivars 'Nakinyika' and 'Sukali Ndiizi'. Six randomly selected transgenic lines of both cultivars were analyzed using *sap*1 specific probes. Different integration patterns were observed in different

lines, indicating that these lines were stably transformed. Transgenic lines NK9, NK14, ND39 and ND49, had at least 1- 4 integration loci (Figure 12). Non-transformed line (NT) did not show any signal, indication that the observed signals were not from the endogenous banana *sap1* gene or genes (Figure 12).

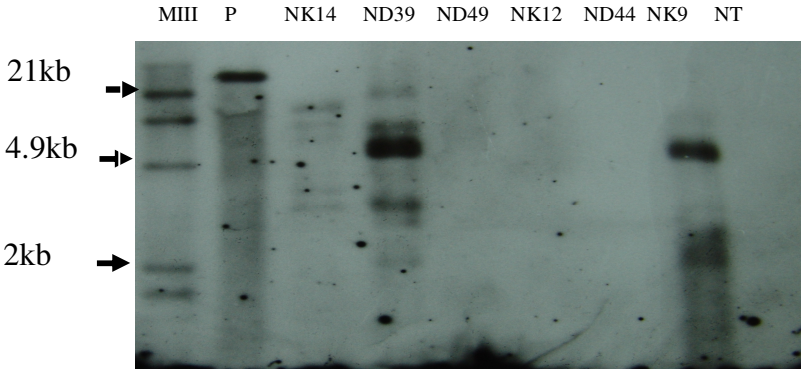

Figure 12. Southern blot analysis using genomic DNA of transgenic banana plants digested with Hind III. Plant genomic DNA pBISAP1 DNA was digested with HindIII. MIII: molecular weight marker; P: pBISAP1 (positive control); Lines NK14, ND39, ND49, NK12, ND44 and NK9 are transgenic plants; NT Non-transgenic plants

Table 2. PCR analysis and integration patterns of randomly selected transgenic lines of 'Sukali Ndiizi' and 'Nakinyika'containing *sap1*and *npt11* genes

Cultivar and line no.	PCR analysis		Southern blot analysis	No. of integration loci
	*Sap*1	*Npt11*	*Sap*1	
Untransformed control	-	-	-	-
NAK 9	+	+	+	1
NAK 14	+	+	+	1
NAK 12	-	-	-	0
ND 39	+	+	+	3
ND 49	+	+	+	4
ND44	-	-	-	0
pB1SAP1	+	+	+	1

- and + denote the absence and presence of PCR (or Southern blot) signal respectively

4.3 Efficacy of *sap1* gene

After *in vitro* screening, plantlets were categorized into three classes. These categories were (1) plantlets with no symptoms (resistant), (2) plantlets with delayed (tolerant/moderately) symptoms (after 37-43 days) and (3) plantlets that quickly developed (susceptible) BXW symptoms (after 12-20 days) (Fig 13). The variable responses to BXW were observed in different transgenic lines. On average, non transgenic plantlets started showing leaf wilting symptoms 16 days after inoculation. Transgenic lines of 'Sukali Ndiizi' showed symptoms within 12-14 days after inoculation. In cultivar 'Nakinyika' symptoms became visible within 16-17 days after inoculation. Three out of fifteen transgenic plants of cultivar 'Nakinyika' did not show BXW symptoms (resistant) whereas for 'Sukali Ndiizi', Two out of fifteen lines were symptom-less. For both cultivars, a total of 7 lines appeared to be tolerant with delayed

symptoms. Other lines which were inoculated quickly got the symptoms and finally wilted. Results of *in vitro* BXW screening are presented in Table 3.

Figure 13. *In vitro* screening of transgenic lines for resistance against BXW after artificial inoculation with *Xanthomonas campestris* pv. *musacearum* (*Xcm*). A represents inoculated untransformed plantlet; B to D show inoculated transgenic plantlets. These pictures were taken 8 weeks after inoculation.

