TRANSGENIC BREEDING OF SWEETPOTATO FOR RESISTANCE TO AFRICAN WEEVIL (*Cylas puncticollis*)

BY

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DECLARATION

I declare that the work presented in this thesis is from my own research and has not been submitted for award of a degree in any other university.

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To Ms Florah L. Ndlovu, Lerato and Laura Ndlovu.

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ABSTRACT

Sweetpotato weevil (*Cylas puncticollis*) Boheman is a serious pest throughout Sub-Saharan Africa. The pest causes up to 28% production losses annually in Uganda. Due to difficulties to implement pest management measures and the low level of resistance to weevils in existing sweetpotato varieties, *cry* genes were introduced, prior to this research, by genetic engineering to express three different Cry proteins known to be toxic to the African weevils. The main objective of this study was to assess the efficacy of sweetpotato expressing Cry proteins to the African sweetpotato weevil and identify candidate sweetpotato genotypes to be incorporated into sweetpotato breeding programme. This study was based on the following specific objectives: i) to evaluate the efficacy of sweetpotato events expressing Cry proteins against the African sweetpotato weevil, ii) to determine the inheritance and expression of *cry7Aa1* gene in F_1 sweetpotato progenies developed through transgenic breeding, iii) to evaluate the agronomic performance of these F_1 genotypes, iv) to identify key non-target organisms exposed to weevil resistant transgenic sweetpotato.

Ten transgenic sweetpotato events expressing Cry7Aa1, Cry3Ca1, and ET33-34 proteins from *Bacillus thuringiensis* (Bt) were evaluated for resistance against *C. puncticollis*. Four bioassays were used to evaluate the activity of the proteins against the weevil. (i) 1st instar larvae in an artificial diet using root powder of transgenic events, (ii) whole root of transgenic events infested with female adults, (iii) root chip, and (iv) small roots of transgenic events both infested with egg-plugs. One event known to have a single copy of the *cry7Aa1* gene was then crossed with three Ugandan sweetpotato cultivars, namely, New Kawogo, NASPOT 1 and Tanzania to assess the segregation pattern of the *cry* gene. In the CIP410008.7 × New Kawogo cross, F_1 progenies were evaluated for Cry protein expression in the storage roots. In addition, these F_1 progenies were accessed to confirm if genetic transformation process generated any unexpected alterations. Vine vigour, vine internode length, vine length, foliage weight, root skin and flesh colour, carotenoid content, number of storage roots and weevil resistance were evaluated in the F_1 genotypes. Furthermore, key non target arthropods which may potentially be affected by cultivation and inclusion of Bt sweetpotato in breeding programmes were also identified.

DAS-ELISA showed variation (0.1 - 0.4 µg g^{-1}) in Cry protein concentration in fresh roots of the transgenic events. The highest protein quantity was observed on the event carrying the *ET33-34* transcript while those carrying *cry7Aa1* and *cry3Ca1* transcripts had low protein quantities. In the insect bioassays, although whole root assay had low weevil handling injuries, this method requires a comparatively large number of adult females for oviposition and roots per event to be tested. The root chip method was the least desirable

because it was prone to fungal and bacterial contamination. The most appropriate method for testing Bt efficacy in sweetpotato is the small root egg-plug bioassay. Generally, none of the transgenic events tested provided weevil control because of low Cry protein expression in storage roots. The F_1 seedlings were analysed for the presence of *cry7Aa1* transgene using PCR. Chi-square test showed that all the three families followed a 1:1 segregation pattern of the transgene. In the CIP410008.7 × New Kawogo family, no significant differences ($p>0.05$) were observed in the protein expression between transgenic progeny with mean values of 0.2 µg Cry protein g^{-1} storage root tissue. The insect bioassay revealed no significant activity against *C. puncticollis* in the transgenic progenies. Despite New Kawogo having high concentrations of resistance compounds (hydroxycinnamic acid esters) synergy between transgenic and natural resistance was not observed in the progeny. Hence, Cry protein expression transmitted as a dominant and Mendelian trait, was not influenced by the genotype of the F1 progeny, and did not synergise with host plant resistance.

Significant variation in vine vigour, vine length, number of roots per plant and carotenoid content ($p<0.05$) was observed in the F_1 progeny of transgenic breeding. Nevertheless, no significant differences were observed for foliage weight and vine internode length. The existence of continuous variation of the measured traits indicated the quantitative nature of most sweetpotato traits. There was also no significant difference in production of roots between the transgenic and non-transgenic F_1 progeny. Hence, we did not observe any unexpected alterations due to the *in-vitro* regeneration and genetic transformation of the sweetpotato crop. In transgenic breeding, the ground, rove, and ladybird beetles were identified as the primary relevant non-target organisms of Bt sweetpotato. These insects are important predators in sweetpotato fields. Additionally, honey bee was also considered as relevant due to its ecological role as a pollinator.

In conclusion, the absence of effective transgenic events to control weevils in storage roots entails that, more research is needed on additional transgenic events with new gene constructs for effective weevil control. The *cry7Aa1* gene inheritance and expression was stable in F_1 progenies, this implies that the transgenic sweetpotato could be used as a valuable insect-resistant germplasm to be employed in transgenic breeding programmes. The existence of continuous variation of agronomic traits observed in the F_1 progeny enables evaluation and selection of sweetpotato clones with farmer preferred traits to be incorporated in a breeding programme. In the likelihood of successful transgenic breeding, there is need to do risk assessment on the non-target organisms at laboratory and field level to access the impact of Bt sweetpotato on the non target organisms.

PUBLICATIONS FROM THIS STUDY

This thesis is based on some unpublished data and the following articles/manuscripts..

1. **Rukarwa, R.J**., Prentice, K., Ormachea, M., Kreuze, J.F., Tovar, J., Mukasa, S.B., Ssemakula, G., Mwanga, R.O.M. and Ghislain, M. 2013. Evaluation of bioassays for testing Bt sweetpotato events against sweetpotato weevils. *African Crop Science Journal* 21(3): 235-244.

2. **Rukarwa R.J.**, S.B. Mukasa, A. Sefasi, G. Ssemakula, R.O.M. Mwanga, and M. Ghislain. 2013. Segregation analysis of *cry7Aa1* gene in F1 progenies of transgenic and non-transgenic sweetpotato crosses. Journal of Plant Breeding and Crop Science 5(10): 209-213. DOI: 10.5897/JPBCS2012.070.

3. **Rukarwa R.J.**, Mukasa, S.B. and Ssemakula, G. 2013. Evaluation of progenies from crosses between Bt and non-transgenic sweetpotato. *International Journal of Agronomy and Agricultural Research* 3(3): 28-37.

4. **Rukarwa R.J.,** Mukasa, S.B., Odongo, B., Ssemakula, G. and Ghislain, M. 2013. Identification of relevant non-target organisms exposed to sweetpotato weevil-resistant Bt sweetpotato in Uganda. *3Biotech.* Doi: 10.1007/s13205-013-0153-1**.**

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Sweetpotato (*Ipomoea batatas* L.) belongs to the Convolvulaceae or morning glory family and is the only economically important species of the *Ipomoea* genus (Woolfe, 1992). The crop is thought to have originated in Mexico and possibly Central America (Zhang *et al.*, 2001). Indigenous South Americans probably cultivated sweetpotato already, since 3000 years BC (Huang and Sun, 2000). It was spread to other parts of the world such as Polynesia and New Zealand in the 8th century. Since then, it has spread to most of the world's tropical, sub-tropical and warmer temperate regions (Janssens, 2001).

Sweetpotato is an important crop in many parts of the world, being cultivated in more than 100 countries (OECD, 2010; FAO, 2012). In terms of annual production, the crop ranks as the fifth most important food crop on a fresh weight basis after rice, wheat, maize, and cassava (Srinivas, 2009). Production is concentrated in East Asia, the Caribbean and tropical Africa, with the bulk of the crop (85%) being grown in China (FAO, 2012). In Africa, production is concentrated in East African countries around Lake Victoria, where Uganda is the second biggest producer with an annual production of 2.65 million metric tons making it forth in the world after China, Nigeria and Tanzania (FAO, 2012).

The crop is grown in different agro-ecological zones and usually plays significant roles in the farming and food systems of developing countries. Desirable attributes of sweetpotato are that it performs well in relatively poor soils with few inputs, has a short growing season and stores well in soil as a famine reserve crop (Mukasa, 2004). Due to its hardy nature and broad adaptability it can provide a sustainable food supply when other crops fail (Thottappilly, 2009; Zhang *et al.*, 2009). Among the major starchy staple crops, sweetpotato has the highest rate of production of calories per unit area per unit time (Woolfe, 1992; Thottappilly, 2009). Sweetpotato is mainly grown for its storage roots and vines. Its storage roots are used for human consumption, animal feed, and seed in temperate regions, while vines are used as animal feed and seed mainly in the tropics (Stevenson *et al.*, 2009). Nutritionally, sweetpotato is high in carbohydrates, vitamin A content and rich in dietary fibre, vitamin C, and vitamin B6 (OECD, 2010). It is consumed

as a vegetable, boiled, baked or often fermented in food and beverages (Panda *et al.*, 2006).

Despite the importance of sweetpotato in developing countries there are several constraints facing production of the crop. The key constraints to sweetpotato production are diseases and insect pests. Sweetpotato weevils constitute a major constraint to sweetpotato production and utilisation worldwide (Fuglie, 2007). In Africa, the principal sweetpotato weevil species are *Cylas puncticollis* Boheman and *C. brunneus* Fabricius, whereas in America and Asia *C. formicarius* Fabricius is the major spp. (Sorensen, 2009). External damage of roots by weevils affects their quality and marketable value while internal damage leads to complete loss. Yield losses due to weevil feeding often reach as high as 60-100% under heavy infestations (Smit, 1997b; Sorensen, 2009) and in Uganda, the pest causes up to 28% production losses annually (Kiiza *et al*., 2009). Even slightly damaged roots are unsuitable for human consumption due to extremely bitter tasting terpenoids produced by the plant in response to weevil feeding (Stathers *et al.*, 2003b). In addition, fungal rotting occurring as a consequence of weevil tunnelling in the storage roots produces several compounds including ipomeamarone which is particularly toxic to animals and possibly humans (Pandey, 2008).

1.2 Problem statement

Sweetpotato weevils attack stems, crowns and roots and their cryptic nature render them difficult to manage (Smit *et al.*, 2001). There is no effective chemical control for the larvae, or other stages found within the plant tissues. Insecticides only kill the adults that migrate in search of feeding or oviposition sites. Therefore, to effectively use insecticides frequent applications are needed to continuously kill adults that emerge from roots or migrate from other fields. However, the use of chemical control is too expensive and impractical for most resource poor farmers. In addition, many insecticides used are broad spectrum, toxic not only to the target pest but also beneficial insects and humans (Damalas and Eleftherohorinos, 2011).

The fungal pathogen, *Beauveria bassiana*, has been used to control sweetpotato weevil with apparent success in Cuba, in combination with other control methods (Stathers *et al.*, 1999) but in Uganda, these pathogens have limited potential for control because small scale farmers are unable to purchase these products. Similarly, sex pheromones of *C.*

formicarius were effective in checking weevil populations in mass trapping trials in Taiwan (Hwang and Hung, 1991), Vietnam (Braun and van de Flieart, 1997) and Cuba (Alcazar *et al.*, 1997) but were not effective in Uganda (Smit *et al.*, 2001). Sweetpotato producers in Sub-Saharan Africa (SSA) are mostly small-scale, resource-poor farmers who grow the crop all year round. Thus, management strategies that have been proven successful in other parts of the world are not necessarily economically suitable and practically feasible for farmers in Africa.

Considerable research has been done to identify host plant resistance to *Cylas* spp. in sweetpotato (Talekar, 1987; Chalfant *et al.*, 1990; Son *et al.*, 1991; Stathers *et al.*, 2003b; Stevenson *et al.*, 2009; Jackson *et al.*, 2012; Muyinza *et al.*, 2012). In Ugandan cultivars, mechanisms of resistance which include antibiosis, antixenosis, and escape have been identified (Magira, 2003) particularly New Kawogo as the best cultivar with some level of resistance to weevils (Muyinza *et al.*, 2012) . Nonetheless, cultivars with sustainable levels of resistance are not yet available (Stevenson *et al.*, 2009). Progress in breeding weevil-resistant cultivars has been slow due to inconsistent resistance displayed by the genotypes across different areas. Additionally, the polyploidy nature of sweetpotato (2n = 6x = 90), outcrossing behaviour, and numerous mating incompatibilities, make conventional breeding for this trait difficult. Given the slow of progress in conventional breeding, the option of using genetic engineering using *Bacillus thuringiensis* (Bt) would provide the resource poor farmers with a better alternative to sweetpotato weevil control. Significant progress has been made in transferring the synthetic *cry* genes derived from Bt into some sweetpotato varieties (Moran *et al.*, 1998; García *et al.*, 2000). However, difficulties in transforming and regenerating a broad range of sweetpotato genotypes currently limits the approach (Kreuze *et al.*, 2009).

Generation of insect-resistant, transgenic crop plants which express insecticidal crystal protein genes of Bt is a standard crop improvement approach (de Maagd *et al.*, 2001). To date, many plants have been transformed with different *cry* genes and showing differing levels of resistance to insect feeding (DeVilliers and Hoisington, 2011). Chinese japonica rice (*Oryzo sativa L.*) expressing a synthetic *cry1Ab* transgene showed significant resistance to feeding by the stripe stem borer (*Chilo suppressalis*) Wang *et al*. (2002). Similar results have been observed with resistance to insect feeding in Cry expressing transgenic eggplant (Arpaia *et al.*, 1997), broccoli (Cao *et al.*, 2002), and potato (Nault,

2001). In addition, Williams *et al.* (2006) also reported significant resistance of maize to southwestern corn borer (*Diatraea grandiosella*) fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*). Successes such as these with regard to insect feeding resistance have led to the development and release of a number of commercial transgenic plants. The most prevalent types of commercial Bt crops are currently maize and cotton with many commercial lines available (James, 2012).

1.3 Justification of the study

Three Bt endotoxins, ET33-34, Cry3Ca1, and Cry7Aa1 have been tested against *C. puncticollis* and *C. brunneus* in artificial diet assays (Moar *et al.*, 2007; Ekobu *et al.*, 2010). All three Bt proteins have been found to be toxic to the *Cylas* spp. at an LC_{50} of less than 1 ppm (Ekobu *et al.*, 2010). As a result transgenic sweetpotato events have been generated using truncated *cry7Aa1*, *cry3Ca1*, *ET33-34*, *cry7Aa1+cry3Ca1* and *cry7Aa1+ ET33-34* genes with the aim of controlling sweetpotato weevils. At present, no work has been done to screen these events for resistance against the African sweetpotato weevil. It is for this reason, important to study the efficacy of the different Cry proteins in transformed sweetpotato against the African *Cylas* spp. as a possible management measure. For these transgenic events to be a useful tool in an integrated pest management programme (IPM) to reduce weevil damage, the roots must have some resistance against the prevalent *Cylas* spp.

Sweetpotato is clonally propagated and a single superior transgenic weevil resistant event could be sufficient for obtaining an improved cultivar for use. However, difficulties in transforming and regenerating Ugandan cultivars have been a barrier to the introduction of the weevil resistance trait in the local germplasm (Moar *et al.*, 2007; Kreuze *et al.*, 2009). Owing to the devastating nature of the weevil, seeking a method to transfer these *cry* genes into the local germplasm is imperative. The transgenes could be introduced into the Ugandan germplasm through hybridisation with the genetically engineered sweetpotato varieties as source germplasm for the *cry* genes. This deliberate method of crossing transgenic events with non-transgenic genotypes is commonly known as transgenic breeding. It has been successfully deployed in potato (Heeres *et al.*, 1997), beans (Pinheiro *et al.*, 2009), cotton (Dong *et al.*, 2004) and maize (Yuan *et al.*, 2009) to develop new varieties with resistance genes from the transgenic genotypes. Equally, this transgenic breeding programme could help in developing new varieties of sweetpotato. It is therefore

necessary to endeavour to cross the recalcitrant Ugandan sweetpotato varieties with transgenic genotypes in order to introduce the weevil resistance trait into the local germplasm pool and broaden the genetic diversity of sweetpotato varieties in Uganda. In this study, New Kawogo with some level of resistance will be crossed with one event to investigate if the resistance by Cry protein would add up to the resistance of the natural host resistance of New Kawogo, or even if it would be synergistic.

To integrate the transgenic plants into any breeding programme, stability of inheritance and expression of the transgenes are critical factors to be considered. Transgenic loci introduced into higher plant species may display unsuitable patterns of inheritance and expression (Yin *et al.*, 2004). Transgenes may deviate from Mendelian segregation depending on several genetic factors. Persistent distortion of transgenes from Mendelian segregation may occur when a transgene is linked to a deleterious locus or the transgene disrupts an endogenous locus which is critical to plant development or gamete transmission (Zhong, 2001). Furthermore, it can also occur when a transgene produces a biochemical product that significantly interferes with the endogenous biochemical pathways for gamete development and transmission (Hood *et al.*, 1997). Some transgenes may get lost or inactivated due to endogenous silencing (Romano *et al.*, 2005). In addition, transgene expression can also be affected by a variety of factors influenced by plant genetic background (Schmidt *et al.*, 2004). Although these cases are rare, the transgene inheritance and expression in the progeny need always to be tested to ensure successful employment of transgenic breeding to develop new sweetpotato varieties adapted to diverse environments and uses with know and stable resistance to weevils.

In exploitation of transgenic Bt sweetpotato in cross breeding with elite varieties for insect resistance, it is also important to assess the morphological and agronomic characteristics for regulatory purposes and variety release. Variation among traits not related to the transgene has been reported due to the *in-vitro* regeneration process. In a study of transgenic rice (R_2 generation), plants were significantly shorter, flowered later, and were partially sterile when compared to their non-transgenic controls (Lynch *et al.*, 1995). Blanche *et al.* (2006) compared transgenic cotton cultivars and their parents. Transgenic cultivars were taller, had greater internodes ratio, larger seed, and lower lint percentages, and in some cases yielded more than their conventional parents. On the contrary, integration of the synthetic Bt fusion gene C*ry1B–Cry1Ab* in the cabbage nuclear genome

did not alter photosynthetic activity (Paul, 2003). Both T0 and T1 Bt transgenic plants flowered normally and set seeds normally like their wild-type counterpart. In sweetpotato, however, research focusing on phenotypic variations in the morphological and agronomic traits between progenies of transgenic and non- transgenic parents is still scanty. Given that sweetpotato is highly heterozygous an understanding of the nature and magnitude of variation among sweetpotato genotypes from such crosses is vital to plan effective transgenic breeding programmes.

In transgenic breeding, the environmental effects of genetically engineered crops that produce the insecticidal Cry proteins of Bt need to be assessed to fulfil regulatory requirements. One of the possible effects of the cultivation of genetically modified (GM) plants is their impact on the occurrence and density of non-target organisms (NTOs) on the cultivated land or the surrounding crop margins, as well on the local as on the regional scale. This impact can be direct uptake of Bt-protein by feeding on parts of the genetically modified plant or through Bt-protein laden prey and subsequent toxic action of the protein against the NTOs. The effects can also be through indirect reduced quality and abundance of susceptible prey, for example, reduced parasitoid and predator densities. The potentially affected NTOs of significance, the relevant exposure pathways, the potential effects, the relevance of the subsequent ecological changes in a given agro-ecosystem and the methods to assess the likelihood and magnitude of such changes are identified (Romeis *et al.*, 2008). Available data and expert opinion are taken into account to identify areas of concern or uncertainty. In sweetpotato, therefore, an inventory of available data is required to ascertain the risk of the genetically-engineered sweetpotato on NTOs. Since Bt sweetpotato was only allowed into Uganda for confined screenhouse trials in 2011, Bt's impact on non-target arthropods in sweetpotato has not been studied. In order, to evaluate which NTOs might be exposed to the insecticidal protein in genetically engineered sweetpotato fields, a review of arthropods associated with sweetpotato fields is needed to prioritise non-target arthropods for risk assessment studies.

1.4 Aim of the study

The main objective of this study was to assess the efficacy of sweetpotato expressing Cry proteins to the African sweetpotato weevil and identify candidate sweetpotato genotypes to be incorporated into transgenic sweetpotato breeding programmes. This thesis is part of a research programme with the ultimate goal of developing locally adapted, farmer

preferred transgenic sweetpotato crop varieties protected from sweetpotato weevil damage.

1.4.1 Specific Objectives

This study was based on the following specific objectives;

1. To evaluate the efficacy of sweetpotato events expressing Cry proteins against the African sweetpotato weevil using different bioassays,
2. To determine the inheritance and expression of *cry7Aa1* gene in F_1 sweetpotato progenies developed through transgenic breeding,
3. To evaluate the agronomic performance of sweetpotato progenies from a cross of transgenic and non-transgenic sweetpotato, and
4. To identify key non-target organisms of weevil resistant transgenic sweetpotato.

1.5 Thesis outline

This thesis has been organised in nine chapters. Chapter 1 covers an introduction, statement of the problem, justification and the objectives of the study. Chapter 2 presents the literature review pertinent to the two weevil species, including advances in using Bt technology, Bt inheritance and expression, an overview of inheritance of agronomic traits in sweetpotato, and environmental risk assessment of non-target organisms of Bt crops. Chapter 3 outlines the evaluation of bioassay methods for testing the transgenic sweetpotato expressing Cry proteins. Chapter 4 outlines the segregation and expression analysis of *cry7Aa1* in transgenic sweetpotato. Chapter 5 outlines the evaluation of some farmer preferred agronomic traits in sweetpotato progeny. Chapter 6 presents the future of Bt crops with a focus on non-target risk assessment and Chapter 7 provides a brief general overview of the main findings, challenges and implications for breeding, and finally Chapter 8 provides the conclusions and recommendations for future research.

CHAPTER TWO

LITERATURE REVIEW

2.1 Sweetpotato weevil (*Cylas* spp.)

2.1.1 Taxonomy of the sweetpotato weevil

Sweetpotato weevil (SPW) belongs to the Brentidae family and Cyladinae subfamily (Wolfe, 1991). At species level classification, there are 29 species names under genus *Cylas* but some of them are junior synonyms (Wolfe, 1991). These species are further divided into three monophyletic groups, according to body shape, length of the hind femora, head structure, and genitalia characteristics. The resulting monophyletic groups are *Cylas brunneus, C. puncticollis* and *C. formicarius.* The *C. formicarius* complex is composed of heterogeneous populations with three names, *C. f. formicarius, C. f. elegantulus,* and *C. f. turcipennis. C. formicarius* considered as the New World species and *C. f. formicarius*, the Old World species (Sorensen, 2009). The *C. brunneus* group includes, *C. brunneus, C. femoralis* and *C. angustatus* (Wolfe, 1991). The *C. puncticollis* group includes *C. puncticollis, C. puncticollis opacus, C. nigrocoerulans, C. compressus,* and C. *hovanus. C. puncticollis* commonly referred to as the "African sweetpotato weevil", which is the most problematic species of sweetpotato within this group (Wolfe, 1991). Despite the complex nature of the classification of SPW, *C. puncticollis* and *C. brunneus* are the widely accepted names of the weevil affecting sweetpotato in Africa and Madagascar.

2.1.2 Geographical distribution

Sweetpotato weevil is found throughout the tropical and subtropical regions where sweetpotato is grown worldwide. *Cylas formicarius* is the most widespread species of SPW, but is rare in Africa whilst *C. puncticollis* and *C. brunneus* are restricted to Africa and Madagascar (Sorensen, 2009). *C. puncticollis* is the most important pest of sweetpotato in tropical Africa, notably Uganda, Rwanda, Kenya and Cameroon (Smit and Matengo, 1995). *Cylas brunneus* is common in West Africa, Central Africa and some countries in East Africa, including Uganda and Rwanda. In Uganda, both *C. puncticollis* and *C. brunneus* can be found infesting the same root in the field (Smit, 1997a).

2.1.3 Life cycle and description of sweetpotato weevil

A complete life cycle of SPWs takes one to two months depending on the prevailing temperature conditions; the higher the temperature, the faster the development (Stathers *et al.*, 2005). The females lay yellowish eggs singly in a cavity excavated in either roots or stems which they subsequently seal the cavity with faecal plug (Smit, 1997b). Roots are most the preferred oviposition sites and most eggs tend to be deposited near the crown of root. On stems, the woodier parts are favoured more than above new growth. Sometimes the adult crawls down cracks in the soil to access roots for oviposition, in preference to depositing eggs in stem tissue. The eggs are oval in shape and their size is about 0.7 mm in length and 0.5 mm in width. Duration of the egg stage varies from about 5 to 12 days with eggs of *C. puncticollis* developing faster than those of *C. brunneus* (Smit and Van Huis, 1998). The larvae of *Cylas* spp. are usually creamy white, with a curved body. They feed and develop within the stems and roots of sweetpotato.

The larva passes through 3-5 instars in a period of 11-33 days (Stathers *et al.*, 2005). The early instar larvae of *C. puncticollis* cannot be distinguished from *C. brunneus* but the last larval instar, pre-pupal stage and pupae of *C. puncticollis* are relatively larger. Pupation occurs inside the stem or root depending on where oviposition occurred. Pupae are similar to adults in appearance, although the head and elytra are bent ventrally. After eclosing from pupae, the adults remain in the stem or root for a few days before emerging from the plant. Just eclosed adults of *C. puncticollis* change colour from white through grey, to completely black, while those of *C. brunneus* reach their final colouration via white and brown. Some *C. brunneus* turn completely black like *C. puncticollis,* while others become brownish. However, in East Africa most *C. brunneus* are bicoloured, having a black head, a reddish brown thorax and black elytra, thus resembling *C. formicarius* (Smit, 1997b). The adults emerge from the root or stem after attaining full colouration. *C. brunneus* has widely separated eyes and two pairs of aedeagal sclerites whereas *C. puncticollis* has narrowly separated eyes and four pairs of aedeagal sclerites (Wolfe, 1991). Average sized adults of *C. brunneus* are generally smaller than the adults of *C. puncticollis*; with the former being 5 mm whilst the latter 7 mm.

Adults of *C. puncticollis* and *C. brunneus* are sexed by the shape of the distal antennal segment which is filiform in males and club like in females (Wolfe, 1991). At 27°C, *C.*

puncticollis has a total developmental period of 20-28 days, whereas *C. brunneus* takes 32-41 days (Smit and Van Huis, 1998). After emergence from the root or stem the females feed for a day before becoming sexually active, oviposition commences shortly after mating. Both *C. puncticollis* and *C. brunneus* females lay around 50-250 eggs in their lifetime (Stathers *et al.*, 2005). Adults are secretive; often feeding on the lower surface of leaves, and are not readily noticed. The adult is quick to feign death if disturbed. The adults of the *Cylas* spp. can fly, but seem to do so rarely and in short, low flights. Adults of *C. puncticollis* have an average life span of 140 days, whereas, *C. brunneus* lives an average of about 92 days (Smit and Van Huis, 1998).

2.1.4 Weevil host range and dispersal

The preferred host plant for *C. puncticollis* and *C. brunneus* is *Ipomoea batatas.* These two *Cylas* spp. existed in Africa well before sweetpotato was introduced during the 16th century, most probably some other plant species could have been their hosts. A range of *Ipomoea* spp. is found in Eastern Africa but no information is available on whether these species are possible hosts of the two weevils (Smit, 1997b). In most cropping systems in East Africa sweetpotato is available throughout the year, and for this reason the need for alternative hosts for survival of weevils is limited. On the other hand, other *Ipomoea* spp. do not produce swollen roots which provide oviposition sites, hence, population build up may be very low. *Cylas puncticollis* has also been reported to feed on sesame and maize (Hill, 2008). The weevils disperse by flying and crawling. With regards to females and males of *C. brunneus* and *C. puncticollis,* males fly more than females. Unmated males kept separate from females fly frequently (Smit, 1997b). The maximum dispersal distance is 500 m in fields with and 1000 m in fields without sweetpotato (Stathers *et al.*, 2005). Nevertheless, their dispersive abilities are probably underestimated because they are most active at night.

2.1.5 Sweetpotato weevil damage

The adult of *Cylas* spp. feed on the epidermis of vines and leaves, scraping oval patches off petioles, young vines and leaves. Feeding inside the vines causes malformation, thickening and racking of the affected vine. Early infestation might lead to poor establishment of the planting material. Foliage may become pale in colour, growth and vigour of the plant can be significantly reduced. Nonetheless, yield losses are seldom serious when the weevil attacks the vines and leaves (Sorensen, 2009). Significant damage

is inflicted when the SPW attacks the underground storage roots. Adults feed on the external surfaces of roots causing round feeding punctures which can be distinguished from oviposition sites by their greater depth and the absence of faecal plug (Stathers *et al.*, 2005). The developing larvae tunnel in the vines and roots causing significant damage. In response to the damage, the root produces bitter tasting terpenoids which render the infested root part inedible (Stevenson *et al.*, 2009). When a root is exclusively infested by *C. puncticollis,* the core of the root might still be untouched whereas *C. brunneus* larvae tunnel further inside the root (Smit, 1997b). Sweetpotato weevils are particularly a serious problem under dry conditions because the insects, reach roots more easily through cracks that appear as the soil dries out. Plants may wilt or die because of the damage to vascular system which reduces the size and number of storage roots produced. While external damage to roots can affect their quality and value, internal damage can lead to complete loss. Losses of marketable yield as high as 60–100% have been reported under heavy infestations (Smit *et al.*, 2001; Sorensen, 2009). In Uganda, a survey on the socio-economic impact of weevils done in Uganda, indicated an average yield loss of over 28% between wet and dry seasons (Kiiza *et al.*, 2009).

2.2 Sweetpotato weevil management and control strategies

Sweetpotato production is greatly threatened by weevil damage in SSA because of prevalent crop continuity. In many tropical and sub-tropical regions, sweetpotato is continuously present at various growth stages in fields (Jansson and Ramman, 1991). Various agricultural practices have been suggested for the management and control of SPWs. These include cultural practices, chemical control, predators and parasitoids, use of sex pheromones and host plant resistances which are discussed further in the next sections.

2.2.1 Cultural control

Cultural control involves changing cultivation practices which directly or indirectly reduce the SPW population (Smit, 1997a). It is usually the most economical and widely acceptable pest control tactic that helps reduce the infestation levels. Cultural control tactics such as crop rotation, intercropping, sanitation, destruction of crop residue, hilling up and mulching have been recommended as primary elements of weevil control (Talekar, 1992). Planting sweetpotato in a rotation with sorghum or rice; far away from other weevil infested sweetpotato fields lead to reduced weevil populations (Smit, 1997a). Talekar (1983) suggested that, sweetpotato should be grown in a field only once every five years.

Intercropping may reduce the accessibility of sweetpotato plants to weevils as these are randomly distributed compared to a monocrop situation where there is uniform distribution of the crop species. Similarly, destruction of crop residue after harvest is important because weevils survive in roots and stems, and infest succeeding or neighbouring sweetpotato plantings. Each sweetpotato chosen for seed should be examined carefully and destroyed if infested. Weevils gain access to roots through soil cracks that form when the soil is dry. Hilling of soil around the sweetpotato plants prevents access of the weevil to the roots. In addition, use of deep-rooted varieties over shallow-rooted varieties is advisable as this prevents easy access of the root by the SPWs. Smit (1997a) noted that these control strategies can individually control weevils when the populations are low but not during long dry periods experienced in East Africa when weevil populations rise to damaging levels. In SSA, in general, farms are closely spaced; some practices require a community effort to effectively reduce weevil populations. An example is the avoidance of spread out and adjacent planting. Some of the cultural control practices, such as hilling up and filling of soil cracks, are very labour intensive. Farmers may not be willing to provide extra labour, especially during periods when other crops also require care, or when the sweetpotato crop is considered to be of minor importance.

2.2.2 Chemical control

Numerous chemicals, applied as fumigants, pre-planting treatments and post-planting foliar applications have been tested for the control of SPW (Sutherland, 1986). Chemical control of adult weevils in the field can reduce the *Cylas* spp. populations, however, insecticides may not adequately control immature larvae of weevil due to the cryptic nature (Sorensen, 2009). Control of the weevil is difficult with conventional spraying, dusting, or fumigation with currently available insecticides once weevils are present within the crown or storage roots. Chemical control requires frequent insecticide applications or use of insecticides with long term residual effect in order to kill newly emerged or migrating adults. In some countries, planting materials are dipped into a synthetic pesticide before planting, which can delay pest infestation for several months (Stathers *et al.*, 2005). According to Allard (1990), deltamethrin and pirimiphos methyl foliar sprays applied three months after planting, followed by four applications at fortnightly intervals resulted in good control of sweetpotato weevils. Similarly, Hwang (2000) also showed that application of chlorpyrifos 5% granules resulted in a lower proportion of damaged roots and a higher control rate (76.8%) as compared to malathion,

phorate, carbofuran and terbufos. Nonetheless, frequent application of insecticides is not cost effective for subsistence farmers due to the low market value for sweetpotato in SSA. This makes chemical control impractical and unsustainable for most poor farmers. Additionally, the problems associated with pesticide residues in food, environmental contamination, and phytotoxicity limits the use of pesticides by farmers (Damalas and Eleftherohorinos, 2011).

2.2.3 Pheromones

Pheromones are semiochemicals secreted by an organism to cause a specific reaction in a receiving organism of the same species. These have been classified on the basis of the type of interaction mediated, such as sex pheromones, alarm pheromones, and epideictic pheromones. Sweetpotato weevil sex pheromones are produced commercially in several laboratories. Male adults are attracted by the sex pheromones and can easily be collected and removed from the field. According to Downham *et al.* (2001) sex pheromones have great potential for suppressing weevil populations by mass trapping males and disrupting mating. Sex pheromones were effective in reducing *C. formicarius* populations in mass trapping trials in Taiwan (Hwang and Hung, 1991), Vietnam (Braun and van de Flieart, 1997) and Cuba (Alcazar *et al.*, 1997). In Africa, Smit *et al*. (1997) identified sex pheromones of *C. puncticollis* and *C. brunneus* as decyl (E)-2-butenoate and dodecyl (E)-2-butenoate, respectively. Unfortunately in Uganda, mass trapping using these sex pheromone traps did not lead to a reduction in weevil damage to roots (Smit *et al.*, 2001). Another restriction of the pheromone approach is the availability and price of the sex pheromone to the resource poor farmers. In Eastern Africa, there is no laboratory where pheromones can be synthesized cheaply for local use.

2.2.4 Host plant resistance

Several attempts have been made to develop weevil resistant sweetpotato clones in breeding programmes around the world because of the high level of economic damage caused by the SPW (Stathers *et al.*, 2003b; Stevenson *et al.*, 2009). Resistance mechanisms, including antibiosis, antixenosis, and escape have been found to exist in the sweetpotato germplasm (Magira, 2003). Plant traits important in weevil resistance have also been identified which include fleshy root density, dry matter and starch content, root depth, vine thickness, and fleshy root chemistry (Chalfant *et al.*, 1990). Sweetpotato contains hexadecylcaffeic and hexadecyl-*p*-coumaric acids which may also be associated

with resistance (Stevenson *et al.*, 2009). These organic compounds are characterised by an orthodihydroxy group associated with insect toxicity in other plant-pest interactions (Kumar *et al.*, 2011). Host plant resistance can be a major line of defence against SPW; however, weevil resistant cultivars are not yet available in spite of over 50 years of research. Progress in breeding weevil-resistant cultivars has been slow because the heritability of the weevil resistant trait is extremely low largely due to the scarcity of varieties with significant levels of resistance (Stevenson *et al.*, 2009). Additionally, sweetpotato is a hexaploid and has problems such as pollen sterility, cross incompatibility and poor seed germination. This makes conventional breeding that relies only on hybridisation to create genetic variability a challenge.

2.2.5 Biological control

Several predators and parasitoids attack the sweetpotato weevil, however, these natural enemies seem to be ineffective at managing weevil populations under field conditions (Jansson, 1991). Three predators have been reported, two ant species *Pheidole megacephala* and *Linepithema humile* formely; *Iridomyrmex humilis* (Mayr) (Castifieiras, 1989) and a predatory maggot, *Drapetis s.s. exilis* group (Family: Empididae) (Rajamma, 1980). Six wasp parasitoids of *Cylas* spp. have been reported (Jansson, 1991), most of which are not effective at suppressing weevil populations (Smit, 1997b). Because several life stages of SPWs are completed underground, within the roots, it is apparently difficult for parasitoids to locate them.

Similarly, several fungal pathogens have been reported to attack *Cylas* spp. (Jansson, 1991). The most predominant fungus isolated from *Cylas* spp. has been *Beauveria bassiana* (Bals.) Vuill. This fungus has been isolated from *C. puncticollis* in Uganda, Kenya, Zimbabwe, and Rwanda as well as from *C. brunneus* in Uganda and Rwanda (Allard and Rangi, 1995). *Beauveria bassiana* has been used with apparent success to control *C. formicarius* in Cuba, in combination with other control methods (Stathers *et al.*, 1999). In Uganda, however, such entomopathogenic fungi have limited potential for control because small scale farmers are unable to purchase these products and grow sweetpotato year-round.

Entomopathogenic nematodes have the great potential for practical biological suppression of sweetpotato weevil. Several strains of *Steinernema carpocapsae* (Nematoda:

Steinernematidae) and *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) penetrate the soil and storage roots, killing weevil larvae (Jansson, 1991). In general, *heterorhabditid* nematodes are more efficacious against sweetpotato weevil than *steinernematids* (Mannion, 1992). These nematodes occur naturally in soil and tend to seek well-concealed hosts, such as the weevil. A desirable attribute in using these nematodes is that they possess high virulence and reproductive rates which kills their hosts quickly preventing the damage caused by the larvae (Jansson and Ramman, 1991). Also, mass rearing and subsequent application of these nematodes is safe and relatively low cost strategy that substantially reduces weevil populations (Woodring and Kaya, 1988). However, several constraints, such as soil moisture, solar radiation, and temperature in the field vary and may limit the effectiveness of entomopathogenic nematodes as biological control agents (Kaya, 1990).

Numerous bacteria are known to attack insects, including members of Bacillaceae, Pseudomonadaceae, Enterobacteriaceae and Streptococcaceae (Lacey *et al.*, 2001). Among the Bacillaceae, *Bacillus thuringiensis* Berliner (Bt) is the most important and best-known bacteria for biological control of insects (Kaur, 2000; Lacey *et al.*, 2001). Jansson (1991) tested the toxicity of a commercially available formulation (FOIL^{TM}) of the bacterium *B. thuringiensis*, and found that it had no activity against *C. formicarius*. Bt was isolated from infected *C. formicarius* larvae in the Philippines (Amalin and Vasquez, 1993). In Kenya, the use of FOIL^{TM} and Novodor, on adults and larvae of *C. brunneus* and *C. puncticollis* did not show an effect on adult and larval mortality of the *Cylas* spp. (as cited in Smit, 1997b). Because of the little impact of the topical sprays on these cryptic insects; sweetpotato plants expressing the Bt *cry* genes have since been developed at confer inherent pest resistance (Kreuze *et al.*, 2009).

Crops that have been genetically engineered for insect resistance have provided farmers with new insect control options. At present the introduction of Bt genes in crop plants ranks among the successful strategies for improving tolerance to certain insects (Sanahuja *et al.*, 2011). The use of Bt crops has led to effective pest control and reduced use of insecticides resulting in higher crop yields (Mutuc *et al.*, 2011). Bt crops are currently being grown in 28 countries and the amount of land set aside for their cultivation is increasing (James, 2012). The total global area devoted to Bt crops in 2012 was over 69 million hectares (41% of all biotech crops), made up of 26.1 million hectares of Bt-only

crops and 43.7 million hectares of crops with Bt stacked with herbicide tolerance (James, 2012).

2.3 *Bacillus thuringiensis*

Bacillus thuringiensis is a soil inhabiting bacterium that forms spores during the stationary phase of its growth cycle. The spores contain crystals that have potent and specific insecticidal activity (Ibrahim *et al.*, 2010). Different strains of Bt produce different types of toxins, each of which affects a narrow taxonomic group of insects. Bt is a member of the *Bacillus cereus* group of Gram positive, spore-forming soil bacteria. The defining feature of Bt is its ability to produce proteinaceous crystals during sporulation known as δ-endotoxins (Bravo *et al.*, 2005). These crystals are predominantly composed of Cry and Cyt toxins (Kaur, 2000; Bravo *et al.*, 2005).

The insecticidal properties of Bt were recognised way before the bacterium was identified, with some suggestions that Bt spores may have already been in use in ancient Egypt (Sanahuja *et al.*, 2011). In the modern era, the bacterium was isolated in 1901 by Shigetane Ishiwatari during an investigation into wilt disease in silk worms, and named it *Bacillus sotto* (Khetan, 2001). The second discovery was by Ernst Berliner, isolate from a diseased Mediterranean flour moth (*Ephestia kuehniella*) in Thuringia, and it was named *B. thuringiensis* (Siegel, 2000). Bt crystal preparations were first used in Europe in 1928 to control the European corn borer, *Ostrinia mubitalis* Hübner, (Lepidoptera: Crambidae) (Siegel, 2000). The first commercial insecticide based on Bt, Sporine, was produced in France in 1938 and used primarily to control flour moths. In the United States, Bt was first manufactured commercially in 1958 and, by 1961 (Khetan, 2001). More Bt toxins were discovered thereafter which led to the revolution of genetically modified crops expressing *B. thuringiensis* genes (Lambert and Peferoen, 1992). Synthetic *cry* genes from *B. thuringiensis*, modified for plant-preferred codon usage, have been introduced in a number of major crops to make them insect resistant (Letourneau *et al.*, 2003). Since 1996, cultivation of insect resistant transgenic crops, known as Bt crops, have expanded around the globe (Kleter *et al.*, 2007; James, 2012). In Africa, South Africa has been the only country on the continent benefiting from commercialization of Bt crops, until 2008 (James, 2012). More recently, Burkina Faso, Sudan and Egypt have started producing Bt cotton and Bt maize (James, 2012).

2.3.1 Bt diversity, structure and toxicity

Taxonomic classification of Bt has been attempted by many researchers using various criteria such as serotyping, phage susceptibility and plasmid profiles, and this has resulted in the classification of approximately 100 sub-species (Sanahuja *et al.*, 2011). Although there is a good correlation between Bt sub-species and insect host range at the family level, the relationship tends to break down at the genus and species levels because most Bt strains can synthesise more than one toxin, resulting in complex and overlapping host profiles (Ibrahim *et al.*, 2010). At the genus and species level, Bt strains are classified functionally on the basis of which toxin proteins they produce, as a logical way to define the host range (Sanahuja *et al.*, 2011). The Bt toxins are described in terms of their amino acid sequences, protein structures and modes of activity in the insect's midgut (Crickmore *et al.*, 1998). Cry toxins have been previously classified into 51 groups and sub-groups (Crickmore *et al.*, 1998). These toxins were separated into six major classes according to their insect host specificities and they include: Group 1: lepidopteran (Cry1, Cry9 and Cry15); group 2: lepidopteran and dipteran (Cry2); group 3: coleopteran (Cry3, Cry7 and Cry8); group 4: dipteran (Cry4, Cry10, Cry11, Cry16, Cry17, Cry19 and Cry20); group 5: lepidopteran and coleopteran (Cry1I); and group 6: nematodes (Cry6). The Cry1I, Cry2, Cry3, Cry10 and Cry11 toxins (73–82 kDa) are unique because they appear to be natural truncations of the larger Cry1 and Cry4 proteins (130–140 kDa). Lately, the known Cry and Cyt proteins fall into 32 sets including Cyt1, Cyt2 and Cry1 to Cry67 based on the homology of amino acid sequences(Crickmore *et al.*, 2013).

Despite their sequence diversity, all Cry proteins share a similar overall tertiary structure, as demonstrated by the Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba (Crickmore *et al.*, 2013). The C-terminal portion is involved in crystal formation but is not part of the mature toxin, as it is cleaved off in the insect gut. The N-terminal portion is the toxin itself, and it comprises three domains (Ibrahim *et al.*, 2010). Domain I which is responsible for membrane insertion and pore formation; is a bundle of seven α-helices, six of which are amphipathic encircling the seventh hydrophobic helix. Domain II consists of three anti-parallel β-sheets with exposed loop regions, and domain III is a sandwich of two antiparallel β-sheets that form a "jelly-roll" topology. Both domains confer receptor binding specificity thus defining the host range (Boonserm *et al.*, 2006).

A current model suggests that domains II and III initially bind to primary receptors (cadherins) which cleave the toxin within domain I and induce oligomerisation, which in turn promotes binding to high-affinity secondary receptors tethered to the membrane via C-terminal glycosylphosphatidylinositol anchors (Soberón *et al.*, 2009). The requirement for oligomerisation has recently been confirmed through the isolation of dominant-negative mutations of Cry1Ab (Rodríguez-Almazán *et al.*, 2009). Additionally, Zhang *et al*. (2006) proposed an alternative model which suggests that initial mid-gut binding triggers a Mg^{2+}dependent signalling cascade that causes G-protein dependent cAMP accumulation and the activation of protein kinase A. Phylogenetic analysis has established that diversity of the Cry family evolved by independent evolution of the three domains and by swapping of domain III among toxins (Sanahuja *et al.*, 2011).

Cyt proteins, on the other hand, have a single α-β domain comprising of two outer layers of α-helix hairpins wrapped around a β-sheet (Li, 1996). Cyt toxin is structurally related to volvatoxin A2, a pore-forming toxin cardiotoxin produced by a straw mushroom, *Volvariella volvacea* (Lin *et al.*, 2004). Cyt toxins are also synthesized as protoxins and small portions of the N-terminus and C-terminus are removed to activate the toxin (Li *et al.*, 1996). The toxic effects of Cyt toxins are different to those of Cry toxins. Cyt toxins directly interact with membrane lipids and insert into the membrane (Bravo *et al.*, 2007). Some Cry and Cyt toxins have synergistic relationships. These synergistic reactions between Cry and Cyt have been shown to promote toxin binding to target membranes and toxicity to mosquito *Culex quinquefasciatus* Say (Diptera: Culicidae) (Pérez *et al.*, 2005). It has been demonstrated that Cyt1Aa protein synergises Cry11Aa toxicity by functioning as a receptor molecule (Pérez *et al.*, 2005). It has been speculated that the mechanism for these synergistic reactions is due to the hydrophobic reactions between toxins and the cell walls of midgut epithelial cells (Berry *et al.*, 2002; Sanahuja *et al.*, 2011).

2.3.2 Transgenic plants with Bt crystal protein genes

Although microbial Bt preparations have been used in agriculture for decades and are the most effective and specific bio-insecticides, they have several limitations for use. Most restricting factors are high cost, instability in rain and sunlight, and inefficiency against pests feeding on internal tissues of the plants (Ely, 1993). The most obvious advantages of Bt based engineered insect resistance in plants include protection of target tissues even

against internal invasion of the pest; weather-independent protection; reduction in the use of chemical insecticides and high target pest specificity.

First attempts to transform plants with *cry* genes did not lead to sufficient expression of the insecticidal proteins. The main reasons for low level expression of transgene is the differences in codon preference and other regulatory sequences affecting expression, which is species specific. For enhanced expression of a foreign protein in a plant system, the codon frequency of a gene may be adjusted to the preferred codon usage frequency of the host plant. Codon optimisation is defined as an experimental strategy in which codons within a cloned gene are changed by *in vitro* mutagenesis to the preferred codons, without changing the amino acids of the synthesised protein. The difference in codon usage between bacteria and plants has been well documented (Fennoy and Baileyserres, 1993) and genome specificity determines the codon usage frequency (Grantham *et al.*, 1980). During the transfer of a gene from a prokaryotic to a plant genome, the prokaryote adapted sequence motifs that are inappropriate for expression in plant systems are changed to the preferred sequence of the plant species. Also, mRNA destabilising and degrading structures are removed (Kang *et al.*, 2004).

The transfer of Bt *cry* genes for expression in plants requires extensive sequence modification (Kumar *et al.*, 2005). Successful expression of Bt genes in plants typically requires optimisation of the *cry* gene to include plant preferred codons, reduction in the overall AT content (typical of bacteria genome) of the gene by increasing the GC content (typical of plant genomes). Also, removal of mRNA destabilizing structures like ATTTA sequences, polyadenylation signal sequences, sites of transcription termination (Misztal *et al.*, 2004) and splicing sequences are essential for proper gene expression in a plant system. Other factors including optimisation of bacterial sequences towards plant codon preference sequences using codon usage frequency tables without altering any amino acid composition are required for proper conditioning of the gene for its expression in plants.