Table 3. Evaluation of transgenic lines for enhanced resistance to *Xanthomonas campestris* pv. *musacearum* using *in vitro* plants

Transgenic Line	Mean* number of days for appearance of disease symptoms	Mean* number of days for complete wilting	Wilting incidence (%)
C	15.65	31	100
T1	_	_	0
T2	38.3‡	_	0
T3	_	_	0
T4	_	_	0
T5	21	32	100
T6	31.33 ‡	53.00 ‡	100
T7	24	32	100
T8	_	_	0
T9	34.00 ‡	52.33 ‡	100
T10	42.6 ‡	-	0
T11	_	_	0
T12	27	40	100

*Mean of two replicates.

†The disease symptoms were chlorosis or necrosis on the leaves of inoculated Plants.

‡Significant differences ($P < 0.05$) in symptoms in a comparison of transgenic Lines with non-transgenic control plants.

Wilting incidence (%) = (Number of plants wilted completely/Total number of Plants inoculated) X100.

The rest of the transgenic plants which were not screened were weaned and maintained in the green house so as to be screened. Later, resistant lines will be evaluated in the field together with the ones worked on in this study.

CHAPTER FIVE

DISCUSSION

5.1 Transformation and regeneration

5.1.1 Transformation efficiency

Transient transformation frequency, using *gusA* or *gfp* reporter gene, is indicative of gene transfer efficiency (Arinaitwe *et al*., 2004). For example, high histochemical *GUS* expression is indicative of high gene transfer efficiency. In this study, over 80% of the cells transformed with *gusA* gene turned blue after the histochemical *GUS* assay; indicating over 80% gene transfer efficiency (Figure 5). High gene transfer efficiency in banana cells transformed using *Agrobacterium*-mediated transformation system was previously reported by (Mohanty *et al.,* 1999), and is associated with high ECS line competency (Yip *et al*., 2006), actively dividing cell-status, and cells on auxin rich medium (Sagi *et al*., 2000). The results obtained confirm that the two ECS lines used were highly competent to *Agrobacterium* infection with high gene transfer efficiency. Since transformation with the *sap*1 gene and *gusA* reporter gene were done under the same conditions, gene transfer efficiency with *sap*1 gene was equally high. Higher gene transfer efficiency in banana cells has been reported by several authors (Ganapathi *et al*., 2001; Khanna *et al*., 2004). High transformation frequency was also confirmed in numerous embryogenic cell clones (stable transformation stage) that survived after the selection process (Fig. 6) and is influenced by the plant genotype, explants type, inoculation and co-culture conditions, transformation competency (Cheng *et al*., 2004) and inoculation or co-cultivation length (Amoah *et al*., 2000).

5.1.2 Selection of transformed lines

After *Agrobacterium* co-cultivation, the banana cells were transferred onto MA3 medium supplemented with 100mg/l Kanamycin. A month after infection, masses of cells turned brown and finally died. Browning is reported to be due to necrotic effects of the selective agent (Sagi, 2000). Although many ECSs are transformed by *Agrobacterium*, not all cells develop into embryos. At each phase of selection the number of surviving cell clones decreased. For example from selective MA3 to RD1 the number of surviving cell clones decreased from 600 to 400 in cultivar 'Nakinyika' as compared to 400-320 in 'Sukali Ndiizi'. This observation implies that a proportion of these cell clones were not transformed. While monitoring *gfp* gene expression in banana cells from transformation till shoot regeneration, Arinaitwe *et al*. (2004), observed similar trends where the cell clones expressing *gfp* gene decreased with time. The explanation given was that some clones were escapes and others were not stably transformed (transiently transformed). Thus cell clones where the gene was successfully integrated into the genome survived and subsequently developed into shoots. Selection at RD1 too provides enhanced selection pressure since single cell clones are transferred onto freshly prepared selective medium.

Development of cell clones depends mainly on the embryogenicity of a particular ECS line. ECS lines that readily for embryos generate numerous embryos during selection and thus enhances the number of surviving cell clones. Variable embryogenicity and survival rates among transformed banana cells were previously observed in different banana cultivars (Arinaitwe *et al*., 2004). Variation of embryogenicity, within the ECS lines may be due to variable GA/ABA rations among individual cells. These ratios determine whether tissue

develops as embryonic or postembryonic (Braybrook *et al*., 2006; Zheng *et al*., 2009). Although not all cell clones subsequently develop into shoots, high embryogenicity and survival rates increase the number of shoots regenerated after the transformation cycle. According to the segregation analysis, genetic control of regeneration by two-dominant genes has been proposed for both the re-generation percentage or efficiency (Reisch and Bing-ham 1980; Wan *et al.,* 1988) and the number of embryos (Hernandez-Fernandez and Christie 1989). However, the ability to form embryos (embryogenicity) seems to be independently controlled (Zheng *et al*., 2009).