Earlier transformation work done on sweetpotato using un-optimised *cry* genes failed to produce adequate gene expression (Moran *et al.*, 1998). Similar examples of no or low gene expression without proper codon optimisation have been reported in potato (Cheng *et al.*, 1992), and tomato (Fischhoff *et al.*, 1987). This low expression was likely due to a low level of Bt-specific mRNA caused by the presence of destabilising motifs in Bt genes and

non-optimisation of Bt gene sequences for plant expression. Nonetheless, when *cry1Ab* was optimized for eggplant using a codon frequency table for rice, low expression was also observed (Kumar *et al.*, 1998). Post-translational factors, such as protein stability, modification and trafficking play crucial role in expression of a heterologous gene (Dai *et al.*, 2005). In the present work, the *cry7Aa1*, *cry3Ca1* and *ET33-34* genes were optimised using the codon usage of sweetpotato (Ghislain *et al.*, 2013) to improve expression of the gene for insect resistance.

2.3.3 Insect resistance management (IRM) in Bt crops

Development of strategies to delay the evolution of pest resistance to Bt crops requires an understanding of factors affecting responses to natural selection, which include variation in survival on Bt crops, heritability of resistance, and fitness advantages associated with resistance mutations. A high dose/refuge insecticide resistance management (IRM) strategy has been implemented for planting Bt crops in the United States and several other countries (Bourguet *et al.*, 2003). This strategy involves to plant "high dose" Bt plants that can kill ≥ 95% heterozygotes for Bt resistance. The high/dose refuge strategy also requires Bt crop growers to plant a specified proportion of their crop to a non-Bt variety of the crop to serve as a refuge for hosting susceptible insects. Bt-susceptible insects should emerge from refuge areas and mate with the rare potentially resistant homozygous individuals that might emerge from the Bt crop so that most offspring will be heterozygous and thus be killed by the high dose Bt plants. Therefore resistance allele frequencies in field populations should remain low for long period of time.

In addition to the high dose/refuge IRM strategy, a gene-pyramiding strategy has been also used for Bt resistance management. This strategy relies on development of transgenic plants that express two or more dissimilar Bt proteins for targeting the same group of insect pests. Mathematical modelling indicates that pyramiding two or more Bt genes with different insecticidal mechanisms into one plant for controlling the same target species should delay resistance development and can be used as a useful supplement for Bt crop IRM (Zhao *et al.*, 2003). For example, genes encoding for Cry1A.105 and Cry2Ab2 proteins have been transferred into corn plants for controlling above-ground lepidopteran corn pests (Monsanto, 2009). The resulting Bt event, MON 89034, has been incorporated into several Bt corn technologies including Genuity VT Triple Pro™ and SmartStax™ (Monsanto, 2010). Such pyramided Bt corn technologies have been commercially

available since the 2010 planting season in the U.S. and Canada (Monsanto, 2010). A study from Ghimire *et al.* (2011) demonstrated that MON 89034 provided complete control of a Cry1Ab-resistant strain of *D. saccharalis*, which was highly resistant to Cry1Ab corn plants. Similarly, the gene-pyramiding strategy has been adopted in Bt sweetpotato for Bt resistance management.

2.4 Regeneration and transformation of sweetpotato

Although protocols have been described for stable integration of transgenes into plants, sweetpotato transformation is still a challenge. This is mainly due to sweetpotato recalcitrance and genotype dependent *in vitro* responses. The first reported transformation of sweetpotato plants was based on the formation of hairy roots using leaf discs as explants by *Agrobacterium rhizogenes* (Otani *et al.,* 1993). Nevertheless, the regenerated transgenic plants presented morphological abnormalities, such as small storage roots and shorter internodes. Later, an *Agrobacterium tumefaciens*-mediated transformation system was developed through either somatic embryogenesis (Liu *et al.,* 2001; Song *et al.,* 2004; Kreuze *et al.,* 2008) or organogenesis (Moran *et al.,* 1998; Luo *et al.,* 2006). As a result some agronomically important genes have been introduced into sweetpotato. Even though stable transformation of sweetpotato has been achieved in some cultivars, efficiencies are generally low (Moran *et al.,* 1998; Otani *et al.,* 2003; Song *et al.,* 2004; Luo *et al.,* 2006).

The sweetpotato transformation systems are relatively slow and laborious because of the heavy reliance on establishment of embryogenic calli which work for only a few genotypes (Yu *et al.,* 2007; Manrique-Trujillo *et al.,* 2013). Not all the cultivated or elite lines of a crop species are amenable to genetic transformation (Visarada *et al*., 2009). African sweetpotato cultivars, in particular have been observed to be recalcitrant and hardly regenerated transformed shoots from calli (Luo *et al.,* 2006). As a result, there is limited development of African sweetpotato cultivars expressing agronomically important traits such as weevil resistance. Nonetheless, some non-African varieties expressing weevil resistance genes are available for weevil control (Ghislain *et al.,* 2013). Unfortunately, these transgenic events are not well adapted to African growing conditions and do not have farmer preferred traits. Hence, there is a need of a series of crossing and selection in transgenic breeding to incorporate the new genes into best African cultivars.

2.5 Transgenic breeding

Transgenic breeding shares the same principles with conventional breeding. Conventional methods of plant breeding include evolutionary methods (germplasm utilisation, pedigree methods, and evaluation of genotype × environment interactions) and revolutionary methods (accelerated introgression of genes and marker-assisted selection). Development of a new cultivar is a sequential and cyclic process of creating new diversity, recombination, selection for superior recombinants, and testing followed by commercialisation. Through transformation, a new genetic variation is created, but it has to be performance-tested like its classically bred counterparts before release and commercialisation. In conventional breeding, the breeder selects for desirable recombination among large segregating population, whereas in transgenic breeding, the breeder looks for a defined trait phenotype and then introgresses the transgenic event into a broad range of desirable genetic backgrounds (Yin *et al.*, 2004). Crossing allows natural flow of genes in random combination, and genetic transformation has the advantage of precision transfer of selected DNA sequences. However, in the case of transgenics, the site of integration of the transgene is usually random, sometimes resulting in alteration of the DNA sequence to be inserted and sometimes disrupting the recipient genome. To integrate the transgenic plants into a breeding programme, stability of inheritance and expression of the transgenes are critical factors to be considered. The potential of transgenic breeding in crop improvement has been well demonstrated in recent commercialisation of varieties and hybrids with novel transgenic traits such as insect resistance (Dong and Li, 2007).

2.5.1 Transgene inheritance and expression

The economical or agronomical applications of the transgene(s) depend on the stable expression of the transgenes from generation to generation. Transgene inheritance studies in plants undergoing sexual propagation have shown the occurrence of both Mendelian and non-Mendelian inheritance (Yin *et al.*, 2004). Transgene copies often integrate at a single genetic locus and are inherited in a Mendelian manner (Miroshnichenko *et al.*, 2007). For example, Register *et al.* (1994) reported maize plants transformed by the biolistic method contained multiple copies of the transgene(s). However, the multiple copies of the transgenes tended to integrate at a single locus, and a Mendelian inheritance pattern was observed in the progeny. The transgene copy number in plants transformed by the *Agrobacterium*-mediated method is often low and inheritance in progeny is often in a Mendelian manner, too (Miroshnichenko *et al.*, 2007). On the other hand, non-Mendelian

segregation had also been observed (Yin *et al.*, 2004; Romano *et al.*, 2005). Segregating patterns of 1:1 and 1:3 were observed in *GUS* transformed maize (Ishida *et al.*, 1996).

Studies have shown that inheritance pattern and performance of transgene(s) may occasionally change from generation to generation. Both Mendelian and non-Mendelian patterns were observed over multiple generations (Pinheiro *et al.*, 2009). When looking at the inheritance pattern over three generations of transgenic *GUS* rice plants by analysing the activity of GUS and II, Peng *et al.* (1995) observed that the T1 generation followed Mendelian inheritance and the T2 and T3 were non-Mendelian with segregation ratio ranging from 1:1 to 1:2 and in some cases 0:all. Similar findings that Mendelian segregation was only in the transgenic T1 rice plants and not the T2 plants were observed by Chen *et al.* (1998). Segregation studies at the expression level, using GFP fluorescence followed by histochemical GUS staining, also showed both Mendelian and non-Mendelian inheritance of GFP and GUS expression in T1 wheat (Miroshnichenko *et al.*, 2007).

There are a number of factors that account for the inheritance pattern and performance of transgenes. Variation in the inheritance pattern could be due to transgene(s) silencing, or unstable transmission of gene(s) (Yin *et al.*, 2004). Register *et al*. (1994) suggested that the nature of the crop genome (maize, in this case, possibly due to epigenetic control) was responsible for the lack of transgene inheritance in the T2 generation. The transformation methods (biolistic vs. *Agrobacterium*-mediated) could also contribute to the integration of transgene(s). The biolistic method often produces plants with higher copy number of transgene(s) and higher frequency of rearrangement of transgene(s) (Pinheiro *et al.*, 2009). The multiple transgene(s) insertion could lead to the silencing of transgene or co-suppression (Hammond *et al.*, 2001). The site of gene insertion is another factor that influences the performance of transgenes (Romano *et al.*, 2005). Ye and Singer (1996) found that silencing of transgene(s) might occur when integration took place in repeat-sequence regions of the heterochromatin (RIGS, repeat-induced gene silencing). Other factors such as selection system (Bhattacharyya *et al.*, 1992), gene elimination (Romano *et al*., 2005), and construct components such as type of promoter and intron (Vain *et al.*, 1996) may all play roles in the inheritance pattern and performance of transgenes.

Understanding the inheritance pattern and performance of transgenes in subsequent generations of transgenic plants allows for selection of lines of desired trait(s). In the

development of agronomically important crops, either a homozygous or hemizygous population is preferred depending on the trait of interest (James *et al.*, 2002). Studies have shown that inheritance pattern and transgene expression could also vary between homozygous and hemizygous progeny plants. Observations of differences between the performance of hemizygous and homozygous plants are controversial since findings from many studies do not agree with each other. Studies examining the difference in transgene expression between homozygous and hemizygous transgenic cereal crops show that, in many cases, the homozygous plants out-performed the hemizygous plant (James *et al.*, 2002). Similarly, Baruah-Wolff *et al*. (1999) found approximately 25% higher firefly luciferase gene activity in association with the homozygous T1 transgenic rice plants.

However, some other reports suggest that a transgene becomes silenced when present in homozygous form but is continuously expressed when present in hemizygous form (James *et al.*, 2002). Transgene silencing associated with the homozygous state have been reported in tobacco transformed by *Agrobacterium*-mediated transformation (de Carvalho *et al.*, 1992; James *et al.*, 2002). de Carvalho *et al.* (1992) detected complete suppression of β-1,3-glucanase transgene expression in homozygous tobacco plants. There are also studies showing no difference in transgene expression level between homozygous and hemizygous plants. Examination of transgene inheritance at the structural and expression levels in white clover showed no significant difference in *gusA* expression levels of homozygous and hemizygous plants (Scott *et al.*, 1998). Considering the plants within each category share the same genetic background, the variation in expression between them is most likely caused by epigenetic effects, such as DNA methylation, which sometimes occur in transgenes. It is known that DNA methylation is a progressive and uneven process and is reset at every generation (Bender, 2004). Thus the variation on the extent and pattern of the transgene methylation among individual offspring plants could generate various transgene expression levels.

2.6 Agronomic characteristics of transgenic plants

A major requirement for transgenic breeding is that transgenic plants bearing the new genetic combinations only differ from untransformed crops in the new traits added by transformation, leaving undisturbed the basic genetic background in which these new traits are expressed. Thus, the agronomic performance of transgenic plants needs to be evaluated in order to establish the impact of transformation on agronomic traits (Zhong, 2001). Mild

genetic changes, sometimes heritable, occur during prolonged *in vitro* culture of plants leading to somaclonal variations. Other genetic drag observed among transgenic plants can be attributed to prolonged exposure to tissue culture or to the position of insertion of transgene (Bhat and Srinivasan, 2002). The genetic base of somaclonal variation is only starting to be known and involve several phenomena such as spontaneous mutations, epigenetic changes (methylations and other chemical modification of the DNA), transposable elements insertion or excisions, and rearrangements of the genome during mitosis. Changes due to somaclonal variation are mild and do not contribute to agronomic superiority in terms of production and productivity. Genetic drag due to position of insertion of transgene leads to undesired levels of performance in the agronomic traits.

Unintended as well as normal behaviour of the transgene progeny was reported in many crops. Rice transgenics expressing high levels of snow drop lectin gene were found to be sterile and stunted (Maqbool *et al.*, 1998). Variation among traits apparently not related to a foreign gene has also been reported. In transgenic rice (R_2 generation), plants were significantly shorter, flowered later, and were partially sterile as compared to their non-transgenic controls (Lynch *et al.*, 1995). Morphologically abnormal flowers that failed to set seeds were observed in soybean (Liu *et al.*, 1996). In cucumber, transgenic plants though produced fruits on self-pollination, failed to set seeds (Mendel, 1995). Blanche *et al.* (2006) compared the transgenic cotton cultivars and their parents. Transgenic cultivars were taller, had greater internodes ratio, larger seed, and lower lint percentages, and in some cases yielded more than their conventional parents. In the case of transgenic sweetpotato progeny, some important agronomic traits may be affected in uncertain ways. Similarly, pleitropic effects of transgenes can affect the performance of transgenic lines as potential parents to be used in a sweetpotato breeding programme.

In addition, sweetpotato is highly heterozygous and many of the agronomic traits show continuous variation. Genotypes differ in root flesh colour, root skin colour, in the size and shape of roots and leaves, the depth of rooting, the time to maturity, disease and insect resistance, and in the texture of the flesh (Woolfe, 1992). Almost all traits are quantitatively inherited, and selection of the best genotypes is important to rapidly aggregate desirable alleles (Jones. *et al.*, 1986). Sweetpotato is propagated asexually so any advances made in breeding can be passed on to the producer and consumer without the need for achieving homozygosity.

2.7 Biosafety regulation of genetically modified crops in Uganda

In Uganda, it is noteworthy that there is no biotechnology and biosafety law in place yet, but a Biosafety Bill is being drafted (Mtui, 2012). Biosafety regulations and, to a degree, biotechnology developments in Uganda are governed in the context of the Cartagena Protocol on Biosafety (GOU, 2004). Biosafety regulation entails identification of risks and development of practices to analyse, manage, and prevent or minimise exposure of humans and the environment to biological hazards. The policies and procedures adopted ensure that the application of modern biotechnology is safe to humans and the environment. Biosafety assessment of genetically modified (GM) crops and their uses is based on an understanding of the technologies used for development of GM crops, the relationship of GM crops to conventionally bred crops, and the relationship of GM crop production practices to current agricultural practices and their impacts.

2.7.1 Concerns related to GM crops in Uganda

There are concerns that the introduction of GM crops might lead to a reduction in biodiversity (the variety of plants and animals in the wild), particularly in areas where a crop originated and a wide range of natural genetic variation is found. There might also be unexpected consequences of gene transfer (or 'gene flow') between plants, for example an irreversible or uncontrollable 'escape' of genes into neighbouring wild plants by pollen. Sweetpotato, however, is not originally from Africa and has no sexually compatible wild relatives in Africa, so the escape of the transgene into the environment is unlikely.

The major concern about use of Bt sweetpotato in reducing losses due to weevils in Uganda is the potential impact on non-target arthropods. Consequently, the key non-target arthropods in major sweetpotato growing regions in the country need to be identified. However, arthropods found in a given sweetpotato habitat may be influenced by the prevailing environmental conditions, the sweetpotato cropping system, the cultivars of sweetpotato and that of the association crops, and also by the agronomic and cultural practices used. In this respect, there is need to i) understand the sweetpotato production systems, especially with respect to sweetpotato weevil damage and management in sweetpotato growing regions in Uganda, ii) identify and determine the relative abundance of the target and non-target arthropods of Bt sweetpotato in the fields, iii) establish a

reference collection of arthropods in the sweetpotato cropping systems and iv) determine the key non-target arthropods on which to focus the impact studies.

Since Bt sweetpotato was only allowed into Uganda for confined screenhouse trials in 2011, Bt's impact on non-target arthropods in sweetpotato has not been studied. Recently, awareness of biosafety issues increased in Uganda through highlighting the possible effects GM crops can have (Mtui, 2012). As pointed out by Sengooba *et al*. (2009), the protocols and guide lines for risk assessment of GM crops in Uganda are still under development. Since Bt sweetpotato is still under contained trials in Uganda, the NTO study and field research on Bt sweetpotato largely contributes to focusing post-release monitoring of potential ecological impact, and possible risk assessment for future release of other Bt events in Uganda and the rest of Africa. Although Bt sweetpotato may be considered as an environmentally friendly alternative to insecticides (Ghislain *et al*., 2013), concerns have been raised that there may be adverse effects of Bt sweetpotato use on non-target organisms.

To study the potential effects of the Bt sweetpotato technology on target and non-target arthropods in sweetpotato based cropping systems, field collections of these organisms and relative abundance of target and non-target organisms in the major sweetpotato agro-ecologies, need to be established. This information is used to (i) identify groups of arthropods that may be adversely affected by Bt sweetpotato, (ii) to quantify the impact of Bt sweetpotato on these non-target species in the greenhouse, prior to field testing, and (iii) serve as a baseline for impact assessment studies to ensure the technology does not adversely affect arthropod diversity while providing weevil control.

2.7.2 Impact of insect-resistant transgenic crops on non-target organisms

The impact of insect-resistant transgenic crops on NTOs is one of the most widely discussed ecological effects. Non-target risk assessment is a process by which risks are identified and the seriousness of the risk is characterised so that decisions can be made on whether or how to proceed with the technology (Andow and Hilbeck, 2004). There are different opinions of how to assess the risk of transgenic crops, but one thing they all have in common is that possible effects must be identified. These are the ecotoxicology model, non-indigenous species model, and the ecological model

2.7.2.1 The ecotoxicology model

This model aims to evaluate the potential non-target effects of chemicals released into the environment and has been suggested for use in evaluation of non-target species effects of GM crops (Andow and Hilbeck, 2004). Universal indicator species are chosen because of their supposed sensitivity to chemical toxins, their wide availability, their ease of culture, and their genetic uniformity (Chapman, 2002). Romeis *et al*. (2008) suggested identifying indicator organisms and developing simple methods that combined suitability and cost effectiveness for ecological risk assessment under field conditions. Such species are supposed to provide information on the likely effects of the chemical on a wider range of species (Andow and Hilbeck, 2004). The most serious problem with this approach is that it is not consistent with the need for case-by-case risk assessment that considers the relevant transgene, crop plant, and environment.

In this model, the primary end point is mortality or some other acute response from short-term exposure to the chemical. These responses, however, reveal little about other ecological impacts at the population, community or ecosystem level. Although acute toxicity testing of the transgene product in the laboratory should be part of initial testing of GM crops, it is insufficient to ensure accurate decision making in risk assessment. It will also be critical to abandon the use of universal indicator species and develop a species selection process that allows risk assessment to adapt on a case-by-case basis to the particular transgene, crop plant, and environment in which the transgenic plant will be grown (Romeis *et al.*, 2011).

2.7.2.2 The ecological model

Species selection based on an ecological model as suggested by Andow and Hilbeck (2004), is case specific, depends on the transgenic crop and its cropping context, and prioritises species that could be adversely affected by the transgenic crop. Species selection follows certain steps: (i) identification and screening for appropriate functional groups of biodiversity, (ii) list and prioritise non-target species and processes for use in a selection matrix, (iii) bio-trophically mediated exposure path ways to transgenic plant and transgene products, (iv) adverse effect scenarios for bio-trophically mediated and other ecological effects, and (v) testing hypotheses and experimental designs to test for adverse effects (Romeis *et al.*, 2008).

An appropriate experimental end point when using the ecological model is generational relative fitness which comprises the relative lifetime survival and reproduction of the non-target species. Survival experiments on the species that will be exposed to transgenic plants should last through one full generation, including all the immature stages (Andow and Hilbeck, 2004). Generational relative fitness is a particularly useful end point, because it relates directly to risk. If the transgenic plant adversely affects a non-target species, its effects will come through some component of relative fitness. The use of the ecological model is the most appropriate to assess the risk of transgenic crops. Several studies have been conducted to identify priority arthropods in sweetpotato fields in Uganda. (Ames *et al.*, 1996; Smit, 1997b; Stathers *et al.*, 2005). In this ecological model priority non-target species are also identified, these will be important in determining the effect of Bt sweetpotato on these non-target species.

2.7.2.3 The need for transgenic resistance

Although sweetpotato plays an important role in the diet and food security of the population in the East African region, the crop yield remains very low as compared to other parts of the world. Its production is mainly constrained by sweetpotato weevils (*Cylas* spp.) that cause yield losses of up to 100%. The use of resistant sweetpotato cultivars is the most sustainable strategy to control sweetpotato weevil for resource poor farmers in sub-Saharan Africa. However, little success has been realised in the development of resistant cultivars because there is no known source of resistance in the available sweetpotato germplasm useful for breeding. SPW control strategies such as cultural, biological, chemical and mechanical methods, may not adequately address the problem of weevil damage experienced by smallholder farmers. Genetic engineering is potentially a more viable option that offers a means to introduce weevil resistance genes into sweetpotato. Transgenic resistance, through the use of transgenic varieties in combination with the other insect control strategies, seems to be the most effective and sustainable option.

CHAPTER THREE

EVALUATION OF BIOASSAYS FOR TESTING Bt TRANSGENIC SWEETPOTATO EVENTS AGAINST SWEETPOTATO WEEVILS

3.1 Introduction

In Africa, *Cylas puncticollis* Boheman and *C. brunneus* F. are the major pest species (Kiiza *et al.*, 2009); whereas in America and Asia *C. formicarius* is the major pest species (Smit, 1997b). All three *Cylas* species attack sweetpotato both in the field and during storage. On established plants, adult weevils attack leaves while the larvae feed on roots and stems; producing larval tunnels followed by pupal chambers. Stem damage is thought to be the main reason for yield loss, although damage to the vascular system caused by feeding, larval tunnelling and secondary rots reduce the size and number of roots substantially (Sorensen, 2009). Pest damage usually continues during storage; therefore, infested roots cannot be stored for a long time. Severe infestations or slightly damaged roots render the crop unpalatable and therefore inedible by humans due to terpenoids produced in response to weevil feeding (Stathers *et al.*, 2003b). Together with other beetle pests, *Cylas* spp. can completely destroy sweetpotato fields (Geisthardt and van Harten, 1992).

The nature of attack by sweetpotato weevils and mode of feeding render them difficult to control due to their cryptic habit which reduces the effectiveness of control (Smit *et al.*, 2001). Sex pheromones of *C. formicarius* were effective in controlling weevil populations in mass trapping trials in Taiwan (Hwang and Hung, 1991), Vietnam (Braun and van de Flieart, 1997) and Cuba (Alcazar *et al.*, 1997), but failed in Uganda (Smit *et al.*, 2001). Hence, weevil control remains top priority in sweetpotato breeding especially in countries like Uganda where yield losses often reach as high as 60-100% under heavy infestations. Despite years of intensive research in conventional plant breeding, varieties with resistance to *C. puncticollis* and *C. brunneus* are not yet available, although low levels of weevil resistance have been recently identified in cultivars (Jackson *et al.*, 2012; Muyinza *et al.*, 2012). Sweetpotato producers in Sub-Saharan Africa (SSA) are mostly small-scale,

resource-poor farmers growing the crop all year round. Hence, management strategies that have been proven successful elsewhere may not be suitable for farmers in SSA.

Weevil control could, however, be achieved by transferring the insecticidal properties of *Bacillus thuringiensis* (Bt) through genetic engineering. Genes expressing insecticidal crystal (Cry) proteins from Bt have been cloned, modified for expression in plants, and inserted into various cultivated crops (Christou *et al.*, 2006). The activity of some *cry* genes against *Cylas* spp. in sweetpotato has been demonstrated in Cuba (Moran *et al.*, 1998). In Africa, previous artificial diet bioassays found three Cry protoxins (Cry7Aa1, Cry3Ca1, and ET33-34) to be active against *C. puncticollis* and *C. brunneus* with LC_{50} values at less than 1 ppm (Ekobu *et al.*, 2010). Genes corresponding to the three proteins have been synthesized and used to transform several sweetpotato varieties with the aim of controlling sweetpotato weevils (Kreuze *et al.*, 2009). The objective of this study was to evaluate the efficacy of transgenic events with genes expressing Cry toxins, with potential for control of weevils in sweetpotato.

3.2 Materials and methods

3.2.1 Plant material

Transgenic sweetpotato events from the United States variety 'Jewel' expressing *cry* genes used in this study were developed by organogenesis (Luo *et al.*, 2006). *In vitro* sweetpotato plants were cultured on propagation medium and grown for 5-6 weeks in a growth chamber. *Agrobacterium tumefaciens* hypervirulent strain EHA105 was used for transformation. Leaf with petioles were submerged in 10 ml of bacterial infection media with the strain EHA105 harbouring different binary vectors and kept for 24hrs in the dark at 25- 27°C without agitation. Following co-culture, the explants were blotted onto sterile filter paper and then transferred onto selective regeneration media. The infected explants after 5-6 days were transferred to the same selective medium containing zeatin 0.2 mg/L. The infected explants were kept in the selective regeneration medium for 6 weeks, changing to fresh medium weekly. Regenerating shoots were harvested and transferred to sweetpotato propagation medium with cefotaxime 200 mg/L. Putative transformed shoots were tested for kanamycin resistance (*nptII* gene expression) using a protocol established for potato transformation (Ghislain, pers. Comm.). Leaf segments were then transferred onto callus inducing media. Each Petri dish contained a negative control (leaf segment

from an untransformed cv. Jewel plant) and a positive control (leaf segment from a transformed cv. Jewel plant obtained previously). These dishes were incubated in a growth chamber. Rapid detection of transformants was done by PCR analysis. Transgene insertions were analysed by Southern blot hybridisation. Out of this, a total of 10 transgenic events from 'Jewel' were made available for this study: four transgenic events with the *cry7Aa1*; three with *cry3Ca1*; and three with *ET33-34* (Table 3.1). Non-transformed 'Jewel' was used as control in all the experiments.

3.2.2 Cry protein quantification

Double antibody sandwich enzyme-linked immune-sorbent assays (DAS–ELISA) for Cry7Aa1, Cry3Ca1 and ET33-34 proteins were performed as described by Clark and Adams (1977). Plates were pre-coated with polyclonal antibodies, prepared from rabbits, against the purified proteins Cry7Aa1, Cry3Ca1 and ET33-34; and diluted (1:1,000) in carbonate buffer (pH 9.6). Samples (1 g from each root of the 10 roots) in extraction buffer (PBS buffer pH 7.4, 2% polyvinyl pyrrolidone (PVP-40), 2% non-fat milk, 0.5 ml l^{-1} Tween-20) diluted 1/10 (w/v), were added to the polystyrene microtitre plate in triplicate and were incubated overnight at 4°C.

Plates were washed four times, then the conjugated antibodies with alkaline phosphotase from bovine intestinal mucosa (BIORAD) diluted 1:1,000, was added to each well and the plate was incubated for 3 hr at 37°C. Finally, the plates were washed and p-nitrophenylphosphate diluted in substrate buffer (BIORAD) was added to each well. The absorbance of the yellow product formed was measured at 405 nm with an ELISA plate reader. A non-transformed Jewel used as a negative control was also included in the experiment. The amount of Cry protein was calculated using a standard curve for each Cry protein (Cry7Aa1, Cry3Ca1 and ET33-34), which were prepared by diluting purified proteins obtained from a previous study (Ekobu *et al*., 2010).

Table 3.1: Transgenic sweetpotato events evaluated for resistance to *C. puncticollis*

Event	Gene construct
CIP 410008.7	5′β-amy/*cry7Aa1*/3′β-amy
CIP410008.9	5′β-amy/*cry7Aa1*/3′β-amy
CIP 410008.14	5′β-amy/*cry7Aa1*/3′β-amy
CIP 410008.17	5′β-amy/*cry7Aa1*/3′β-amy
CIP 410009.12	5′SPOA1/*cry3Ca1*/3′SPOA1
CIP 410009.14	5′SPOA1/*cry3Ca1*/3′SPOA1
CIP 410009.15	5′SPOA1/*cry3Ca1*/3′SPOA1
CIP 410010.14	5′SPOA1/*ET33-34*/3′SPOA1
CIP 410010.18	5′SPOA1/*ET33-34*/3′SPOA1
CIP 410010.19	5′SPOA1/*ET33-34*/3′SPOA1

N.B. β-amy and SPOA1 refer to regulatory elements of the sweetpotato β-amylase and sporamin genes, respectively.

3.2.3 Sweetpotato weevil rearing

A sweetpotato weevil colony was established from a field collected population (about 500 insects) and maintained in the laboratory on storage roots in collapsible cages (1450C, BioQuip Products, Inc, CA) at 25±2°C and 70±10% RH.

3.2.4 Artificial diet bioassay

Transgenic and non-transgenic storage roots were harvested four months after planting. Samples were frozen at -80°C following harvest and then lyophilised until dry. Lyophilised material was ground to a fine powder using a laboratory miller and was stored at -20°C until use. Artificial diet was prepared as described by Ekobu *et al*. (2010). For each treatment, 200 ml of the diet was used. The diet + sweetpotato root powder was homogenised to give a final concentration of 8% (wt/vol) lyophilised tissue in the diet.

Formulated diets were poured equally into three 90-mm diameter sterile glass petri dishes and allowed to cool at room temperature. The solidified diet was left at 4°C until the next morning. Ten first-instar, *C. puncticollis* larvae per petri-dish were placed in small (2-3 mm in depth) burrows dug into the diet by using a pair of flat-headed forceps. The experiments were replicated three times in a completely randomised design and conducted for 15 days at 22-24°C and 70±10% RH.

Larval mortality was determined after 15 days by observing no larval movement. All surviving larvae from a particular treatment were weighed together on an electronic

balance to within 0.10 mg (Mettler, Toledo AG204); then this value was divided by the total number of larva weighed, to get average weight.

3.2.5 Whole root assay

Screenhouse-grown storage roots from transgenic and non-transgenic control were used in the assay. Roots were harvested approximately four months after planting. Ten *C. puncticollis* female adult (2-3 week-old), were placed in individual 1 litre polystyrene jars with tissue paper at the base (Stathers *et al*., 2003). Sexing of weevils was conducted by using the shape of the distal antennal segment; males have a filiform shape and females have club like shape (Smit, 1997b). Single un-infested transgenic and non-transgenic roots were introduced into each jar representing a replicate. After 24 hr, the adult females were removed. Roots were then incubated until weevils emerged. Insect counting commenced when adults started emerging. Newly emerged adults were recorded and removed daily. This was done until weevil emergence stopped. This bioassay was replicated three times, in a completely randomised design including entire events and the control. The experiment was conducted at 25±2°C and 70±10% RH and was repeated three times. In this bioassay, the total number of progeny that emerged and their median development period were derived for each replicate. The median development period was calculated as the number of days from oviposition to first emergence of progeny.

3.2.6 Root chip bioassay

Roots were sliced into thin chips (1 cm thick), and each chip had two small holes (approximately 2-3 mm deep) for introducing eggs (Vasquez and Gapasin, 1980). Eggs were obtained by exposing cultivar NASPOT 1 storage roots (a known susceptible variety) to a large number of females for 24 hr for oviposition. Egg plugs were cut from the non-transgenic cultivar and needle nosed forceps were used to gently transfer the plugs to the transgenic root chips. Root chips were placed between microscope slides. Both ends of the slides (bioassay block) were tied with rubber bands. Each event had six chips and each chip was treated as a replicate. The bioassay blocks were then placed in plastic containers. At 5 days after the eggs were deposited, root chips were examined for hatched eggs. Non-viable eggs or rotten root sections were discarded. Root chips were dissected to locate larvae and pupae. Root chips were changed, when there were signs of contamination or drying. Larval and pupal survival was recorded. Bioassays were replicated four times in a completely randomised design at 25± 2°C and 70±10% RH.

3.2.7 Small root egg-plug bioassay

Twenty four hour old eggs were introduced into small transgenic roots measuring 3 cm diameter, as adopted from Mao *et al*. (2001) . Roots were provided with initial holes for insertion of eggs. Eggs were obtained from cv. NASPOT 1. Needle nosed forceps were used to gently transfer egg plugs to transgenic roots. Each small root was infested with five egg plugs. After placing the egg plugs into the holes, they were sealed with masking tape. Roots were placed in 1 litre polystyrene jars with tissue paper at the base for incubation. Five small roots were used per event and control. Each small root was treated as a replicate. At 5 days after the eggs were deposited, small roots were examined to determine if eggs had hatched and larvae had burrowed into the sweetpotato. Unviable eggs were not considered in this experiment. At 21 days after infestation, root sections were dissected to locate pupae. Larval and pupal survivals were recorded. Roots from all events and controls were tested in a completely randomised experimental design at 25±2°C and 70±10% RH.

3.2.8 Statistical analysis

Total mortality, percent mortality and percent pupation of *C. puncticollis* were calculated on artificial diet incorporated with the transgenic root powder. In whole sweetpotato root assay the number of adult weevils that emerged was recorded, and median weevil development period was calculated. Pupation rate was recorded in the small sweetpotato root infested with 24 hr egg plugs. All percentage data were transformed using the Arcsine square root before analysis. All data collected were subjected to analysis of variance at $p<0.05$ using GenStat 14th Edition software, version 14.1.0.5943 (VSN International Ltd, 2011).

3.3 Results

3.3.1 Cry protein quantification

DAS-ELISA analysis showed differences in Cry protein concentration among the sweetpotato events (Figure 3.1). Two events carrying ET33-34 had the highest protein concentration among all events tested. CIP410010.18 had a concentration of 0.394 µg g^{-1} of root fresh weight followed by CIP410010.19 with 0.11 µg g^{-1} of root fresh weight. The rest of the tested events carrying the other proteins had concentration below 0.1 µg g^{-1} of

root fresh weight. Cry3Ca1 proteins were not detected in all the three events that were tested.

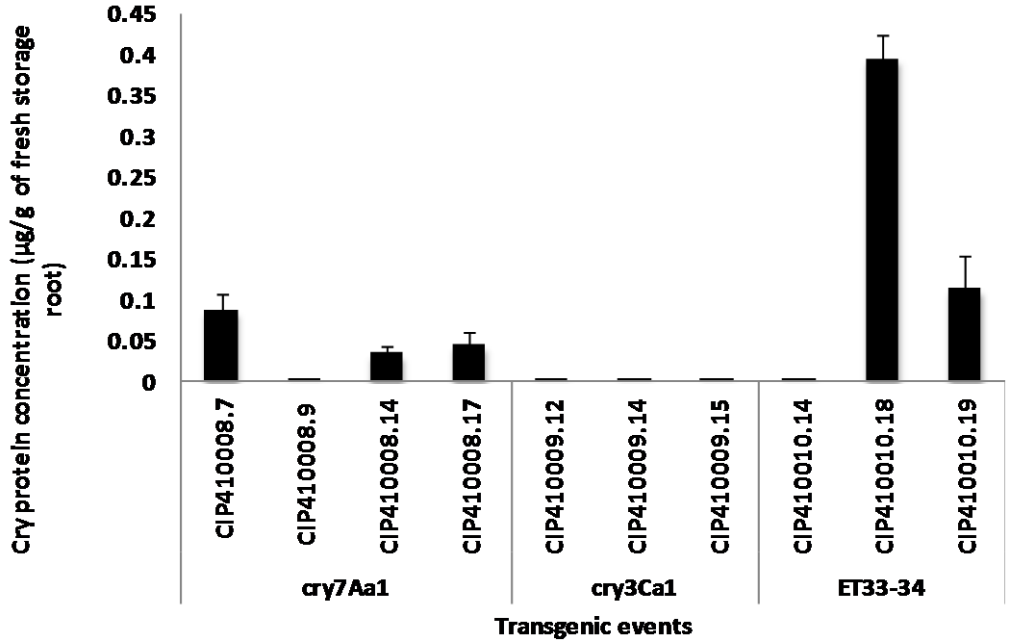


Figure 3.1. Concentration of Cry7Aa1, Cry3Ca1 and ET33-34 proteins in storage roots of different transgenic events based on DAS-ELISA assay. Protein concentration is expressed in µg g^{-1} fresh root tissue.

3.3.2 Artificial diet bioassay incorporated with transgenic root powder

Mortality of larvae in artificial diet with transgenic sweetpotato powder did not vary from that of the control, reared on a toxin-free diet ($p = 0.28$; Table 3.2). There was no adverse effect of powder from the eight transgenic events tested on pupation of *C. puncticollis.* Neither were there significant differences in pupation rate ($p = 0.86$; Table 3.2). Larvae were delicate to handle and easily died during placement on diet. In general, the larvae that fed on the artificial diet appeared healthy and active in all treatments. The average weight of *C. puncticollis* larvae ranged between 1.0-1.3 g in all experiments 15 days post incubation (Table 3.2).

Table 3.2: Mortality and development of *C. puncticollis* on artificial diet with lyophilised root powder of transgenic and non-transgenic sweetpotato genotypes

Genotype	**Mortality rate (% ±SE)**	**Pupation rate (% ±SE)**	**Larval weight (mg)**
Transgenic:			
CIP410008.7	26.7(±6.7)	23.61 (±6.05)	1.1
CIP410008.9	23.3(±3.3)	30.36(±3.72)	1.2
CIP410008.17	16.7(±3.3	31.94(±3.68)	1.0
CIP410009.5	30.0(±5.8)	28.97(±2.41)	1.2
CIP410009.12	16.7(±3.3)	36.11(±7.35)	1.2
CIP410010.14	26.7(±3.3)	27.38(±1.19)	1.3
CIP410010.18	30.0(±5.8)	24.21(±5.51)	1.0
CIP410010.19	30.0(±5.8)	29.56(±8.96)	1.2
Non-transgenic:			
Jewel (control)	23.33(±3.3)	26.19(±7.31)	1.1

There was no significant difference in mortality rate and pupation rate ($p > 0.05$) between the transgenic and non-transgenic root powder in artificial diets.

3.3.3 Whole root bioassays

Seven transgenic events evaluated under laboratory conditions displayed no significant variation in resistance to *C. puncticollis* as compared to the non-transgenic control (Table 3.3). Visual observation revealed that the adult weevils fed in the same way on transgenic roots as on non-transgenic roots. When roots were incubated after oviposition by female adults, new adult weevils began to emerge 23-24 days post infestation in all events. Peak emergence occurred 26-31 days for all the experimental sweetpotato genotypes. Adult emergence was recorded 40 days after set-up. There was no significant difference ($p = 0.94$) between the transgenic and non-transgenic storage roots in the total number of weevils that emerged (Table 3.3). The median development time on Cry-containing plant material was not significant ($p = 0.51$).

Table 3.3: Effect of transgenic and non-transgenic sweetpotato roots on *C. puncticollis* emergence and development

Genotype	Number of weevil adults emerged (±SE)	Median development period (days) (±SE)
Transgenic:		
CIP 410008.7	75.3±3.18	28.0±0.0
CIP 410008.14	76.3±4.37	27.0±0.6
CIP 410009.12	79.0±4.04	27.0±0.6
CIP 410009.15	77.3±7.22	27.7±0.9
CIP 410010.14	82.0±4.16	26.7±0.3
CIP 410010.18	74.0±10.12	27.7±0.3
CIP 410010.19	71.3±5.17	27.3±0.3
Non-transgenic:		
Jewel (Control)	77.7±4.67	28.0±0.6

There was no significant difference between number of weevil adults emerged and median development period ($p > 0.05$) between the transgenic events and the control.

3.3.4 Root chip bioassay

Mortality rates of up to 100% were recorded in some replicates for weevils on root chips making it difficult to compare statistically. In addition, root chips were prone to damage by fungi and desiccation. Mechanical injury inflicted on larvae during examination when the larvae had to be excavated from tunnels of dry root chips and transferred to fresh root chips caused up to 70% mortality. Mortality was highest during the first larval instar and reduced as the larvae matured. No mortality was observed once the insect reached pupation stage. Very few weevil larvae (up to 30%), which pupated emerged as adults.

3.3.5 Small root egg-plug bioassay

Transgenic events had no significant effect on larval survival and pupal development. Visual observation after 21 days showed that the weevils were not affected by Cry proteins being expressed in roots. Some transgenic lines had higher pupation rate than the non-transgenic control. However, there were no significant differences in the pupation rates between the transgenic events and the non-transgenic Jewel ($F = 0.56$; $df = 39$; $p = 0.76$). Highest pupation rate of 96% was observed on event CIP410009.12 and low pupation rate (82%) was observed in event CIP410008.14 (Table 3.4)

Table 3.4: Pupation rate of *C. puncticollis* at 21 days in the small root egg plug bioassay

Genotype	Pupation rate (%) (±SE)
Transgenic:	
CIP 410008.7	92±4.9
CIP 410008.14	82 ±7.8
CIP 410009.12	96 ±4.0
CIP 410009.15	87 ±8.3
CIP 410010.14	95 ± 5.0
CIP 410010.18	86 ± 5.8
CIP 410010.19	91 ± 5.6
Non-Transgenic:	
Jewel (Control)	88 ± 8.0

N.B. There was no significant difference in pupation rate ($p > 0.05$) between the transgenic events and the control.

3.4 Discussion

Although the *cry* genes were expressed under the control of tissue specific promoters, the Cry proteins concentration was low in the roots, and below detection levels like in the case of Cry3Ca1 events (Figure 3.1). Sporamin and β-amylase promoters could not confer high levels of Cry protein accumulation in sweetpotato roots despite reports of enhanced accumulation of other proteins in potato (Hong *et al.*, 2008). When Cry proteins were quantified in the storage roots, they were observed to be less than 0.5 µg g^{-1} of fresh storage root flesh. Despite, the presence of the *cry* genes in some events, the Cry protein levels were too low to be detected in the roots. There is a likelihood of very low or total lack of expression. The low or failure of gene expression was probably due to the fact that the sweetpotato root is a low protein storage organ (OECD, 2010).

The artificial diet bioassay was relatively fast; it needed only 15 days to observe the effects of Cry proteins on sweetpotato weevil. In addition, this technique allowed for relatively easy testing of small quantities of tissue. This would be especially important when test material is limiting (from a single transgenic event). However, the reagents used for making the artificial diet are relatively expensive and the diet does not adequately account for sweetpotato/Bt interactions. When insects are not handled delicately especially the 1st instar larvae using proper forceps or fine brushes, it is likely that they could be injured and the bioassay effects could be magnified. The effectiveness of Cry protein in the diet could be more difficult to observe than in storage roots because the protein is diluted by about three-fold (80 g in 1 litre of diet compared to about 250 to 300 g dry

matter in 1 Kg of storage roots according to OECD (2010). Furthermore, due to practical constraints to maintain the media liquid before pouring in petri-dishes, the root powder had to be added at higher temperature (55 °C), which could have inactivated the Cry protein unlike when adding purified Cry protein at 50 °C previously described by Ekobu *et al*. (2010).

The other methods tested used actual fresh root tissue as compared to lyophilised tissue. These methods had an advantage that the weevils were exposed to the root with the actual amount of Cry protein that will be expressed under certain field conditions. In addition, plant tissues are likely to contain natural defensive compounds, which may act additively or synergistically with the Cry proteins and increase resistance to the pests (Coombs *et al.*, 2005). For instance, the whole root bioassay described in this study, has significant advantages when it comes to handling and contamination as compared to all other bioassays. This method, however, does not allow for determining larval mortality directly. It concentrates on the number of weevils that emerge after infestation and the mean development days of the weevil to establish the level of resistance of the events. This is based on the assumption that a few insect progenies would emerge out of a resistant genotype and insect progeny development would take a longer time in a resistant than in a susceptible genotype.

Adults which fed and oviposited on transgenic roots provided no evidence of non-preference to feed and oviposit on transgenic roots. This could be due to the fact that the adults were not sensitive to the Cry proteins expressed in sweetpotato. Even though no indication of a feeding deterrent behaviour was observed, this aspect could be given more attention, as reduced feeding and decreased food utilisation could result in reduced fitness leading to population reduction.

The sweetpotato root chip bioassay facilitated observations of the probing activity and other aspects of the weevil's behaviour within the bioassay block other than mortality. Nonetheless, this method was not suitable because larval mortality was too excessive to give reliable results on efficacy of transgenic roots. The high mortality in this study was primarily due to high levels of fungal contamination observed and also the injury inflicted on the weevil during changing of the root chips. Vasquez and Gapasin (1980) also

reported high mortality of the weevil due to injury inflicted to the insect during rearing of the sweetpotato weevil on the bioassay blocks.

The small root egg-plug bioassay was an improved version of the root chip method to overcome the above mentioned disadvantages of the root chip bioassay. Escapes and damage of weevil larvae were reduced due to no transfers of the larvae to the new roots and also the contamination by fungi and bacteria was reduced. The eggs used in this technique hatched and larvae went directly into the root for the whole incubation period and developed normally with no hindrance to development. In addition, a major advantage of this technique was the ability to test the efficacy of selected events focusing on mortality and sub lethal effects of the Cry protein to the weevil. This bioassay was suitable for evaluating resistance of transgenic sweetpotato roots to sweetpotato weevil because the number of eggs infested per root was known and the number which hatched and fed on the root flesh would easily be noticed. Additionally, injury inflicted to the insect is reduced, and the weevil is exposed to higher levels of Cry protein than in artificial diet bioassays.

An ideal bioassay method, however, is one that is fast, labour efficient, and closely correlated with field control (Perez *et al*., 1997). According to these criteria, small root-egg plug bioassay would be preferable because it is easier to get as many eggs as possible and infest the roots, than the whole root bioassay where a large number of females need to be sexed for the experiment for oviposition on each experimental root. The whole root bioassay, nevertheless, is more suitable for testing a small number of events where a large number storage roots required can be easily handled to reach statistical significance.

Overall, these bioassay methods revealed no significant activity of the three Cry proteins against *C. puncticollis* when transformed sweetpotato storage roots and root powder were used. This lack of efficacy was due to either a low level of accumulation or inactive form of the Cry proteins in these transgenic events (Figure 3.1). For the Cry7Aa1 and Cry3Ca1 events, the quantities were too low as compared to the LC_{50} reported by Ekobu *et al*. (2010) to expect control of the weevil. However, for ET33-34, at least one event was close to the LC_{50} (0.394 µg g^{-1} of fresh storage root flesh) previously defined by Ekobu *et al*. (2010) and would be expected to show at least partial activity. Thus, the low accumulation cannot be the sole reason for lack of efficacy. However, the comparison with the LC_{50} is inappropriate because the artificial diet used three fold less dry matter, stressed larva, and

pro-toxins which are more stable and typically less toxic than truncated protein. Although several reasons can be responsible for lack of activity of the Cry proteins, information from the literature is scarce concerning expression of Bt Cry proteins in storage roots. In sweetpotato, the accumulation of the Cry3A1 was estimated to be 0.5 to 1.5 µg g^{-1} fresh storage root flesh (Moran *et al*., 1998). These authors observed partial resistance in the field using *Cylas formicarius*. Hence, more research is needed on additional transgenic events to determine whether the Cry proteins in sweetpotato roots are functional or alternatively new gene constructs need to be tested in order to lead to higher accumulation of Cry proteins in the storage root of sweetpotato for effective weevil control.

CHAPTER FOUR

TRANSGENIC BREEDING OF SWEETPOTATO FOR ENHANCING WEEVIL RESISTANCE USING A TRANSGENIC EVENT WITH *cry7Aa1* GENE AND CONVENTIONALLY BRED CULTIVARS

4.1 Introduction

Although traditional breeding has significantly contributed to trait improvement in sweetpotato in the last several decades, producing new varieties with desirable traits remains difficult due to the high levels of male sterility, self and interspecific incompatibility, and hexaploid nature of the sweetpotato, which results in the strong segregation of hybrid progenies and loss of many valuable traits. Progress in breeding for weevil-resistant cultivars has been slow due to inconsistent resistance displayed by genotypes across different areas (Stevenson *et al.*, 2009). Low levels of weevil resistance have recently been identified in some cultivars (Jackson *et al.*, 2012; Muyinza *et al.*, 2012). The option of using genetic engineering using insecticidal genes from *Bacillus thuringiensis* (Bt) could provide breeders or even the resource poor farmers with a new source of weevil-resistance. Introduction of novel insecticidal genes by transgenic approach is a proven technology to increase crop productivity in crop plants (Falco and Silva-Filho, 2003; Christou *et al.*, 2006; James, 2012).

To date, many plants have been transformed with different *cry* genes for insect resistance which include commercial varieties from cotton, and maize (James, 2012). The activity of some *cry* genes against *Cylas* spp. in sweetpotato has been demonstrated in Cuba (Moran *et al.*, 1998). Recently, sweetpotato has been engineered with a synthetic *cry7Aa1* gene for sweetpotato weevil control (Kreuze *et al.*, 2009; Ghislain *et al.*, 2013). However, the effect of *cry7Aa1* genes expressed in sweetpotato events on *Cylas* spp. has been found to be low in the first transgenic plants tested (Rukarwa *et al.*, 2013). Hence, the need to combine transgenic and natural host resistance so as to observe additive effect or synergism in the sweetpotato roots for weevil control. Combining natural host plant resistance and engineered resistance conferred by a Bt gene in sweetpotato breeding against sweetpotato weevil may increase the efficacy and stability of resistance.