5.1.3 Shoot regeneration

Shoot initiation and development from embryogenic cell clones remain a challenging step in plant genetic engineering. Although hundreds of cell clones are recovered after the selection process, a small proportion of up to 70% develop into shoots (Sagi *et al*., 1995). In the current study, shoot regeneration frequencies of 62% and 38% were observed in cultivars 'Nakinyika' and 'Sukali Ndiizi' respectively. Shoot regenerability in plant cell cultures is reported to be influenced by embryogenicity and the duration in culture (Arinaitwe *et al*., 2008). Thus freshly initiated ECS lines are highly regenerable than ECS lines that have been in culture for over a year. The number of shoots regenerated per cultivar is known to be influenced by the number of clones picked from embryo germination medium onto shoot germination medium, success of the transformation event and effective control of *Agrobacterium* by using an appropriate antibiotic at lower concentrations, during selection (Yip *et al*., 2006; Liau *et al*., 2003). The results of this study were similar to previous studies where shoot basal discs were used and exposed to same selection. In that study, a total of 28 out of 260 (10.8%) were regenerated (Yip *et al*., 2006).

5.2 Molecular characterization of transgenic lines

5.2.1 PCR analysis

Using gene specific primers (*sap*1-specific primers) and npt11, PCR analyses were performed to confirm its presence in the banana genome. Out of 29 the lines randomly selected and tested, 26 lines showed the presence of *sap*1 and *npt11*gene; a frequency of 89%. Three lines didn't amplify for both *npt11* and *sap*1. Probably the DNA contained Taq polymerase inhibitors or this could be due to the presence of contaminants within the DNA samples or these could have been escapes. No amplification was identified in the non transformed plants for both transgenes, hence the primers were gene specific.

5.2.2 Southern blot analysis

The results show that *sap1* gene was integrated into the banana genome with lines 9, 14, 39 and 49 having at least 1- 4 integration sites, thereby confirming their transgenic nature. The pattern of bands differed in these transgenic lines, indicating that these lines represent different transgenic events. The number of integrations varied between one and four. Similar results were reported in several other monocots including rice, wheat and barley by Dai *et al*. (2001). Line ND 49 gave the highest number of integrations followed by ND 39 whereas Lines NK 14 and ND 9 both had one integration locus of *sap*1 gene. Integration patterns of 1 to 4 have been observed in different banana genome types transformed using *Agrobacterium*-mediated transformation procedure (Arinaitwe *et al.,* 2008). In this study, the integration pattern observed was simpler than what is observed (over 7 integrations) in transformants generated through particle-mediated transformation system (Wakita *et al*., 1998). Although previous studies reported the ferredoxin-like gene as ubiquitous in the plant kingdom (Yip *et*

al., 2006), it was not detected in non transformed banana genomic DNA. Southern blot analysis of the putative positives confirmed stable integration of the *sap1*gene into the banana genome. The transgenic lines showed 1 to 4 integration patterns which are characteristic of *Agrobacterium*- mediated transformation. The integration patterns were also generally random, implying different transformation events.

5.3 Efficacy of *sap1* gene

The efficacy of *sap*1 gene was tested using *in vitro* assay of the transgenic lines of the banana cultivars 'Nakinyika' and 'Sukali Ndiizi' for resistance against BXW. Two weeks after inoculation, leaves of transgenic plants exhibited HR-like symptoms. These results were similar to (Yip *et al*., 2006) who observed the same. In contrast, randomly selected lines of the two cultivars that were inoculated with 100µl of the bacterial suspension *Xanthomonas campestris* pv. *musacearum* produced symptoms of chlorosis and necrosis within 12-14 days for 'Sukali Ndiizi' and 16-17 days for 'Nakinyika' after inoculation. Susceptible lines wilted and died 30-40 days and 32-47 days after inoculation for cultivars 'Sukali Ndiizi' and 'Nakinyika,' respectively. Control plants, inoculated with sterile water remained healthy. This was similar to what was observed by (Tripathi *et al.,* 2008; Tripthi *et al*., 2010). Previous studies report that *sap1* transgene activity against *Erwinina carotovora* (*EC*) and *Pseudomonas syringae* pv *tabaci* in tobacco plants have shown hypersensitive response (HR) and suppressed bacterial growth during compatible pathogen attack (Huang *et al*., 2004). In this study, inoculated transgenic lines demonstrated resistance to *Xcm* at variable levels (Yip *et al*., 2006; Tang *et al*; 2001). The strongest resistance was observed in two lines of 'Sukali Ndiizi', and three of 'Nakinyika'. Basing on the previous reports (Tang *et al*., 2001; Liau *et*