Conventional breeding using a transgenic event as a parent, referred to as transgenic breeding, is common for crops such as maize, soybean and cotton but rare for clonally-propagated crops (Wu *et al.*, 2002; Yong *et al.*, 2006; Arvinth *et al.*, 2009). Successful transmission and stable expression of transgene to the progeny is important in breeding programmes involving transgenics for further improvement by hybridisation and clonal selection. Genotype-dependent genetic transformation in sweetpotato is a major barrier to obtaining superior cultivars with the desired transgenic trait. In addition, genotypes currently amenable to genetic transformation are not necessarily adapted to growing conditions in SSA. The introduction of transgenic parents into the breeding programme would generate new varieties with the genetically engineered trait.

Successful application of genetic transformation of any plant involves not only the production of primary transformants showing stable expression of inserted genes but also the inheritance and continued expression of those genes in subsequent generations. Various factors have been reported to affect expression and inheritance of transgenes including number of copies of the transgene and the gene insertion site (Kohli *et al.*, 2010). For breeding purposes, more information is needed for early selections of transformants that consistently express transgenes at high levels and reliably transmit high levels of expression to their progeny. Changes in gene expression in progeny in development of commercial cultivars could lead to problems in variety registration, seed certification and value for cultivation and use. Loss of gene expression would inevitably reduce the value of a cultivar to the end users. Studies on *cry* gene transmission to the sexual progeny have never been conducted in sweetpotato. In the present study, inheritance and expression of the transgene in the progeny was investigated and the possibility to enhance weevil resistance by combining low level of resistance from conventional and transgenic sources was assessed under the current Ugandan biosafety regulation.

4.2 Materials and methods

4.2.1 Parental genotypes

The transgenic event from Cv. Jewel expressing *cry7Aa1* gene for resistance to sweetpotato weevil was produced as described by Luo *et al*. (2006). The cultivar was

transformed using *Agrobacterium tumefaciens* strain EHA105 with the pCIP78 plasmid construct containing the *cry7Aa1* gene under the control of the promoter including the 5' and 3' untranslated and the poly-adenylation signal sequences of the sweetpotato *β*-amylase gene. The cassette also contained the *nptII* as a marker gene under the control of the Nopaline synthase (*Pnos*) promoter (Figure 4.1). CIP410008.7 was selected for this study after undergoing several cycles of vegetative propagation and phenotypic evaluation. Transgene integration patterns and copy number for this event were also determined from Southern Blot analysis and transgene expression was determined by DAS-ELISA.

The event CIP410008.7 was used as a female parent in crosses with Ugandan Cvs New Kawogo, Tanzania and NASPOT 1. Tanzania and New Kawogo are farmer preferred cultivars grown in Uganda because of their wide adaptability and high dry matter content (Mwanga *et al.*, 2001). NASPOT 1 is also a dominant Cv. in the central and eastern parts of Uganda because of its high dry matter content and moderate resistance to sweetpotato virus disease (Mwanga *et al.*, 2003).

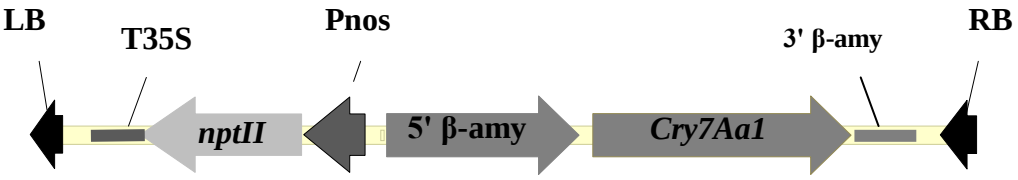


Figure 4.1. Expression cassette of event CIP410008.7

4.2.2 Progeny generation and management

Crossing the sweetpotato parents was done in a Biosafety Level II screenhouse at the National Crops Resources Research Institute (NaCRRI) as a biosafety regulatory requirement in Uganda. The transgenic Jewel was used as female parent and the Ugandan cvs as male parents in bi-parental crossings. In this experiment the transgenic plants were maintained in a screenhouse and subsequently grafted to *Ipomoea setosa* to induce flowering. Pollen of the male parents was collected from a field crossing block outside the screenhouse. Hand pollination of event CIP410008.7 was carried out in the morning before 10.00 am to minimise the rate of flower abortion. Four to five weeks after pollination the ripe seed capsules were harvested and dried for a week. Seeds were removed from capsules and soaked in concentrated sulphuric acid (98% H_2SO_4) for 45

minutes to break dormancy. This was followed by rinsing 4-5 times with water for 5 minutes and subsequently sown in nursery boxes.

4.2.3 PCR analysis of *cry7Aa1* gene

Genomic DNA was isolated from all sweetpotato leaf samples using the CTAB method (Stacey *et al.*, 2000). PCR amplification of the 608 bp DNA fragment of the *cry7Aa1* gene was carried out in a Multigene Thermocycler (Labnet International, Inc. NJ, USA) using 5′-ACAACTCATCACCATACCAAAC-3′ and 5′-AAGAGCAAGATGCAAGTTTG-3′ as forward and reverse primers, respectively. All the PCR experiments took place in a total volume of 25 μl containing 50 ng of total plant DNA made as follows: 12.5 μl of Ready mix Taq® DNA polymerase (Sigma-Aldrich, Poole, UK) and primer F (0.5 μl) and primer R (0.5 μl) and adjusted to 25 µl with nuclease free water. PCR conditions were as follows: initial denaturation of DNA at 93^{o}C for 2 minutes and then amplified through 35 thermal cycles of 93^{o}C for 15 seconds, 55^{o}C for 30 seconds, 72^{o}C for two minutes and ending by a final extension step at 72^{o}C for 7 minutes. The PCR amplicons were separated by electrophoresis on 1% agarose gel in Tris-EDTA buffer stained with ethidium bromide and visualised under UV light. The presence or absence of the transgenes in the hybrids was used to confirm the transgenic and non-transgenic progenies.

4.2.4 Cry protein quantification

Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was carried out for detection and quantification of Cry7Aa1 protein in root tissue samples of transgenic progenies as described by Clark and Adams (1977). ELISA microtitre plates were precoated with polyclonal antibodies prepared from rabbits to the protein, Cry7Aa1 and diluted (1:1000) in carbonate buffer (pH 9.6). The F_1 root tissue samples in extraction buffer (PBS buffer pH 7.4, 2% polyvinyl pyrrolidone (PVP-40), 2% non-fat milk, 0.5 ml/l Tween-20) diluted 1/10 (w/v) were added to the polystyrene microtitre plate in triplicate and were incubated overnight at 4°C. Each sample extraction was repeated three times. The plate was washed 3 times, then the conjugated antibodies with alkaline phosphotase from bovine intestinal mucosa (BIO-RAD, Hercules, CA) diluted 1:1000 was added to each well and the plate was incubated for 3 hr at 37 °C. Finally, the plate was washed and the substrate p-nitrophenylphosphate diluted in substrate buffer (BIO-RAD, Hercules, CA) was added to each well. The absorbance of the yellow product formed was measured at 405 nm with an ELISA plate reader (BIO-RAD, Hercules, CA). A non-transformed Jewel

used as a negative control was also included in the experiment. The amount of Bt protein was calculated using a standard curve to Cry7Aa1, which was prepared by diluting purified Cry7Aa1 proteins.

4.2.5 Insect bioassay

Insect eggs used in this study were obtained by exposing cultivar NASPOT 1 storage roots (a known susceptible variety) to a large number of females for 24 hrs for oviposition. The egg plugs were cut from the non-transgenic cultivar and gently transferred using a needle nosed pair of forceps to the F_1 transgenic roots and their parents. This method was adopted from Mao *et al*. (2001) with some modifications. The roots were punched with initial holes for entry of the eggs. Each root was infested with five egg plugs. After placing the egg plugs into the holes, they were sealed with masking tape. The roots were placed in 1 litre polystyrene jars with tissue paper at the base for incubation. Five roots were used per F_1 progeny and their parents. Each root was treated as a replicate. At 5 days after the eggs were deposited, the roots were examined to determine if the eggs had hatched and the larvae had burrowed into the sweetpotato. At 21 days after infestation, root sections were dissected to locate pupae. Pupation rates were recorded. Roots from all events and controls were tested in a completely randomised experimental design at 25±2°C and 70±10% RH.

4.2.6 Statistical analysis

The segregation data were analysed by the χ^2 test at $P < 0.05$ to determine if the observed segregation ratios of *cry7Aa1* fitted the expected Mendelian 1:1 phenotypic ratio. ELISA data were subjected to analysis of variance at $p<0.05$ using GenStat 14th Edition (VSN International Ltd, 2011). In the insect bioassay, pupation rate was recorded and percentage data were transformed using the Arcsine square root before analysis and were subjected to analysis of variance at 5% level of significance using GenStat.

4.3 Results

4.3.1 Sweetpotato progeny development

In this study, all parental genotypes (transgenic and non- transgenic) failed to flower under screenhouse conditions five months after planting. Flowering in the transgenic parent was only achieved after grafting to the wild relative *Ipomoea setosa* (Figure 4.2A). The Ugandan cultivars, however, failed to flower even after grafting to *I. setosa*; pollen was

obtained from a field crossing block. Pollination was done on fully bloomed flowers (Figure 4.2B). After pollination the initial fruit grew and produced round shaped green capsules with a diameter of 1-2 cm (Figure 4.2C). Seeds that were removed from the capsules were dark brown, small with a diameter of 3-4 mm (Figure 4.2D).

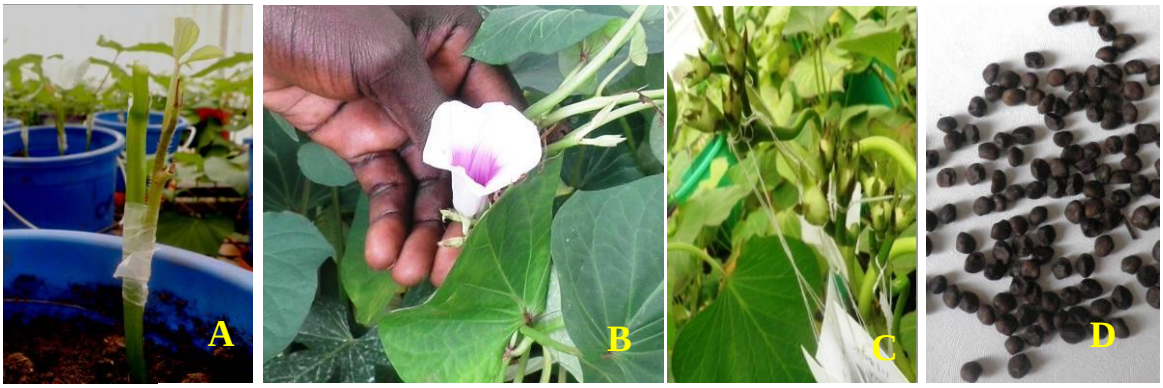


Figure 4.2. Sweetpotato breeding process (**A**) sweetpotato scion grafted on *I. setosa* root stock; (**B**) sweetpotato fully bloomed flower (**C**); capsules of sweetpotato produced from the crosses; (**D**) sweetpotato seed.

The crosses between event CIP410008.7 and the three Ugandan cultivars produced varying numbers of seed capsules and seeds. The number of capsules produced from a cross between CIP410008.7 and New Kawogo were higher than NASPOT 1 and Tanzania. The average number of seeds per capsule ranged from 1.10 to 1.68 (Table 4.1). Not all sweetpotato flowers that were pollinated produced capsules that could be harvested. When the transgenic event was crossed with the pollen from the Ugandan cultivars, most flowers aborted. Practically all crosses produced much less than the potential four seeds per capsule. Each cross produced 1-2 seeds per capsule. After scarification with sulphuric acid to break the seed dormancy and floating in water, all the seeds planted germinated. Seeds that were light or deformed were discarded.

Table 4.1: Sweetpotato capsule and seed production from crosses between CIP410008.7 and three Ugandan cultivars

Family	Number of capsules collected	Total seeds collected	Mean number of seeds per capsule
CIP 410008.7 × NASPOT 1	13	19	1.46
CIP 410008.7 × New Kawogo	52	57	1.10
CIP 410008.7 × Tanzania	19	32	1.68

4.3.2 Segregation of *cry* gene

The F_1 progenies were evaluated using PCR to check the segregation of the weevil resistance gene. PCR analysis of *cry7Aa1* transgene using the gene-specific primers in samples of progenies from crosses between CIP410008.7 and Ugandan cultivars is shown in Figure 4.3. In some sweetpotato progeny samples evaluated, the amplification produced a DNA band sized 608 bp and some did not show any amplification.

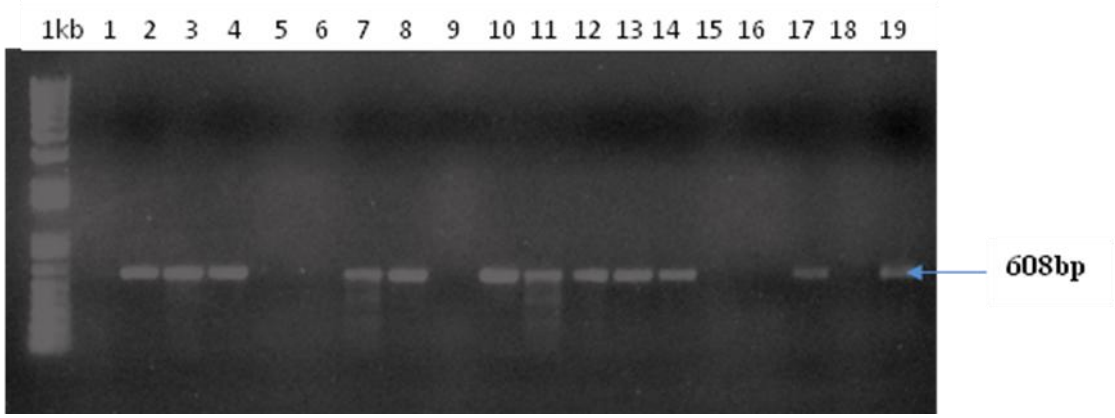


Figure 4.3. PCR analysis for the F_1 plants resulting from crosses between CIP410008.7 and Ugandan cultivars. The pair of primers used amplified the *cry7Aa1* gene in plants yielding a 608-bp fragment**. 1 kb ladder, 1-17** plant numbers, negative control **(18)** and positive control **(19).**

In all the 86 plants tested, at least 50% of progenies in each of the three families carried the *cry7Aa1* gene. Crosses of CIP410008.7 × New Kawogo, CIP410008.7 × Tanzania and CIP410008.7 × NASPOT 1 produced 25, 13 and 8 progenies carrying the *cry* gene derived from the transgenic parent, respectively (Table 4.2). Based on the Chi square (χ^2) test of the three families, the segregation pattern obtained was in accordance to the expected Mendelian segregation ratio of 1:1 (transgenic to non-transgenic plants).

Table 4.2: Segregation of *cry7Aa1* gene based on PCR analysis of the F_1 progenies of sweetpotato.

Family	Number tested	Observed negative	Observed positive	χ^2 (1 : 1)	P-value
CIP 410008.7 × NASPOT 1	18	10	8	0.22	0.637
CIP 410008.7 × New Kawogo	53	28	25	0.17	0.680
CIP 410008.7 × Tanzania	25	12	13	0.04	0.841

4.3.3 Cry protein quantification in progeny

To confirm PCR results, as well as to determine whether the *cry7Aa1* transgene was expressed in the sexual hybrids, DAS-ELISA was performed to examine Cry7Aa1 protein accumulation in roots from Cry7Aa1 positive progeny. There was no significant difference *in cry7Aa1* transgene expression levels ($p>0.05$) observed in the offspring produced with mean Cry protein accumulation values ranging from 0.12-0.20 µg Cry protein g^{-1} root tissue (Figure 4.4). These values are not significantly different from the Cry protein accumulation of 0.163 µg Cry protein g^{-1} root tissue seen in the transgenic parental control, CIP41008.7, which was included in the assay. The inclusion of positive controls of known Cry protein concentration allowed a standard curve to be calculated to which sample values were compared. Although there was variation in the actual Cry protein accumulation values among progeny, expression levels in this study was low.

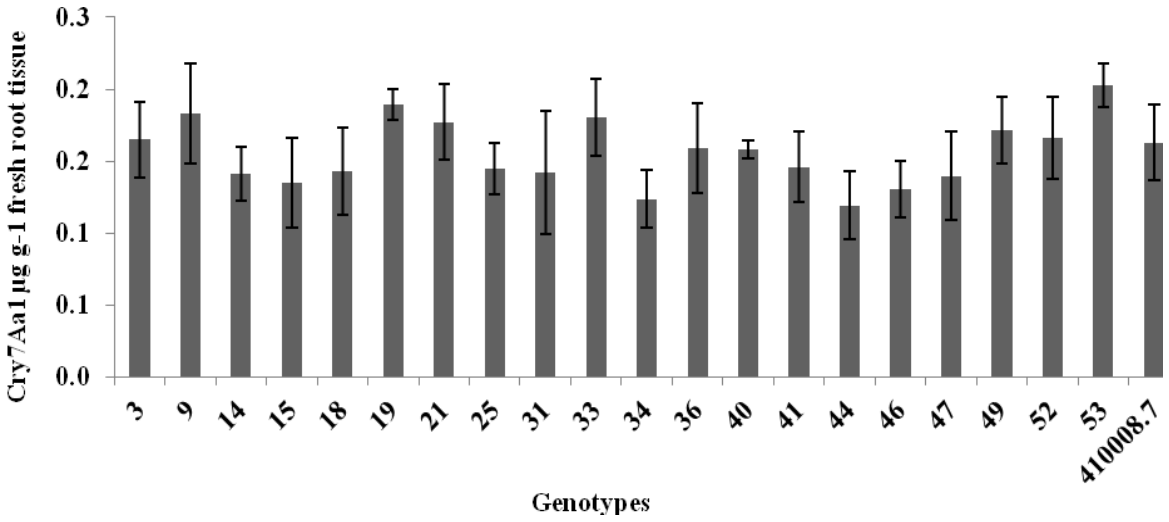


Figure 4.4. Cry protein concentration in plant tissue of F_1s derived from the cross between CIP410008.7 × New Kawogo.

4.3.4 Insect bioassay

In the bioassays, the transgenic progeny and its parents had no significant effect on larval survival and pupal development. Visual observation after 21 days showed that the weevils were not affected by Cry proteins expressed in roots. Percent pupation varied considerably, on all treatments, but was not significant ($p = 0.35$). Maximum pupation of 100% was observed in one of the tested progenies JNK18 (Table 4.3).

Table 4.3: Pupation rate of *C. puncticollis* at 21 days in the small root egg weevil bioassay using transgenic progeny and their parents.

Genotype	Pupation rate (±SEM)
JNK 3	84(±7.48)
JNK 9	84(±4.00)
JNK 14	76(±7.48)
JNK 15	80(±6.32)
JNK18	100(±0.00)
JNK 19	80(±6.32)
JNK 21	84(±7.48)
JNK 25	92(±4.90)
JNK 31	76(±4.00)
JNK 33	80(±6.32)
JNK 34	76(±7.48)
JNK 36	80(±6.32)
JNK 40	84(±7.48)
JNK 41	76(±4.00)
JNK 44	80(±8.94)
JNK 46	84(±7.48)
JNK 47	72(±8.00)
JNK49	80(±6.32)
JNK52	88(±4.90)
JNK53	76(±7.48)
CIP41008.7	80(±6.32)
New Kawogo	76(±4.00)

4.4 Discussion

Transgenic sweetpotato expressing Cry proteins were allowed into Uganda sorely for contained screenhouse trials. The Biosafety regulation in Uganda requires strict containment of transgenic events until these are fully evaluated for potential risks on the human health and the environment, at which stage regulatory authorities can deregulate and authorise their planting for commercial purposes. The containment measures are established to prevent gene flow between transgenic and non-transgenic plants. As a result all experiments were carried out in a totally enclosed facility with no control of the temperature and relative humidity. This had some implications on the success of sweetpotato transgenic breeding under screenhouse.

The failure of parental genotypes to flower under screenhouse environment observed in this study was probably due to exposure to unfavourable environmental growing conditions. Usually the success of flowering initiation and flower development is

influenced by environmental conditions such as light intensity, relative humidity and temperature (Rossel *et al.*, 2008). A photoperiod of 11.5 hr day length or less, temperatures of 20-25°C and relative humidity of 75% usually promote flowering in sweetpotato. Similarly, some of the flowers aborted probably due to the environmental conditions to which the parental genotypes were exposed. The feasibility of transgenic breeding under containment is difficult; one way which is practical in the crossing of sweetpotato is in the field. For this reason the transgenic parent should first be approved and put in a field crossing block so as to get maximum flowering. For cultivar purity purposes, isolation in time and space should always be observed in the field.

The low seed numbers obtained in the crosses was probably due to various degenerative processes which occurred during the flower and ovule development stage that resulted in low capacities of the capsule and seed formation. Serious physiological problems, occurring primarily as post pollen barriers to fertility, often impede seed production in sweetpotato. Problems of incompatibility and sterility impede controlled pollination in this crop. Besides difficulties in flower setting in sweetpotato, the flower that developed readily formed seed upon fertilisation despite extensive previous reports of complex incompatibilities and sterility mechanisms (Murata and Matsuda, 2003). The polyploidy nature of sweetpotato (2n = 6x = 90) and numerous mating incompatibilities, make crossing this crop difficult (Murata and Matsuda, 2003).

Sweetpotato is considered an autohexaploid with hexasomic inheritance (Kumagai *et al.*, 1990). The segregation pattern of traits in sweetpotato is more complex because there more than two homologous chromosomes that can pair during meiosis (Kumagai *et al.*, 1990). A transgene present in just one parent is expected to segregate in a 1:1 ratio if present as a single copy (simplex) or multiple copies inserted at a single locus or a 4:1 ratio if present as a double copy (duplex) at different loci. Southern blot analysis showed that the *cry* gene was integrated as a single copy in the genome of transgenic event CIP410008.7. The *cry* gene dominant allele controlling the resistance was expressed as a simplex configuration (Rrrrrr) in the genome of transgenic sweetpotato. The Ugandan cultivars that had no gene had an allellic composition at the locus as rrrrrrr which is recessive. The presence of the *cry* gene in this study followed a simplex hexasomic model of inheritance. Similar results were reported by Okada *et al*. (2002), who evaluated the

offspring from crosses between transgenic and non-transgenic sweetpotato and concluded that *hpt* and *CP* gene were transmitted in a Mendelian pattern.

The Cry protein expression values ranged from 0.12 to 0.20 µg Cry protein g^{-1} root tissue, and were not statistically different among the F_1 progeny. However, there are several proposed factors which may affect the expression of a foreign gene. Dunsmuir *et al*. (1989) described a source of variation known as a position effect, in which the expression of an introduced gene is affected by its inserted location in the existing plant genome. Somaclonal variation has also been attributed to variation in transgene expression levels. The pressures exerted by callus growth and regeneration in tissue culture often alter plant development which could affect the performance of a transgene directly or indirectly as in the methylation of a promoter region. However, since the hybrid progeny examined in this study are F_1 plants derived from a parental primary transformant, neither of these factors would be expected to affect their expression. All hybrid progeny inherited the same transgene from the parent (CIP410008.7), and thus the most notable source of variation from a plant breeding standpoint would come from meiotic recombination.

The mean Cry7Aa1 accumulation levels from the individual progenies in this study indicate that the *cry7Aa*1 transgene is stably expressed in the hybrid progeny that harbour the transgene. This is not unusual, as stable transgene expression in sexual hybrids has been reported in many instances. In a study examining inheritance and expression of neo and 30gusA genes in transgenic rice, transgene expression did not significantly differ within hybrid progeny groups of generations T2 and T3 (Peng *et al.*, 1995). Another study involving transgenic rice showed that *cry1Ab* transgene expression was quantitatively stable through six sexual generations (Wu *et al.*, 2002). Fearing *et al*. (1997) reported that expression of Cry1Ab protein was shown to be stable over four backcross generations in a study examining protein concentrations in both leaves and pollen of transgenic maize. In recent years, Choi *et al*. (2009) also reported that expression of both *uidA* and *sgfp* (S65T) in barley did not significantly differ within hybrid progeny groups of generations F_2, F_3 and F_4 progenies. Because of these examples, the protein expression seen in this study is not unique to the crosses involved.

The insect bioassay revealed no significant activity of the Cry protein against *C. puncticollis* when transgenic progeny and the parental genotypes were compared. This

lack of efficacy was due probably to a low level of accumulation of the Cry protein observed in the transgenic parental genotype (Rukarwa *et al.*, 2013) and its progeny. Despite New Kawogo having high concentrations of the hydroxycinnamic acid ester compounds (resistance compounds) as described by Stevenson *et al*. (2009), synergy between transgenic and natural resistance was not observed. New Kawogo's natural resistance has been reported to have deterrent effects on adult feeding and oviposition (Stevenson *et al.*, 2009). In this study, egg plugs (no choice test) were used to infest the roots, this could be the possible reason for not observing significant resistance in the progenies.

The data in this study illustrates the feasibility of crossing transgenic and non-transgenic sweetpotato to obtain transgenic progeny following the established segregation ratios. The F_1 sweetpotato progenies that produced DNA bands sized 608bp positively contained *cry* gene derived from transgenic event CIP410008.7. As the *cry7Aa1* gene expression remained stable in progenies, the transgenic sweetpotato could be used as a valuable insect-resistant germplasm to be employed in traditional breeding programmes. Crosses between commercial sweetpotato cultivars would theoretically produce a high diversity for many characters. Each F_1 progeny has the potential to become a new variety. In the case of crosses between the event and Ugandan cultivars, it is expected that superior characteristics which are farmer preferred can be collected from the parents. The sweetpotato lines produced from this study that positively contained *cry7Aa1* gene need to be evaluated further for their resistance to the weevil both in the laboratory and under field conditions as well as further evaluation of various agronomic traits. In practice, large numbers of progenies are required for the breeder to have the opportunity to select for other characteristics other than the presence of the transgene. Several rounds of crossing and selection are needed. Clearly this is not achievable under screenhouse conditions as required by current biosafety regulation. Hence, two options exist to make transgenic breeding feasible for sweetpotato. The first option is using a deregulated transgenic event and it can be used in the field crossing blocks at the breeding station. However, it takes three or more years to have an event deregulated after the risk assessment and submission of a regulatory dossier. The second option is to introduce a regulatory procedure for transgenic breeding with non-authorised events which allow confined field crossing blocks that are isolated from other conventional crossing blocks with barriers for the pollinating bees.

CHAPTER FIVE

EVALUATION OF PROGENIES FROM CROSSES BETWEEN Bt AND NON-TRANSGENIC SWEETPOTATO

5.1 Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is an important crop worldwide which is cultivated in more than 110 countries. Nine African countries namely Uganda, Nigeria, Tanzania, Angola, Burundi, Mozambique, Madagascar, Rwanda and Ethiopia are among the top 10 sweetpotato producers in SSA (FAO, 2012). Sweetpotato is mainly grown for its storage roots and vines. Its storage roots are used for human consumption, animal feed, while vines are used as animal feed and seed, mainly in the tropics (Stevenson *et al.*, 2009). In spite of the economic value of sweetpotato, its production is limited by insect pests and viral diseases (Fuglie, 2007). Severe weevil infestations or slightly damaged roots render them unpalatable and inedible to humans due to terpenoid production in response to weevil feeding (Stathers *et al.*, 2003b). There is no effective control measure for the larvae, or other stages found within the plant tissue. Given the lack of progress in conventional breeding exploring the option of using genetic engineering using *Bacillus thuringiensis* (Bt) would provide the resource poor farmers with a better alternative to sweetpotato weevil control. However, difficulties in transforming and regenerating a broad range of sweetpotato genotypes currently limits the approach (Kreuze *et al.*, 2009).

Due to genotype dependent responses in transformation, highly responsive genotypes for *in vitro* culture are used for introduction of transgenes in crop germplasm. For instance, in rice, genotypes belonging to the *japonica* subgroup and a few *indica* genotypes like IR65, IR72, and Radon and aromatic varieties like ‘Basmati 370’ and ‘Pusa Basmati-1’ are model genotypes for genetic transformation (Ashikari *et al.*, 2004). In cotton, the ‘Coker’ genotype is the most responsive *in vitro*; in maize ‘A188’, and its derivatives; ‘Bobwhite’ lines in wheat; and ‘Golden Promise’ and ‘Igri’ in barley are are model genotypes for genetic transformation (Visarada *et al.*, 2009). Similarly, in sweetpotato, an American cultivar, Jewel, is the most amenable genotype for *in vitro* genetic manipulation as compared to African cultivars (Luo *et al.*, 2006). As a result several events expressing Bt genes for sweetpotato weevil control are available from this model genotype.

Unfortunately these transgenic events are not well adapted to African growing conditions and do not have farmer preferred traits. Hence a series of crossing the transgenic events with desirable agronomically superior lines and varieties is required to put the new genes into best cultivars.

In transgenic breeding, it is important to assess the morphological and agronomic characteristics of the progenies for regulatory purposes and variety release. Variation among traits apparently not related to a foreign gene has been reported. In transgenic rice (R_2 generation), plants were significantly shorter, showed delayed flowering, and were partially sterile as compared to their non-transgenic controls (Lynch *et al.*, 1995). Blanche *et al*. (2006) compared the transgenic cotton cultivars and their non-transgenic counterparts. Transgenic cultivars were taller, had greater internodes ratio, larger seed, and lower lint percentages, and in some cases yielded more than their conventional parents. On contrary, integration of the synthetic Bt fusion gene c*ry1B–cry1Ab* in the cabbage nuclear genome did not alter photosynthetic activity (Paul, 2003). Both T0 and T1 Bt transgenic plants flowered normally and set seeds normally like their wild-type counterpart. In sweetpotato, however, research focusing on phenotypic variations in the morphological and agronomic traits between progenies of transgenic (Jewel) and non-transgenic parents is still lacking. Given that sweetpotato is highly heterozygous and understanding of the nature and magnitude of variation among sweetpotato genotypes from a cross between model lines and elite lines for traits of economic importance is vital to plan effective transgenic breeding programmes.

The objective of the present work was to evaluate the morphological and agronomic traits of progeny derived from a cross between transgenic and non-transgenic agronomically superior genotypes grown under screenhouse conditions.

5.2 Material and methods

5.2.1 Plant material

A transgenic parental sweetpotato event, CIP410008.7, carrying *cry7Aa1* gene and Ugandan cultivar, New Kawogo (has some host resistance against the weevil (Stevenson *et al.,* 2009), were crossed under screenhouse (Table 5.1). Botanical seed obtained from crosses between CIP410008.7 and New Kawogo were scarified in concentrated sulphuric

acid, floated in water and germinated in sterile soil, in seed boxes, and spaced 15 cm x 3 cm in screenhouse. The plants were fertilised and irrigated according to screenhouse standard regimes. Each progeny was assigned an identification code starting with JNK (Jewel × New Kawogo) and a number. The F_1 progeny were transplanted into pots in the same screenhouse. The pots were arranged in a randomised complete block design with three replications. The experiments were maintained under natural light in screenhouse. Relative humidity ranged from 65 to 90% and temperature averaged 27±5 °C during the day and 20±5 °C at night. The trial was harvested when the plants were 7 months old.

Table 5.1: Morphological and agronomic descriptors of the sweetpotato parental genotypes.

Descriptor	**Cultivar**	
	Jewel	**New Kawogo**
Plant type	Semi-erect	Spreading
Root skin colour	Orange	Red
Root flesh colour	Orange	White
Vine vigour	Good	Good
β-carotene content, 100 g^{-1}	11.03 mg	0.0 mg

5.2.2 Evaluation of F_1 progeny

Characterisation was based on aerial and below ground parts of all progeny using standard International Potato Centre morphological and agronomical descriptors. Quantitative measurements were taken for: (a) plant type: determined by length of the main vines: erect (<75 cm), semi-erect (75-150 cm), spreading (151-250 cm), extremely spreading (>250 cm); (b) vine internode length: very short (< 3 cm), short (3-5 cm), long (6-12 cm), very long (>12 cm); (c) foliage weight; (d) number of roots/plant; (e) vine vigour: based on visual appearance using a scale of 1 to 9 where: 1-2 represents very low vigour (very weak); 2-4, low vigour (weak); 4-6, intermediate vigour (good vigour); and > 7 (high vigour); (f) root and skin colour and (g) β- carotene content - expressed as mg 100 g^{-1} fresh weight (FW) of the root; β-carotene value was recorded as per the sweetpotato colour chart developed by Burgos *et al*. (2009).

5.2.3 Statistical analysis

Plant type, vine internode length, foliage weight, number of roots, vine vigour, root and skin colour and β- carotene content; data were classified and subjected to ANOVA using

GenStat (VSN International Ltd, 2011) at $p<0.05$; t-test was used to compare the number of roots per plant of transgenic and null segregants.

5.3 Results

Four F_1 genotype plant types were observed erect (13.7%), semi erect (64.2%), spreading (18.9%) and extremely spreading (3.8%). The frequency distributions showed that majority of the clones were semi erect type showing an affinity to the female parent. Only 18.9 % of the clones showed affinity to the male parent which has a spreading growth habit. The two extreme types; erect and extremely spreading were also found at 16.98% and 5.66%, respectively (Figure 5.1).

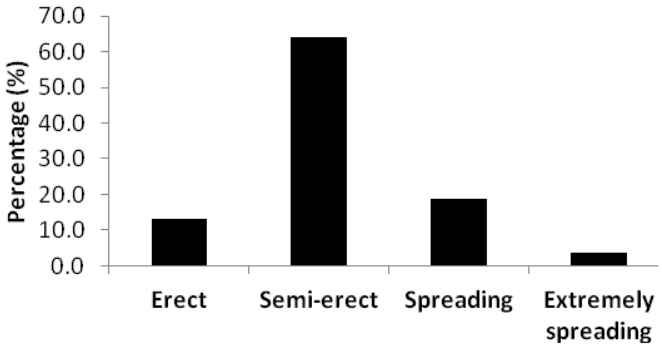
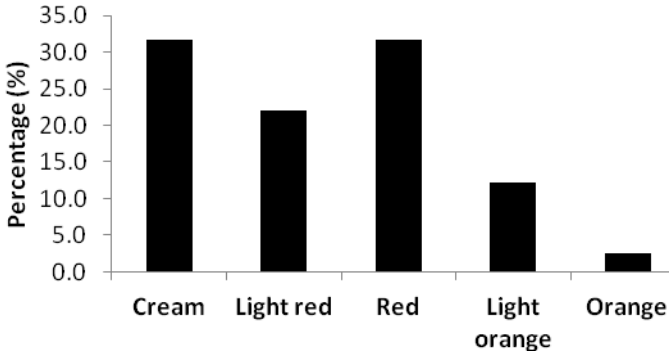


Figure 5.1. Growth habit of the F_1 (Jewel × New Kawogo) genotypes.

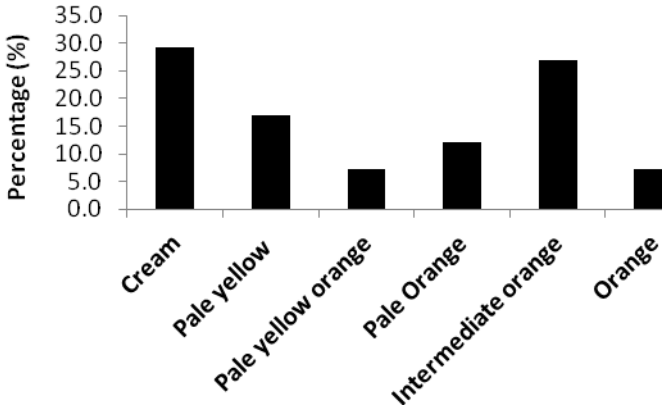
The root skin colour in the hybrid progeny segregated into a variety of colours varying from cream, light red, red light orange and orange. The progenies were predominantly cream and very few were light orange and orange (Figure 5.2a). Various flesh colours were exhibited by the progenies such as cream, pale yellow, pale yellow orange, pale orange, intermediate orange and orange (Figure 5.2b). Orange colour was predominant in the progeny with pale yellow orange (7.3%), pale orange (12.2%), intermediate orange (26.8%) and orange (7.3%) colour, respectively, which constituted about 53.7% of the total progeny. Cream colour was noticed in 29.3% of the progeny while 17.1 % of the genotypes exhibited a pale yellow colour .The flesh colour observed in the progeny as per the sweetpotato colour chart, had β-carotene values ranging from 0.0 to 11.03 mg 100 g^{-1} fresh weight (FW). Of the 53 hybrids, 4.9 % possessed a high value of 10.0 – 12.0 mg 100 g^{-1} FW; 9.8% had 6.1 – 8.0 mg 100 g^{-1} FW; 14.6% had 6.1 – 8.0 mg 100 g^{-1} FW and the rest of the hybrids had less than 2.0 mg 100 g^{-1} FW (Figure 5.2c). Hybrids JNK 48 and JNK 50 possessed highest β-carotene value of 11.03 mg 100 g^{-1} FW which is similar to the

maternal parent CIP410008.7 (Table 5.2). A total of eight F_1 progenies had no β-carotene, like the paternal parent, New Kawogo.

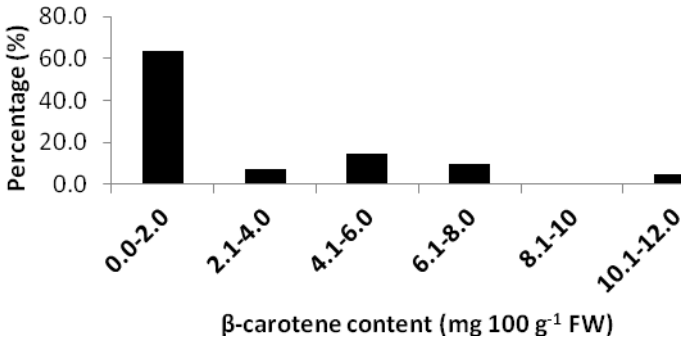
Though not statistically different, vine weight and average internode length were observed to vary according to morphological classification (Table 5.2). Majority of the clones were found to possess vine weight ranging from 0.3 – 0.4 kg (70%) with a few weighing higher between 0.4-0.5 kg (Figure 5.3a). The average internode length (cm) was obtained by measuring the mean length of the internode between the nodes of the mid-section of four randomly sampled vines from each of genotype. It varied across the progeny, majority of the internodes were short (81.1%) and a few very short (13, 2%) and long (5.7%) respectively (Figure 5.3b). However, there was significant variation (p=0.01) observed between the progeny in vine vigour (Table 5.2). High vine vigour value of 7.0 was recorded in JNK 7 and majority of the progeny were above average vine vigour with JNK 43 having the lowest vine vigour value (3.3) (Table 5.2). Similarly, there was significant variation in the number of roots/ plant in all 53 genotypes.



a) Root skin colour

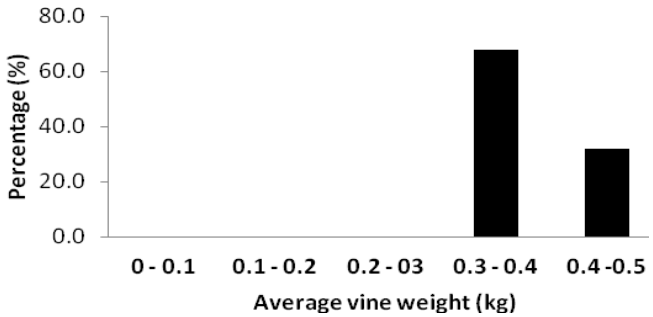


b) Root flesh colour

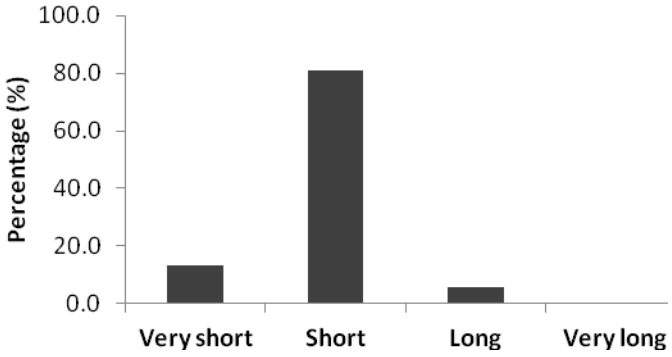


c) Root β-carotene content

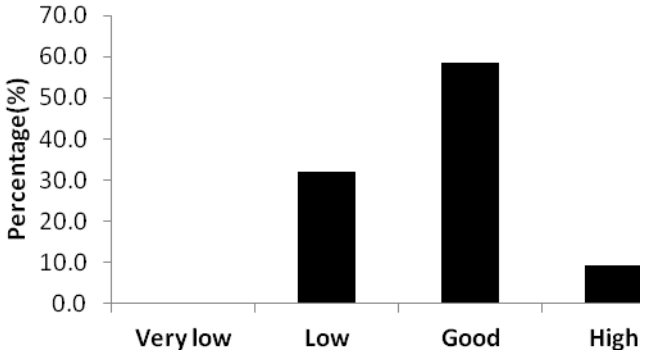
Figure 5.2. Root characteristics of the 53 F_1 genotypes (**a**) root skin colour, (**b**) root flesh colour and (**c**) root β-carotene content**.**



a) Vine weight (kg)



b) Vine internode length



c) Vine vigour

Figure 5.3. Vine characteristics of the 53 F_1 genotypes (**a**) average vine weight, (**b**) vine internode length, and (**c**) vine vigour.

Table 5.2: Morphological and agronomic traits of the F_1 progeny

Genotypes	Vine vigour	Vine length (cm)	Internode length (cm)	Foliage weight (Kg)	Number of roots	β-carotene content (mg/100^{-1} FW)
JNK 1	4.3	105.7	3.8	0.45	2.3	0.15
JNK 2	5.0	138.7	4.9	0.39	1.7	1.76
JNK 3	5.3	130.3	3.5	0.41	1.0	1.65
JNK 4	4.7	221.3	2.9	0.38	2.3	0
JNK 5	4.3	152.0	3.0	0.41	2.0	1.65
JNK 6	6.7	133.7	3.3	0.43	1.3	0.02
JNK 7	7.0	98.7	4.2	0.38	0.0	-
JNK 8	4.7	105.7	3.0	0.35	2.7	1.38
JNK 9	4.3	107.3	4.2	0.38	2.0	1.76
JNK 10	4.0	215.7	3.5	0.39	0.0	-
JNK 11	4.7	79.0	2.2	0.41	2.3	4.92
JNK 12	4.7	143.7	4.5	0.43	2.3	0
JNK 13	5.7	115.7	4.0	0.39	0.0	-
JNK 14	5.0	124.0	4.7	0.37	3.0	0.12
JNK 15	5.0	90.3	4.2	0.39	1.7	4.92
JNK 16	5.3	167.0	3.5	0.42	0.0	-
JNK 17	5.0	76.7	3.3	0.37	3.0	0
JNK 18	6.0	123.7	3.3	0.38	0.0	-
JNK 19	5.7	252.7	5.2	0.43	2.3	0.02
JNK 20	4.0	127.0	3.7	0.37	0.0	-
JNK 21	4.3	121.7	4.4	0.37	1.7	0.02
JNK 22	4.7	121.0	3.0	0.41	1.7	4.41
JNK 23	4.7	58.3	3.2	0.36	1.3	0.12
JNK 24	3.7	99.7	3.6	0.41	1.3	0.15
JNK 25	5.7	197.0	5.2	0.39	2.7	0.12
JNK 26	5.7	120.7	5.0	0.46	0.0	-
JNK 27	4.0	110.7	3.8	0.37	0.0	-
JNK 28	4.0	103.0	3.0	0.36	1.3	4.92
JNK 29	4.7	107.3	3.0	0.43	0.0	-
JNK 30	5.3	104.0	3.1	0.37	0.0	-
JNK 31	4.7	92.7	2.2	0.41	2.0	0
JNK 32	4.3	101.0	2.8	0.39	2.3	0
JNK 33	4.0	75.0	4.0	0.39	2.0	3.03
JNK 34	4.0	96.3	3.7	0.40	1.3	3.03
JNK 35	5.0	74.0	4.1	0.41	1.7	0.02
JNK 36	6.0	72.7	3.8	0.37	2.0	0.12
JNK 37	4.0	64.3	4.2	0.39	0.0	-
JNK 38	5.3	131.7	4.3	0.39	1.3	0
JNK 39	5.0	120.0	4.7	0.39	3.0	0.12
JNK 40	6.0	102.3	3.4	0.37	2.3	0.12
JNK 41	5.3	93.7	3.2	0.45	2.3	6.12
JNK 42	4.7	101.7	4.0	0.43	3.0	0.12
JNK 43	3.3	73.7	3.9	0.38	0.0	-
JNK 44	4.3	125.0	3.0	0.34	3.0	7.23
JNK 45	4.3	102.7	4.0	0.40	1.7	0
JNK 46	4.0	186.3	3.9	0.39	2.7	0
JNK 47	5.7	190.0	2.8	0.38	2.0	1.38
JNK 48	3.7	103.3	3.5	0.43	2.7	11.03
JNK 49	4.0	121.3	2.9	0.37	1.0	3.03
JNK 50	4.0	59.0	2.9	0.39	1.3	11.03
JNK 51	4.0	81.0	4.1	0.39	1.7	5.46
JNK 52	4.0	106.0	3.8	0.41	1.0	7.23
JNK 53	3.7	266.7	3.3	0.40	1.0	4.61
Mean	**4.74**	**120.6**	**3.67**	**0.39**	**1.60**	**2.22**
LSD $_{(0.05)}$	**1.74**	**80.42**	**1.96**	**0.07**	**1.98**	**0.04**
p-value	**0.01**	**<0.001**	**0.49**	**0.56**	**0.001**	**<0.001**

In the previous experiments, PCR confirmed that the transgene cassette was stably inherited in the F_1 progeny and showed segregation at 1:1 ratio ($\chi^2 = 0.17$, p = 0.68) (give reference). There were 25 transgenic progeny and 28 null segregants (non-transgenic). A t-test done on number of roots/plant of transgenic progeny (group 1, *n* = 25) compared with the null segregants (group 2, *n* = 28) gave a p-value of 0.44. The mean of group 1 was 1.64 (SD = 0.93; SEM = 0.19) while that of group 2 was 1.43 (SD = 1.05; SEM = 0.20). The t-test demonstrated that there were no significant differences in the number of roots/plant between the transgenic and non-transgenic segregants.

5.4 Discussion

The introduction of a transgene into a recipient genome is a complex event depending on the transgene and the host genome interaction. The results from the previous study showed that *cry7Aa1* gene was successfully transmitted from the transgenic parent to some of the progeny as expected. Sweetpotato is a highly heterozygous hexaploid (*x* = 15, 2n = 90), as a consequence, F_1 progeny resulting from crosses between sweetpotato clones results in high levels of segregation (Mwanga *et al.*, 2002). Equally, this was observed in this study irrespective of the presence of the transgene. The use of a transgenic parent in transgenic breeding had no adverse effect on the intrinsic morphological and agronomic traits in sweetpotato. Similar results were reported by Okada *et al*. (2002), who evaluated the offspring from crosses between transgenic (carrying hygromycin-resistant (*hpt*) and SPFMV-S coat protein (*CP*) genes) and non-transgenic sweetpotato.

Most progenies in this study possessed semi-erect growth habit with a few erect, spreading and extremely spreading. The erect and semi-erect sweetpotato types may be preferred by farmers in crop mixtures when the companion crops are dwarf and shade tolerant. The spreading types are considered more suitable for intercropping, since they have greater potential of utilising available sunlight than the erect and semi-erect types. Additionally, spreading sweetpotato varieties have the ability of spreading rapidly over the soil surface and intercepting all the available light, thus preventing and suppressing weeds, thus providing suitable soil environment for proper root development. In sweetpotato, vine length is highly heritable and maternally inherited (Wilson *et al.*, 1989), its improvement coupled with transgenic weevil resistance would be compliant with farmer preference and needs.

Skin colour preferences in sweetpotato have been reported to differ among farmers depending on location (Bashaasha *et al.*, 1995; Kapinga and Carey, 2003). In this study, skin colour was found to be more variable, raising potential to breed and select transgenic cultivars with skin colour preferred by a wide range of farmers. Rees *et al*. (2003) reported that the colour characteristic of sweetpotato roots constitutes an important factor in the choice of sweetpotato by consumers. The transgenic parent used in cross breeding was orange fleshed yet most consumers prefer varieties with white or yellow flesh which contain very little carotene (Takahata *et al.*, 1993). This variability observed in the F_1 progenies allows for selection of genotypes with little or no carotenoids and with inherent transgenic insect resistance. In addition, varieties of sweetpotato with high carotenoid content tend to have lower dry matter content. African consumers prefer cultivars with high dry matter content (Kapinga and Carey, 2003), and orange-fleshed varieties have low dry matter content. Crossing a transgenic parent which is an orange-fleshed and one which is not increases the possibility of having a cultivar which has transgenic resistance and high dry matter content after selection.