al., 2003; Tripathi *et al*., 2008), and the results of this study, *sap*1 gene provides variable protection to plants against wilt-causing pathogens.

Similar resistance trends were previously reported in transgenic lines of rice containing *sap*1 gene (Tang *et al*., 2001). In that study, only lines 2, 6, 8, and 9 showed strong resistance whereas other lines showed variable resistance levels. Lines of Cana lily and rice containing *sap1* gene exhibited HR-like necrosis as observed by Yip *et al*., (2006), and Tang *et al*., (2001) respectively. Results obtained in this study and previous reports in rice (Tang *et al*., 2001), and Cana lily (Yip *et al*., 2006) indicate that *sap*1 gene provides variable resistance, thus evaluation of substantial number of transgenic lines is desirable.

Though some transformed lines did not show resistance, this does not mean that they didn't carry the gene. The possible reasons for this could be attributed to the way the gene was positioned in the plant. In that, if the gene is positioned in the non coding region it will not be transcribed so in this way, the gene will not be active. Unlike on the other hand if it's in a coding region, then it will be transcribed and therefore active and so resistance will be expressed. The other reason could be based on the integration site in that the lesser the number of integration sites, the more stable it becomes and vice versa.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

Banana Xanthomonas wilt, caused by the gram-negative bacterium, is a devastating disease of banana causing yield losses of up to 100% in Uganda (Tushemereirwe *et al*., 2003). For many years, researchers have used conventional breeding to improve banana yields, especially by looking for traits which confer resistance to banana pests (Nematodes and weevils) and diseases like, Black Sigatoka, and Fusarium wilt. No edible banana cultivar is resistant to BXW (Tripathi *et al*., 2008) hence resistance is needed from external sources. Wide crossing with distant banana cultivars, for example Calcutta 4, generates hybrids with inferior cooking characteristics.

The results from this study showed successful transformation of ECSs from cultivars 'Sukali Ndizi' and 'Nakinyika' with *sap*1 gene from sweet pepper. *Invitro* BXW-screening results show enhanced protection from BXW. Transient *gusA* expression confirmed ECS lines (ECS-299 ND) and (ECS-274 NAK) from cultivars 'Sukali Ndizi' and 'Nakinyika' respectively, were competent to *Agrobacterium* infection. Shoot development from transformed Embryogenic Cell Clones (ECs) confirmed the regenerability of both ECS lines used.
In this study, 83 and 135 transgenic lines of banana cultivars 'Nakinyika' and 'Sukali Ndiizi' were generated.

Molecular characterization of selected transgenic lines confirmed both the presence and stable integrations of transgene *sap*1 in the banana genome. PCR analyses showed the presence of *sap1* gene in the genome of the regenerated banana lines. Integration sites of two to four were

observed, after Southern blot analyses with gene specific probes. *Sap*1 gene from sweet pepper can protect banana plants from BXW.

Invitro evaluation of randomly selected regenerated banana lines, containing *sap*1 gene from sweet pepper showed enhanced resistance against BXW. Thus *sap*1 gene has a potential to protect banana plants against Banana Xanthomonas wilt. However, it is important to evaluate these lines under natural *Xcm* infection in the field since the development of transgenic lines resistance against BXW is highly desirable.

Furthermore, a few lines have been characterized, so it is important to do molecular characterization of all the lines prior to their evaluation in the field. Combination of *Sap*1 gene with other genes that have different modes of action would provide strong and durable resistance against Banana Xanthomonas Wilt (BXW).

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