The carotenoid rich genotypes obtained in this study, however, indicate the possibility of significantly improving the Vitamin A nutrition in Africa. According to Faber *et al*. (2010), increasing the consumption of the orange-fleshed sweetpotato (OFSP) roots and food could provide a significant proportion of the required dietary vitamin A intake. In general, vitamin A intake is often inadequate in children because of the seasonality of foods, early abandonment of exclusive feeding, and the practice of not giving vitamin A-rich foods to young children under 5 years. Foods such as dairy and meat products containing pre-formed vitamin A are often too expensive for the majority of people in tropical and sub-tropical Africa (Faber *et al.*, 2010). Plant foods like OFSP which contain concentrated pro-vitamin A carotenoids can make a tremendous contribution to improved human health in these areas.

Vine weight and average internode length varied in all progenies though were not statistically significant. The high ploidy level in sweetpotato could be responsible for variation in these traits due to increased mutations associated with polyploidy (Osborn *et al.*, 2003). The existence of a continuous variation for these characters reflects their quantitative nature of inheritance. Most of the F_1 genotypes displayed good vine vigour. Vine vigour is of importance for propagation of planting materials, survival and in general

indicates tolerance to stress conditions. Because conditions at resource-poor farms are often not ideal, low vine vigour is an especially undesirable trait. Vine vigour is also important for future bulking of planting material for production of new varieties.

Storage root production in sweetpotato has been reported to be inconsistent from plant to plant; some plants have few or no storage roots while others have four or more marketable ones (Firon *et al.*, 2009). However, the underlying genetic mechanisms and the factors that promote storage root formation, the most important physiological process in sweetpotato, are not clearly understood. In this study, majority of the genotypes produced roots with varying numbers (Table 5.2). Nonetheless, some of the progenies failed to produce roots seven months post planting instead developed many fibrous roots. These particular genotypes present interesting information that requires further investigation before being considered as useful genetic material for breeding purposes. When focusing on yield, these genotypes are most likely not to be considered in the next level of evaluation for breeding purposes. The high yielding genotypes obtained in this study could be used in the breeding programmes to upgrade the yield of other transgenic events.

In the screenhouse and laboratory evaluation, the sweetpotato F_1 genotypes showed variation in plant type, internode length, foliage weight, number of roots, vine vigour, root and skin colour, and β- carotene content. The results presented here show that the transgene do not necessarily have an effect on morphological and agronomic traits. Since sweetpotato is a hexaploid, significant variation in all traits studied indicated segregation of many genes for a given trait. Evaluation and characterisation of sweetpotato clones could be used to identify and select genotypes from crosses involving transgenic and non-transgenic parents with farmer preferred traits in a breeding programme. Our experiments were done under controlled environments because of the regulatory requirements; further field trials along with additional transgenic events would provide additional information on the effect of the transgene on agronomic performance and weevil resistance.

CHAPTER SIX

IDENTIFICATION OF NON-TARGET ORGANISMS EXPOSED TO WEEVIL-RESISTANT *Bt* SWEETPOTATO IN UGANDA

6.1 Introduction

Sweetpotato (*Ipomoea batatas*) is an important crop in tropical areas of the world. In Uganda, sweetpotato is grown as a staple food in low-input farming systems (Smit, 1997b; Stathers *et al.*, 2003a). For some farmers, the crop also supplements family income. Strategies to reduce losses due to pests would impact directly on livelihood of millions of rural households by enhancing food security. Sweetpotato weevils, *Cylas puncticollis* Boheman and *C. brunneus* F., are the major production constraints in sub-Saharan Africa (SSA), whereas in the Americas and Asia, *C. formicarius* F. is the major pest (Sorensen, 2009). In areas where weevils are endemic, production losses range between 60-100% (Smit, 1997b). In Uganda, a survey on the socio-economic impact of sweetpotato weevils indicated an average yield loss of over 28% between wet and dry seasons (Kiiza *et al.*, 2009). Even low levels of *Cylas* spp. infestation can reduce root quality and marketable yield because the plants produce unpalatable terpenoids in response to weevil feeding. In addition, fungal rotting occurring as a consequence of weevil tunnelling in the storage roots produces several compounds, including ipomeamarone which is particularly toxic to animals (Pandey, 2008). Hence, control of weevils through host plant resistance would bring significant benefits to low-input farmers (Qaim, 2001).

Considerable research has been conducted to identify host plant resistance to *Cylas* spp. in sweetpotato and significant progress has been made to release varieties less affected by weevils (Stathers *et al.*, 2003a; Jackson *et al.*, 2012; Muyinza *et al.*, 2012). However, improved varieties with high levels of *Cylas* spp. resistance are not yet available. Progress in breeding weevil-resistant cultivars has been slow due to the genetic complexity of the crop (hexaploid and highly heterozygous) and lack of attractiveness of deep-rooting varieties which is the most effective breeding target to avoid weevil infestation (Stathers *et al.*, 2003a). Another option could be to breed for enhanced accumulation of the biochemical component of resistance of the variety New Kawogo, but its inheritance and

impact on nutritional quality of the storage roots remain to be elucidated (Stevenson *et al.*, 2009). Conversely, genetic transformation for insect resistance is one of the attractive options to improve sweetpotato production as has been witnessed in insect-resistant (Bt) varieties of maize and cotton in sub-Saharan Africa (Thomson, 2008). High levels of resistance have been achieved against Coleopteran pests by expressing toxins derived from *B. thuringiensis* in crop plants (Betz *et al.*, 2000; Qaim *et al.*, 2008).

Accordingly, in Uganda Cry proteins were tested for activity against the African sweetpotato weevil resulting in the identification of three samples of *B. thuringiensis* (Bt) endotoxins; Cry7Aa1, ET33/34, and Cry3Ca1 which were found to be active against *C. puncticollis* and *C. brunneus* in artificial diet assays (Ekobu *et al.*, 2010). To that end, the corresponding genes were introduced into sweetpotato via *Agrobacterium tumefaciens* genetic transformation (Ghislain *et al.*, 2013). Assuming weevil pests will be controlled through the expression of the Cry proteins in sweetpotato, it is important to assess the impact of these proteins on other organisms in the sweetpotato growing environments, like other insect control technologies. Therefore, the objective of this study was to identify NTOs that are associated with Bt sweetpotato in the likely event of release progenies arising from transgenic breeding.

6.2 Methodology

Expert consultation and published literature were used to identify the key non-target arthropods in major sweetpotato growing regions in Uganda, and to evaluate which NTOs would be at risk by exposure to the genetically modified plants. Non-target risk assessment is a process by which risks are identified and the seriousness of the risk is characterised so that decisions can be made on whether or how to proceed with the technology. The process requires a framework for non-target organisms risk assessment of Bt crops, problem formulation, established assessment endpoints and selection of species for testing**.**

6.2.1 Framework for non-target organisms risk assessment of Bt crops

Environmental risk assessment of a Bt crop considers the impact of the Cry protein on the target pest but also on non-target organisms either directly or indirectly (OECD, 2007). To identify relevant non-target organisms, it is important to understand the mode of action of

Cry proteins. All Cry toxins characterised so far bind to specific receptors on the plasma membrane of mid-gut epithelium cells in susceptible insects which form oligomeric trans-membrane pores causing osmotic lysis (Aronson and Shai, 2001; Bravo *et al.*, 2007). Some Cry proteins have multiple receptors, or may bind to multiple sites on a single receptor and it has been demonstrated that receptor binding is necessary but not sufficient for toxicity (de Maagd *et al.*, 2001). Experiments using sub-lethal concentrations have also revealed that there may be other relevant interactions between Cry proteins and their target insects (Aronson and Shai, 2001). Zhang *et al*. (2006) also suggested that toxicity could be related to G-protein mediated apoptosis following receptor binding instead of forming oligomers resulting in pore formation. Preliminary research results indicate that the Cry proteins used to control weevils in sweetpotato have similar mode of action as the other Cry proteins (Hernandez-Martinez *et al.*, 2010).

Non-target organisms (NTO) are species not targeted for control using a particular Cry protein expressed in transgenic plants but which may become exposed to it by feeding directly on plant tissues or indirectly on herbivores or parasites, or by direct ingestion via the environment; such as in the soil or water (Groot and Dicke, 2002). Although all organisms of relevance to sweetpotato are arthropods, NTO will be used when discussing non target arthropods due to the global acceptance of NTO. Non target risk assessment is a process based on scientific principles that aim at the evaluation of the potential adverse effects of transgenic plants on the non-target organisms of environmental relevance (OECD, 2007; Romeis *et al.*, 2008).

6.2.2 Problem formulation in NTO risk assessment

The initial step of risk assessment is problem formulation which is an important step that leads the risk assessment process to successful risk characterisation (Raybould *et al.*, 2007; Romeis *et al.*, 2011). The Environmental Protection Agency (EPA) of the United States elaborated in 1998 guidelines on ecological risk assessment which sets the basis for NTO risk assessment (EPA 1998). Problem formulation, in an ideal sense, develops a concise problem statement, a risk hypothesis, a conceptual model and an analysis plan. The risk hypothesis represents an assumption regarding the cause-effect relationships among attributes of the risk characterisation, including sources, exposure routes, endpoints, responses, and measures relevant to the risk assessment. The conceptual model describes key relationships between a transgenic plant occurring in the environment and

its linkages to an assessment endpoint (Raybould, 2007). It sets the problem in perspective and establishes the proposed relationships that need evaluation and the analysis plan establishes the appropriate risk formulation to be considered in the risk characterisation.

In Uganda, the concern is that Bt sweetpotato plants may have an adverse effect on biodiversity and it's functioning at several levels, through interactions with populations of other species associated with Bt sweetpotato fields. Because the environment is to be protected from harm according to protection goals set out by Ugandan legislation (The National Environment Act-Cap 153 1995), protection of species richness and ecological functions should be considered in this risk assessment. The receiving environment is the Bt sweetpotato cultivated fields and the wider environment (other adjacent Bt or non-Bt cultivations). For the benefit of sustainable production, the interest is to maintain a certain level of biodiversity in sweetpotato fields, providing essential functions such as biological control of pests, decomposition of plant materials and maintenance of soil quality and fertility. Bt sweetpotato varieties need to be evaluated to determine if they are directly and/or indirectly (through food web interactions) potentially harmful to species guilds involved in ecosystem functions. Problem formulation in our case is the identification of potential hazards such as exposure to the Cry proteins through a comparison of the Bt sweetpotato with their conventional counterpart.

6.2.3 Assessment endpoints in NTO risk assessment

A second conceptual element of the NTO risk assessment is the assessment endpoint which is an explicit expression of the environmental value to be protected (EPA, 1998). This necessitates defining species and ecosystem functions that could be adversely affected by the Bt plant, and that require protection from harm. Assessment endpoints are made operational into quantitatively measurable endpoints. An appropriate measurement endpoint for NTO testing is relative fitness or some component of relative fitness, which is the relative lifetime survival and reproduction of the exposed versus unexposed non-target species (Andow and Hilbeck, 2004). It is therefore required that NTO tests consider both toxic effects (mortality, longevity) and sub-lethal effects. The sub-lethal effects assessed through growth pattern, development rate, reproduction parameters (number and size of offspring, percentage of eggs hatching, sex ratio of progeny, age of sexual maturity), and, when appropriate, behavioural characteristics (searching efficiency, predation rates, food choice). In field conditions, the abundance and species diversity of certain groups of NTO

at a relevant life stage are typical measurement endpoints. The choice of specific measurement endpoints shall be done according to the problem formulation on a case-by-case basis (Romeis *et al.*, 2011).

An appropriate assessment endpoint for initial testing in Bt sweetpotato will be the relative survival and reproduction of NTOs. These parameters are a particularly useful measurable assessment endpoint in relation to Bt sweetpotato because they relate directly to risk. Survival experiments should last at least through all relevant developmental stages of the selected NTO including adult parameters such as age specific mortality. In principle, the duration of the test should correspond to the time the non target species would be exposed to the Bt sweetpotato plants or crop residues. In our case, NTO survival experiments would be conducted through all developmental stages including adult life stage parameters such as age specific mortality. If the Bt sweetpotato has a negative impact on NTOs in the field, its effect could be observed at any developmental stage during their life cycle. Usually, inappropriate assessment endpoints may misdirect research or regulatory efforts, and may even lead to the imposition of unnecessary controls to reduce risk.

6.2.4 Species selection

Non-target organisms in Bt crop fields feeding directly or indirectly on the crop or residues are exposed to the Cry protein expressed in the pest-resistant plant. Hence, NTO risk assessment has to be done for some of these species when there is a reasonable doubt that they may suffer a negative impact due to a real exposure. For practical reasons, only a small fraction of all possible terrestrial organisms can be considered for regulatory testing. Therefore, to assess the effect of insect resistant plants on NTO, appropriate species should be selected (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2008). It is necessary to select suitable species which can act as surrogates for species that should, but cannot, be tested (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2013).

The use of appropriate surrogates is a widely accepted concept for scientific experimentation and enables one to design high-quality and repeatable laboratory (and semi-field) studies with clear measurement endpoints and the ability to extrapolate results to other species. Non-target species subject to the NTO risk assessment should be chosen from different ecological functions such as herbivores, pollinators, predators and parasitoids of pest organisms, and decomposers in the soil (Romeis *et al.*, 2006). The NTO

risk assessment may also consider species with special aesthetic, economic or cultural value or species of national importance. These species are regionally specific and can be evaluated within the ecological risk assessment independent of their ecological function. In order to reflect biogeographical variation, it is crucial to determine what relevant species are likely to occur in the cropping systems where the transgenic plant is expected to be grown.

6.3 Results

Over the years field experiments have been conducted in Uganda by National Agricultural Research Organisation (NARO) scientists in different sweetpotato growing districts to determine pests and beneficial organisms associated with sweetpotato (Ames *et al.*, 1996; Smit, 1997b; Stathers *et al.*, 2005; Sorensen, 2009). Insects representing more than 30 species of 8 orders and in different developmental stages were found to be prevalent in sweetpotato fields (Table 6.1). Their levels of abundance differ according to the seasons and agro-ecological zones. Individuals belonging to 8 species of 6 families were noted as major pests of sweetpotato and are those farmers have to monitor as part of a sustainable integrated pest management (IPM) system in sub-Saharan Africa (Stathers *et al.*, 2005).

Furthermore, individuals of 19 species were minor pests while 9 species belonging to 9 families were represented by beneficial insects. The beneficial insects noted were pollinators, decomposers, predators and or parasitoids of insect pests. Among the homopterans pests observed, white fly (*Bemisia tabaci*) and aphids (e.g. *Myzus persicae*) are vectors of viral diseases. Most of the minor pests observed are cosmopolitan, polyphagous and are pests of other crops. These include *Phyllophaga* spp., *Hapatesus* spp., *Leucinodes orbonalis, Spodoptera* spp., *Omopyge sudanica, Macrotermes bellicosus, Gryllus* spp., *Zonocerous variegates, Attractomorpha psitticina, Locusta migratoria, Bemisia tabaci, Myzus persicae, Macrosiphum euphorbiae, Leptoglossus gonagra* and *Nezara viridula*.

Table 6.1: Insects associated with sweetpotato fields in Uganda

Order	Family	Species	Common Name	Importance	Prevalence
Coleoptera	Brentidae	*Cylas puncticolis*	African Sweetpotato weevil	Major Pest	Common
		Cylas brunneus	African Sweetpotato weevil	Major Pest	Common
	Scarabeidae	*Phyllophaga* spp.	White grub	Pest	Common
	Meloidae	*Epicauta* spp.	Blister beetle	Pest	Common during flowering
	Curculionidae	*Peloropus batatae*	Peloropus Weevil	Pest	Fairly common
		Blosyrus obliguatus	Rough Sweetpotato weevil	Major Pest	Fairly common
		Alcidodes dentipes	Striped Sweetpotato weevil	Pest	Fairly Common
	Coccinellidae	*Delphastus catalinae*	Ladybird beetle	Predator	Common
	Chrysomelidae	*Aspidomorpha* spp.	Tortoise shell beetle	Major Pest	Common
	Elateridae	*Hapatesus* spp.	Wireworm	Pest	Common
	Carabidae	*Poecilus chalcites*	Ground beetle	Predator	Common
	Staphylinidae	*Aleochara bilineata*	Rove beetle	Predator	Common
Lepidoptera	Nymphalidae	*Acraea acerata*	Sweetpotato butterfly	Major Pest	Common
		Synanthedon dascyeles	Clearwing Moth	Major Pest	Common
	Crambidae	*Leucinodes orbonalis*	Eggplant fruit borer	Pest	Rare
	Noctuidae	*Agrotis subterranea*	Granulate cutworm	Pest	Fairly common
		Spodoptera spp.	Armyworm	Major Pest	Common
	Sphingidae	*Agrius cingulata*	Sweetpotato hornworm	Major Pest	Fairly Common
		Agrius convolvuli	Sweet potato moth	Pest	Fairly Common
		Hippotion celerio	Taro hawkmoth	Pest	Fairly Common
Isoptera	Termitidae	*Macrotermes bellicosus*	Termite	Pest	Common
Orthoptera	Gryllidae	*Gryllus* spp.	Field cricket	Pest	Common

	Pyrgomoriphidae	*Zonocerous variegatus*	Elegant grasshopper	Pest	Common
	Acrididae	*Attractomorpha psitticina*	Slant-faced grasshopper	Pest	Rare
		Locusta migratoria	Migratory locust	Pest	Rare
Hemiptera	Aleyrodidae	*Bemisia tabaci*	Sweetpotato whitefly	Pest/vector	Common
	Aphididae	*Myzus persicae*	Aphid	Pest/vector	Fairly common
		Macrosiphum euphorbiae	potato aphid	Pest	Common
	Coreidae	*Leptoglossus gonagra*	Squash bug	Pest	Common
	Pentatomidae	*Nezara viridula*	Green stink bug	Pest	Common
	Reduviidae	*Sycanus* spp.	Assassin bug	Predator	Common
Dermaptera	Forficuliidae	*Doru taeniatum*	Earwig	Predator	Common
Hymenoptera	Ichneumonidae	*Charops* spp.	Ichneumon wasp	Parasitoid	Common
	Braconidae	*Meteorus autographae*	Braconid wasp	Parasitoid	Common
	Apidae	*Apis mellifera*	Honey bee	Pollinator	Common
Diptera	Tachinidae	*Caricelia normula*	Tachinid fly	Parasitoid	Common

In addition, nine spp. of non-insect organisms (Table 6.2) were also found to be common in sweetpotato fields. Three of these non-insects were beneficial while five are pests of sweetpotato. The identification of the arthropod complex in sweetpotato fields helps to ascertain value or risk of each species. Some samples of arthropod specimen mentioned in this paper have been preserved in the Entomology laboratory at National Crop Resources Research Institute (NaCRRI), Namulonge.

Table 6.2: Non insect arthropods associated with sweetpotato fields in Uganda

Order	**Family**	**Species**	**Common Name**	**Importance**	**Prevalence**
Haplotaxida	Lumbricidae	*Eisenia foetida*	Earthworm	Decomposer	Common
Araneae	Oxyopidae	*Oxyopes* spp.	Lynx Spider	Predator	Common
	Glycosidase	*Lycos* Spp.	Wolf Spider	Predator	Common
Nematoda	Hoplolaimidae	*Rotylenchulus reniformis*	Reniform nematode	Pest	Common
	Meloidogynidae	*Meloidogyne arenaria*	Root knot nematode	Pest	Common
Rodentia	Muridae	*Mus musculus*	Mouse	Pest	Common
.		*Spalax* spp.	Rat	Pest	Common

Diplopoda	Odontopygidae	*Omopyge sudanica*	Millipede	Pest	Common

6.4 Discussion

Based on the considerations addressed on the identification of the functional groups of arthropods associated with sweetpotato fields and categorisation of NTO, focal species are selected from each functional category of NTO group. The functional groups commonly associated with sweetpotato fields are pollinators, decomposers, predators and parasitoids. The following criteria should be considered in choosing the appropriate focal test species.

The mode of action and specificity of the insecticidal protein and the impact of that protein on non-target species closely related to the target pest: Cry proteins identified as active against *Cylas* spp. (Cry7Aa1, ET33/34, and Cry3Ca1) are typically of the type affecting primarily Coleopteran species (Crickmore *et al.*, 2013). Hence, the most likely affected NTO to initially evaluate should be within Coleoptera. Therefore, ground beetles, (Carabidae: *Poecilus chalcites*), rove beetles (Staphylinidae: *Aleochara bilineata*), and ladybird beetles (Coccinellidae: *Delphastus catalinae*) may be considered, that occur in the same taxonomic order (Coleoptera) as the target species. Weevil species such as striped sweetpotato weevil (*Alcidodes dentipes*), rough sweetpotato weevil (*Blosyrus obliguatus*) and peloropus weevil (*Peloropus batatae*) belonging to the same superfamily (Curculionoidea, as the target pests) are either considered as minor pests or not ecologically relevant. There are few examples of cross-order activity for Cry proteins (Tailor *et al.*, 1992; van Frankenhuyzen, 2009). However, previous research has shown that that this cross-order activity does not threaten the environmental safety of Bt-based pest control, because Cry proteins tend to be much less toxic to taxa outside of the primary specificity range (van Frankenhuyzen, 2009). Furthermore, the large body of published literature provides no indication that the currently grown Bt crops cause direct adverse effects on arthropods that are not closely taxonomically related to the target pest (Romeis *et al.*, 2006; Wolfenbarger *et al.*, 2008; Duan *et al.*, 2010).

Exposure based on habitat and field abundance: Relevant NTO should represent species that are abundant in the crop and have known relevant routes of exposure to the insecticidal protein (Romeis *et al.*, 2013). Exposure could be direct, from deliberate or incidental feeding on crop tissues or decaying plant material, or be indirect, from feeding

on herbivores that feed on the crop. For example, testing ground beetles (Carabidae) is relevant for coleopteran insecticidal proteins produced in sweetpotato but their exposure is low since these insects are primarily predators of organisms unaffected by Cry proteins in sweetpotato fields, living especially at the soil surface or within the soil where the roots are located. The same can be said of the rove beetle (Staphynilidae). Ladybird beetle (Coccinellidae) adults are active fliers and feed on pollen; they are unlikely to be affected because Bt protein expression in the pollen is low and because nectar is a plant secretion, not a tissue and has no cellular content (Ferry *et al.*, 2007). The ladybird larvae feed primarily on aphids feeding on sweetpotato leaves and exposure of the larvae to the Bt toxins is considered to be relatively low as aphids contain no or only trace amounts of the toxins due to the fact that they feed on the phloem sap which does not contain Cry proteins (Raps *et al.*, 2001; Romeis and Meissle, 2011). Although no NTO risk assessment for Bt crops have been conducted on *Delphastus catalinae*, other Coccinellidae species have been shown to be unaffected by Coleopteran pest resistant Bt crops (Duan *et al.*, 2002; Ferry *et al.*, 2007; Li and Romeis, 2010; Álvarez-Alfageme *et al.*, 2011). In the case of maize, Cry3Bb1, Cry34Ab1/Cry35Ab1 proteins impact on ground, rove and ladybird species was reviewed and essentially found not to persist in the environment due to rapid degradation in the soil (Wolt *et al.*, 2007). These Cry proteins are closely related to those currently used to engineer resistance to sweetpotato weevils (Cry7Aa1 and Cry3Ca1 are close to the Cry3Bb1 and the ET33/ET34 to the Cry34Ab1/Cry35Ab1) (Crickmore *et al.*, 2013). Furthermore, these Cry proteins used to control weevils in sweetpotato have similar mode of action as the other Cry proteins (B. Escriche, University of Valencia, Spain, pers. comm.). Therefore, Bt sweetpotato is unlikely to cause harm to the above mentioned species.

Ecological and taxonomic diversity: Relevant NTO may include a broad range of invertebrates, particularly economically or socially beneficial species that represent diverse habitats. In our case; honeybee (*Apis mellifera)* is the main pollinator of sweetpotato which may forage for sweetpotato pollen and therefore could be exposed to Cry proteins. Earthworms (*Eisenia foetida*) are important decomposers and sweetpotato butterfly (*Acraea acerata*) feeds on plant canopy and would be surrogate to Lepidopteron arthropods which feed on the sweetpotato canopy. In all three cases, Coleopteran-specific Cry proteins are unlikely to cause any harm because of their target specificity. Indeed, a meta-analysis of Cry protein impact on honey bees resulted in no harm as well as a recent

study including several Cry proteins (Duan *et al.*, 2008; Hendriksma *et al.*, 2012). Similarly, field studies have also shown no significant differences in earthworm populations in fields planted with Cry1Ab1 or Cry3Bb1 proteins (van der Merwe *et al.*, 2012).

Ability to conservatively estimate field exposure: In the laboratory the potential level of exposure of test species to insecticidal proteins in the field has to be identified. Farmers rarely cultivate only one landrace in one area even when they have adopted an improved variety they will maintain some level of diversity because production is not targeting a single use. It is unlikely that a Bt sweetpotato improved variety will displace landraces because these are grown for their culinary or tasting properties. Therefore, an accurate estimate of exposure of the relevant NTO will be difficult to agree on. Hence, the concentration of the insecticidal protein in the plant tissue on which the NTO feeds provides a worst-case estimate of the environmental exposure concentration. Such data for the Bt sweetpotato is not yet available, because Bt sweetpotato is still under development in Uganda.

Whether a suitable test system exists for laboratory analysis: Relevant NTOs adaptable to a laboratory bioassay system and suitable protocols are necessary for testing. When feasible, the organism life stage that is most susceptible to the insecticidal protein should be tested. Protocols typically include information on test endpoints, positive/negative controls, acceptable control mortality, sample sizes and statistical power analyses. For a number of chosen species on Bt sweetpotato, standard testing protocols are not yet available but a number of protocols are available from tests conducted with other crops and/or related invertebrate species which could be adapted for testing the effect of insecticidal proteins being expressed in sweetpotato.

In general, non target organisms that are related taxonomically to the target pests are most likely to be affected in the same way by the Bt Cry protein (Romeis *et al.*, 2008). In the case of Bt sweetpotato, the rove, ground and ladybird beetles are the primary relevant NTO. Accepting a much lower probability of impact, the honey bee, as the main pollinator of sweetpotato and a charismatic insect, may be looked at as an NTO for Bt sweetpotato. However, numerous impact studies have been published over the last decade and have been subject to meta-analyses drawing very clear conclusions of non-impact of Cry

proteins on NTO (Marvier *et al.*, 2007; Wolt *et al.*, 2007; Duan *et al.*, 2008; Wolfenbarger *et al.*, 2008). Species that are not exposed to the Cry proteins or from kingdoms never reported to be affected by other Cry proteins do not need to be tested to draw a negligible-risk conclusion (Peterson *et al.*, 2011; Prischl *et al.*, 2012).

Once the relevant non target species are selected and their surrogates identified, these would be evaluated moving through the tiered testing procedure that has been recommended and well accepted by regulators and risk assessors (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2006; USEPA, 2007; Romeis *et al.*, 2008). In the case of ground, rove and ladybird beetles, these can be used directly as test species. The procedure starts with laboratory tests (lower tier), followed by semi-field (glasshouse or screenhouse) and field (higher tier) tests if necessary (Figure 6.1). However, the tiers should not be just considered as sequential steps in a linear approach, because the response of arthropods between the tiers is necessary during the assessment, to determine whether to stop or proceed to the next tier (Kos *et al.*, 2009). Lower tier tests serve to identify potential hazards and are typically conducted in controlled conditions. Lower-tier tests are designed to measure a specific endpoint under worst-case conditions using protein concentrations that are normally 10-100 times higher than those present in plant tissues (USEPA, 2007).

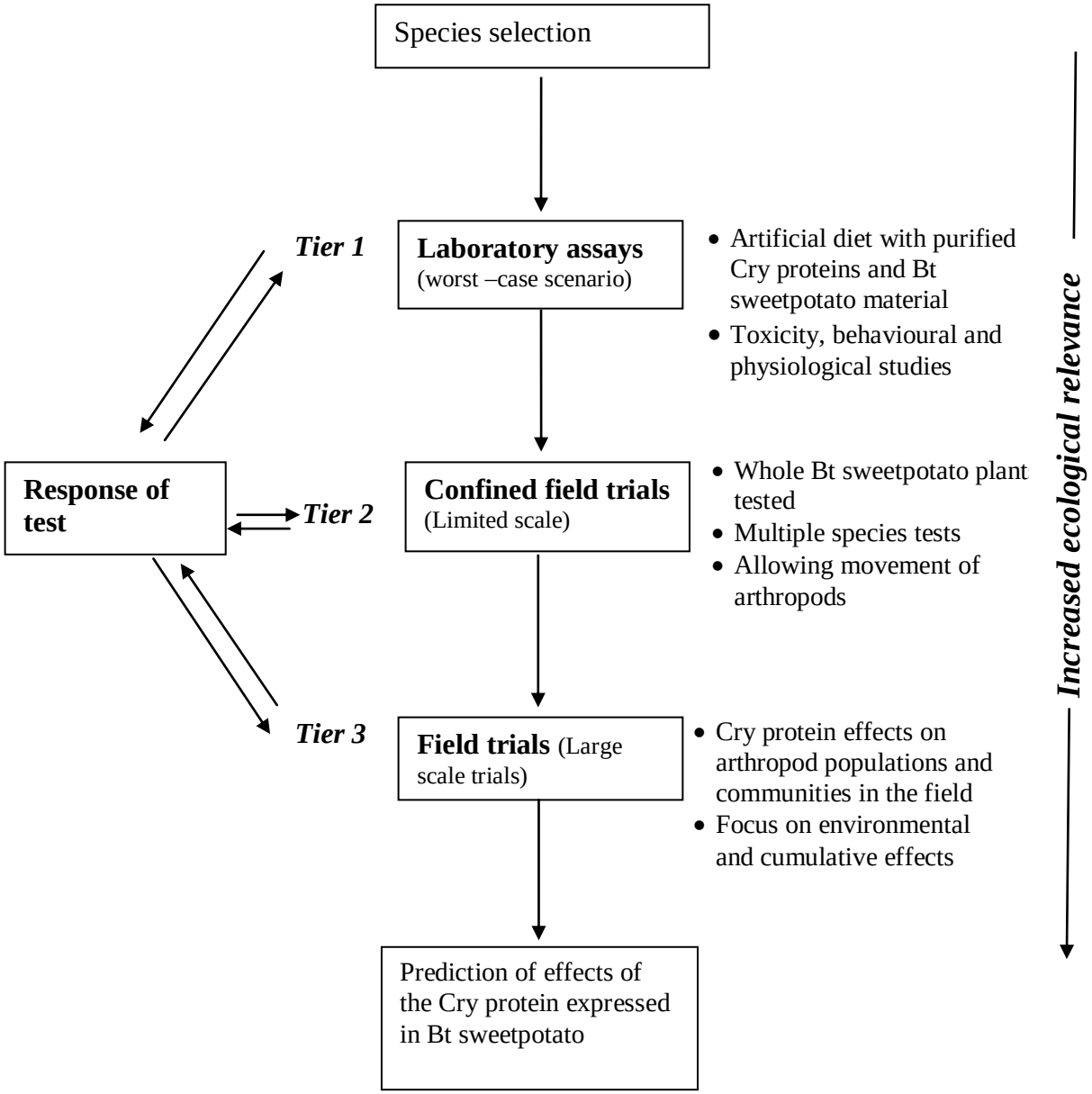


Figure 6.1. Tiered approach for testing effect of Cry proteins non- target organisms found in Bt sweetpotato fields

Lower tier studies must be properly designed and executed to maximize the probability that compounds with adverse effects are detected. The confidence in the conclusions drawn from these studies mainly depends on the study's ability to detect potential hazards, if present (Romeis *et al.*, 2008; Duan *et al.*, 2010). The Cry protein level of the Cry7Aa1, ET33/ET34, and Cry3Ca1 will first be determined for the transgenic events causing mortality in both the storage roots and leaves, and then 10-100 times higher than those present in these tissues will be evaluated in artificial diet bioassays. The use of storage

root-specific promoters (sporamin and β-amylase) in sweetpotato is expected to reduce the amount of Cry proteins in leaves.

6.5 Conclusion

This study provides the scientific rationale for risk assessment of Bt sweetpotato to assist regulatory decision making. The ground, rove, and ladybird beetles have been identified as the primary relevant NTOs which may potentially be affected by cultivation of Bt sweetpotato. Honey bee may be considered as relevant due to their ecological role but there is solid scientific evidence from literature indicating no harm. Potential hazards are evaluated with representative surrogate/indicator species that are selected case by case for their suitability and amenability to test relevant risk. For effective NTO assessment threshold, values need to be defined that elicit the advance to higher tiers as has been done for environmental risk assessments of conventional pesticides. These values will be available when a transgenic event with efficacy to control weevils will be available. At this point it is too speculative to estimate what this threshold could be based on solely the LC_{50} which is currently the only toxicity value known. Tissue specific or enhanced promoters and different Cry protein combinations will influence this threshold value. It is important to note that defining the threshold values is not solely a scientific question but also depends on whether policy-makers are concerned about under- or over-estimating risks considering that sweetpotato is an introduced crop and that Bt sweetpotato will bring food security benefits to vulnerable populations. The NTO testing approach presented above minimises the likelihood of unexpected negative impact on other organisms and help decision-makers to authorise the release of weevil resistant sweetpotato plants with confidence that it will not have undesirable effects on NTOs.

CHAPTER SEVEN

GENERAL DISCUSSION

Cry proteins have been tested for activity against sweetpotato weevil and were highly effective against the weevil when used in *in vitro* insect bioassays (Ekobu *et al.*, 2010). Not only did the delta-endotoxins cause mortality of the weevil larvae and inhibit the growth, but also did this at low concentrations (less than 1.0 µg g^{-1}). This high level of activity, even at extremely low doses made the *cry* genes of interest for *in planta* pest resistance studies. The hypothesis was that if the plant expresses low levels of protein in the tissue, the presence of such highly active proteins could significantly decrease the plant's susceptibility to weevil attack. Thus, the corresponding genes were introduced into sweetpotato via *Agrobacterium tumefaciens* genetic transformation (Kreuze *et al.*, 2009; Ghislain *et al.*, 2013).

Although the *cry* genes were expressed under the control of storage root specific promoters, the Cry protein concentration was low in most roots tested. Sporamin and β-amylase promoters did not confer high levels of Cry protein expression in sweetpotato roots despite previous reports of enhanced accumulation of other proteins in potato (Hong *et al.*, 2008). Cry protein quantification in most events was less than 0.5 µg g^{-1} of fresh storage root flesh, with other events having undetectable levels of protein. Transgene expression in plants has been reported to be highly variable, even among plants independently transformed with the same construct (Hobbs *et al.*, 1993; Mlynarova *et al.*, 1994). The variation in protein expression was probably due to positional effects of the transgene in different events. Transgenes integrate at different chromosomal locations and if integration occurs in a transcriptionally active area, the resulting expression may be influenced by proximal regulatory sequences (Topping *et al.*, 1991; Down *et al.*, 2001; Husnain *et al.*, 2002). As a result large numbers of transgenic events need to be produced and tested in order to find a rare one accumulating significant Cry protein to kill the weevils. Some events such as CIP410008.9, CIP410009.12, CIP410009.14, CIP410009.15 and CIP410010.14 failed to accumulate enough protein. This may be due to the transgene having integrated in a low-transcriptionally active region of the genome, a weak activity of the promoters, and also a general low transcriptional activity in this organ naturally poor in protein.

Given that *Cylas* spp. usually infest the roots of host plants and the larvae develop to adult stage inside the roots, insect-resistance assays were carried out using transgenic sweetpotato roots and sweetpotato root powder in artificial diet assay. Efficacy of Cry proteins expressed in sweetpotato storage roots against *Cylas puncticollis* was investigated using four different bioassays. Ten transgenic events expressing Cry7Aa1, Cry3Ca1, and ET33-34 proteins were evaluated for activity against sweetpotato weevil. The bioassays used in this study were as follows: (i) First instar larva in artificial diet using storage root powder of transgenic events; (ii) whole sweetpotato roots of transgenic events infested with female adults; (iii) sweetpotato root chip and (iv) small sweetpotato root (about 3 cm diameter) infested with 24 hr egg-plugs. The results indicate that whole-root bioassay has less handling injuries to the weevil as compared to other methods and is most convenient to test the whole insect cycle from oviposition to emergence. However, this assay requires a large number of adult females for oviposition and roots per event to be tested to get efficacy results with statistical significance. The small root egg plug bioassay is the most suitable and economical in terms of root number and experimental insects needed to assess larval mortality and development.

Although different insect bioassay methods were used, the results revealed no significant activity of the three Cry proteins against *C. puncticollis* when transformed *I. batatas* fresh roots and root powder were used. This lack of efficacy was attributed to low level of accumulation of the Cry protein in these transgenic events. Previous work on successful Coleopteran pests control using Cry proteins indicated that, high levels of Cry protein expression *in planta* is effective for pest control. The expression of Cry3A protein in sweetpotato root conferring limited field resistance in one trial in Cuba was estimated to be 0.5 - 1.5 µg g^{-1} of fresh storage roots (García *et al.*, 2000). Similarly in maize, Cry3B1 protein in the roots ranged between 3.2 - 66 µg g^{-1} of root tissue for effective control of corn rootworm in MON863 (USEPA, 2003).

Even though there was no effective control of the weevil by the Cry protein in the tested events, understanding inheritance of the *cry* genes is vital for future sweetpotato breeding. The successful transmission and stable expression of transgene to the progeny is important in breeding programmes involving transgenics for further improvement by hybridisation and clonal selection. Transgenic plants sometimes cannot be brought to cultivation

immediately after production because not all the cultivated or elite lines of a crop species are amenable to genetic transformation. Genotypes currently amenable to genetic transformation are not adapted to growing conditions in SSA. Hence a series of crossing and selection is required to introgress the new genes into the elite African varieties.

Transgenic sweetpotato expressing Cry proteins were allowed into Uganda for confined screenhouse trials only. As a result one transgenic event, CIP 410008.7, which had one copy of the transgene integrated in its genome, and Cry7Aa1 protein accumulation of 0.09 µg g^{-1} FW of root tissue, was chosen and used as a maternal parent in transgenic breeding. Transgenes are inherited sexually as a dominant trait (Christou *et al.*, 1989; Misra, 1990; Pawlowski and Somers, 1996; Theuns *et al.*, 2002), with inheritance conforming to Mendelian ratio. Transgene inheritance followed the Mendelian pattern in all the crosses with commercial cultivars (New Kawogo Tanzania and NASPOT 1) as confirmed by PCR analysis. Similar results of Mendelian segregation of transgenes were reported by Okada *et al*. (2002), who evaluated the offspring from crosses between transgenic (R_0 lines) and non-transgenic sweetpotato. Because the maternal parent has a single copy of the transgene, there is no reason to expect a different copy number in the transgenic progeny. However, southern blot assay are usually necessary to determine the copy number of the transgene in progeny. Single copy transgenic lines are desirable for a number of reasons, which permit simple structural documentation and potentially greater stability in gene structure and expression (Yao *et al.*, 2006).

Low Cry toxin expression detected in the maternal parent (0.09 µg g^{-1} FW of root tissue) used could have been due to numerous factors such as the codon usage, the A+T content, the molecular context at the translational start, mRNA sequence and structure (Perlak *et al.*, 1991; Ely, 1993; Corbin *et al.*, 2001). Low expression was still observed in transgenic sweetpotato even when the *cry* genes were codon optimised closer to sweetpotato codons and had a tissue-specific promoter (Sporamin) known to cause higher expression in the roots. The use of coding sequences of the truncated protein instead of the full length protein could have caused low expression in the roots. Even though, the sporamin promoter was considered for driving protein expression in transgenic sweetpotato roots as they are sink tissues for Cry protein, from the results this promoter was not as robust in causing higher expression in the sweetpotato roots.

There were no significant differences in Cry protein expression among F_1 progenies derived from transgenic sweetpotato crossed to conventional sweetpotato varieties. This was not unusual, as stable transgene expression in sexual hybrids has been reported in several instances. In a study examining inheritance and expression of neo and 30gusA genes in transgenic rice, transgene expression did not significantly differ within hybrid progeny groups of generations T2 and T3 (Peng *et al.*, 1995). Another study involving transgenic rice showed that *cry1Ab* transgene expression was quantitatively stable through six sexual generations (Wu *et al.*, 2002). Fearing *et al*. (1997) reported that expression of Cry1Ab protein was shown to be stable over four backcross generations in a study examining protein concentrations in both leaves and pollen of transgenic maize. Accordingly, the uniform protein expression observed in this study is not unique to the crosses involved. Furthermore, this indicates that genetic background may not necessarily affect transgene expression in sweetpotato. On the contrary, some studies have shown that genetic background affected the expression of transgenes. Sachs *et al*. (1998) reported that genetic background greatly impacted on the content of Cry1Ab toxin protein in F2 population derived from Bt transgenic cotton crossed to conventional insect-resistant cotton isolines. In addition, Schmidt *et al*. (2004) also reported that transferring transgenes to distinct genetic backgrounds could lead to different gene expression levels.

There are several proposed factors which may affect the expression of a foreign gene in transgenic progeny. Dunsmuir *et al*. (1989) described a source of variation known as a position effect, in which the expression of an introduced gene is affected by its inserted location in the existing plant genome. Originally, this effect was attributed to the DNA regions surrounding the inserted gene, however, this variability in transgene expression was not reduced when the transgene construct included as much as an 8 kb flanking region around the gene of interest (Dunsmuir *et al.*, 1989). Thus, the factors that cause this type of variation must be able to operate over longer genomic distances. Somaclonal variation has also been attributed to variation in transgene expression levels. The pressures exerted by callus growth and regeneration in tissue culture often alter plant development which could affect the performance of a transgene directly or indirectly as in the methylation of a promoter region. However, since the hybrid lines examined in this study were F_1 plants derived from a parental primary transformant, neither of these factors would be expected to affect their expression. All hybrid lines inherited the same transgene from the parent (CIP410008.7), and thus the most notable source of variation from a plant breeding

standpoint comes from meiotic recombination. In a relatively large sample population, the effect of recombination on transgene expression would presumably be insignificant

The transgene introgression seems not to have affected the morphology and agronomic performance of the sweetpotato progeny. Similarly, Graeber *et al*. (1999) observed no difference between Bt transgenic corn (*Zea mays* L.) and non-transgenic corn with respect to agronomic performance. High variation was observed in all morphological and agronomic traits measured in sweetpotato progeny. Variation among the clones necessitates selection of the best events with farmer preferred morphological and agronomic traits. The high ploidy level (2n = 6x = 90) in sweetpotato could be responsible for variation in these traits due to increased mutations associated with polyploidy (Osborn *et al.*, 2003). Sweetpotato is propagated asexually so any advances made in selection and breeding can be passed on to the producer and consumer without the need for achieving homozygosity.

Crossing New Kawogo and a transgenic event (CIP410008.7) was intended to increase inherent insect resistance in the progeny because both forms of resistance, genetically engineered and classically bred, are likely to provide long-term control of weevils. In Uganda, farmers have consistently reported that the sweetpotato cultivar, New Kawogo, suffers lower sweetpotato weevil damage by harvest time compared to other commercially important varieties (e.g. NASPOT 1 and Tanzania), widely grown in the country (Stevenson *et al.*, 2009). Previous studies also show that New Kawogo contains higher concentrations of the hydroxycinnamic acid ester compounds which are responsible for weevil resistance (Stevenson *et al.*, 2009). The insect bioassays that were carried out, however, revealed no significant resistance of the transgenic progeny and their parental genotypes against *C. puncticollis*. Despite New Kawogo having high concentrations of insect resistance compounds, there seemed to be no synergy between transgenic and natural resistance in the progeny. Natural resistance was quantitatively inherited with low heritability and might have been inherited in very small quantities the tested progeny. New Kawogo's natural resistance has been reported to have deterrent effects on adult feeding and oviposition (Stevenson *et al.*, 2009). In this study, egg plugs were used to infest the roots, instead of using root cores for oviposition and feeding behaviour studies, thus, possibly, this is the reason we did not observe significant resistance.

Before any new transgenic sweetpotato crops can be cultivated commercially, risks to human health and the environment have to be assessed and evaluated as a regulatory requirement in Uganda. One crucial part of the environmental safety assessment of transgenic insect-resistant crops is the evaluation of potential risks to non-target species. The focus is usually on organisms providing ecosystem services, like decomposition, pollination and biological control. The characterisation of risk includes the determination of exposure to the active compound and the hazard of being exposed (Romeis *et al.*, 2008). Many beneficial species could be exposed to the insecticidal protein by feeding directly on transgenic sweetpotato. When natural enemies feed on prey which has previously consumed Bt plants, the insecticidal protein is transferred along the food chain (Romeis *et al.*, 2011). Additionally, species living below ground encounter Bt protein remaining in plant residues, residue-leachates or root exudates (Saxena *et al.*, 2002; Icoz and Stotzky, 2008). However, a certain species is only at risk if the transgenic protein shows toxicity at a realistic level of exposure (Raybould, 2007). Toxicity is usually tested on a range of selected species using laboratory assays (Romeis *et al.*, 2008). Accordingly, the ground, rove, and ladybird beetles were identified as the primary relevant NTOs which may potentially be affected by cultivation of Bt sweetpotato. Honey bees could be considered as relevant due to their ecological role (main pollinator) but there is solid scientific evidence from literature indicating no harm.

CHAPTER EIGHT

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Improvements in genetic transformation technology have allowed the genetic modification of most important food crops. Insect-pest resistance genes from microbial origin are useful to develop transgenic crops. The *cry* genes have proved to be important alone or in combination against Coleopteran pests. The activity of some *cry* genes against *Cylas* spp. in sweetpotato was demonstrated in Cuba (Moran *et al*., 1998; Garcia *et al*., 2000). However, in Africa, bioassays of sweetpotato events expressing Cry7Aa1, Cry3Ca1, and ET33-34, did not show efficacy against the weevil, a result partly ascribed to the low level of Cry protein accumulation in the root tissue. The results also showed that efficacy of transgenic events against the sweetpotato weevil are best assayed by using the small sweetpotato root infested with 24 hr egg plugs as first screening test. Those transgenic events found to be effective against the weevil can then be screened using the whole root bioassay to characterise better which step(s) in the weevil biology is affected.

The crosses in transgenic breeding were done to understand the inheritance and expression pattern of *cry* genes in sweetpotato. The knowledge attained could help in future of sweetpotato breeding, since directly transformed African genotypes are not yet available for field testing. The data illustrates the feasibility of crossing transgenic and non-transgenic sweetpotato to obtain transgenic progeny following the established segregation ratios. There were no significant differences in Cry protein expression among hybrid sweetpotato derived from the transgenic event crossed to different conventional sweetpotato varieties. This is not unusual, as stable transgene expression in sexual hybrids has been reported in many instances. In the preliminary screenhouse and laboratory evaluation of morphological and agronomic traits, the sweetpotato F_1 genotypes showed variation in plant type, internode length, foliage weight, number of roots, vine vigour, root and skin colour, β- carotene content and resistance to *C. puncticollis*. Since sweetpotato is a hexaploid, significant variation in all traits studied indicated segregation of many genes for the given trait.

Although the transgenic sweetpotato is not yet grown commercially in Uganda, it is important to identify non-target organisms which are likely to be affected by the Bt sweetpotato in the field. Non-target arthropod species commonly found in sweetpotato fields that are related taxonomically to the weevils were identified through expert consultation and literature review and are expected to be evaluated initially. The review indicates the presence of relevant non-target Coleopterans that could be affected by Bt sweetpotato varieties: ground, rove, and ladybird beetles. These insects are important predators of insect pests in sweetpotato fields. Additionally the honey bee, is the main pollinator of sweetpotato and used for honey production. The NTO testing approach presented here aims at minimising the likelihood of unexpected negative impact on other organisms and help decision-makers to authorise the release of weevil resistant sweetpotato with confidence that the crop will not have undesirable effects on non-target arthropods.

8.2 Recommendations

Since the first generation of transgenic sweetpotato expressing Cry proteins did not perform with the desired level of resistance to *C. puncticollis*, the future prospect is to evaluate more transgenic events carrying *cry* genes because this increases the possibility of finding those with higher Cry protein accumulation. Additionally, it will be useful, in the long term, to identify alternative tissue specific promoters that can drive strong expression of transgenes in the sweetpotato roots. Furthermore, the evaluation of F_1 progeny was done under controlled environments because of the regulatory requirements in Uganda; further field trials along with additional transgenic events are required to provide additional information on their performance in the field and study the effect of the transgenes on agronomic performance.

With regards to transgenic crops, the current biosafety regulations require that GM crops be evaluated to identify potential risks that their release would trigger. When Bt sweetpotato effective against the sweetpotato weevil is available in Uganda, there is need to do risk assessment on the non-target organisms at laboratory and field level to access the impact of Bt sweetpotato on these arthropods. However, years of research have indicated that there are no significant risks associated with current commercial GM crops. The biosafety regulation needs to continuously adjust to scientific progress rather than being frozen in time when little was known about transgenic crops. In this regard,

transgenic breeding will need a more agile regulatory environment if the full potential of this technology is deemed to contribute to Ugandan agriculture improvement.

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