

Full Length Research Paper

Segregation analysis of *cry7Aa1* gene in F₁ progenies of transgenic and non-transgenic sweetpotato crosses

Rukarwa R. J.¹, Mukasa S. B.^{1*}, Sefasi A.², Ssemakula G.³, Mwanga R. O. M.⁴ and Ghislain M.⁵

¹School of Agricultural Sciences, Makerere University, P.O. Box 7062, Kampala, Uganda.

²International Potato Center, Applied Biotechnology Laboratory, P. O. Box 1558 Lima,

Peru. ³National Crop Resources Research Institute, P. O. Box 7084, Uganda.

⁴International Potato Center – Kampala, P. O. Box 7065, Kampala, Uganda.

⁵International Potato Center, Nairobi, P. O. Box 25171, Nairobi 00603, Kenya.

Accepted September 11, 2013

Sweetpotato weevils are the most devastating pests of sweetpotato causing yield losses ranging from 60 to 100%. Their cryptic nature, where the larvae is found within plant tissues render them difficult to manage especially using chemicals control. Development of weevil resistant sweetpotato was conducted by crossing a transgenic event CIP410008.7 as a female parent with three Ugandan cultivars as male parents. Crossing event CIP410008.7 with New Kawogo, Tanzania and NASPOT1 gave 57, 32 and 19 seeds respectively. A total of 86 F₁ progenies were analysed for the presence of *cry7Aa1* using polymerase chain reaction (PCR). Expected 608 bp bands were amplified in progenies that contained the cry gene. The gene was integrated at different frequencies in the F₁ progenies of different families: CIP410008.7 x New Kawogo (47.2%), CIP410008.7 x Tanzania (52%) and CIP410008.7 x NASPOT1 (44.4%). Chi-square test showed that all the three families followed a 1:1 segregation *cry7Aa1* gene ratio. This study shows the transfer of a transgene from genetically modified event into elite sweetpotato lines.

Key words: Weevil resistance, transgenic plants, sweetpotato, *cry7Aa1*.

INTRODUCTION

The African sweetpotato weevils, *Cylas puncticollis* B. and *C. brunneus* F. are highly destructive pests of sweetpotato throughout sub-Saharan Africa (Fuglie, 2007; Andrade et al., 2009). They attack sweetpotato both in the field and during storage causing substantial losses of up to 60 to 100% (Sorensen, 2009). Stem damage to the vascular system caused by larval feeding and tunnelling reduces the size and number of roots. Weevil feeding on storage roots induces terpenoid production that makes even slightly damaged roots unpalatable (Stathers et al., 2003).

The use of resistant varieties is the most appropriate

way to control sweetpotato weevils because it is more effective and safe for the environment. However, cultivars with reliable 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 behavior, and numerous mating incompatibilities, make conventional breeding difficult. Given the lack of progress in conventional breeding, exploring the option of genetic engineering using *Bacillus thuringiensis* (Bt) could provide the resource

*Corresponding author. E-mail: sbmukasa@agric.mak.ac.ug.

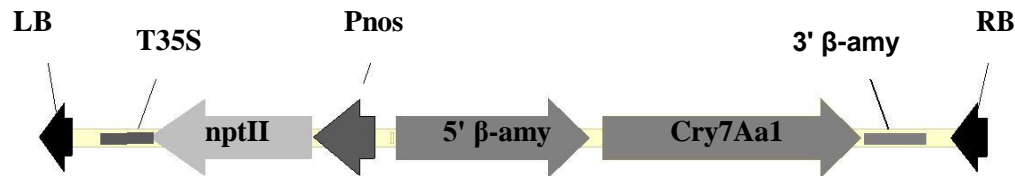


Figure 1. Map of genetic elements in pCIP78 transferred into sweetpotato for expression of the Bt protein, *Cry7Aa1*.

poor farmers with a better alternative to sweetpotato weevil control.

Significant progress has been made in the recent past in transferring Bt cry genes into the American sweetpotato cultivar Jewel (Kreuze et al., 2009). However, this cultivar is not well adapted to sub-Saharan Africa. Transformation of cultivars adapted to Africa using the protocol for Jewel would thus be a suitable option. Unfortunately, the response of sweetpotato to *in vitro* regeneration and transformation conditions is cultivar-specific. Farmer preferred African sweetpotato cultivars have been reported to be recalcitrant and hardly regenerate transformed shoots from calli (Sefasi et al., 2012). As a result, there is limited development of African sweetpotato cultivars expressing agronomically important traits such as weevil resistance. Crossing adapted African cultivars with characterised transgenic events carrying cry genes can circumvent the problem of *in vitro* recalcitrance of elite cultivars and facilitate the introgression of the transgene. Furthermore, use of these transgenic cultivars as parents in breeding programs has some advantages over transformation of existing elite African cultivars which requires several years of evaluation to ensure that introduced gene is stably expressed and no other detrimental phenotypic effects are induced by transformation process. In this case, large numbers of transgenic progeny can be produced from crosses involving transgenic parents, and these can be evaluated following conventional selection programmes. However, in order for transgenic crops to be acceptable for commercial exploitation it is essential that transgenes should be predictable and stably incorporated.

Thus the aim of this study was to introduce a weevil resistant gene (*cry7Aa1*) from transgenic event CIP410008.7 of cultivar Jewel into African cultivars New Kawogo, Tanzania and NASPOT1, and verifying the integration and segregation in F1 progenies of different families.

MATERIALS AND METHODS

Parental plants

The transgenic sweetpotato from Cv. Jewel expressing *cry7Aa1* gene for partial 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 a β -amylase promoter. The cassette also contained the *nptII* as a marker gene under the control of the Nopaline synthase (*Pnos*) promoter (Figure 1). One of the resulting transformed sweetpotato events, 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 three Ugandan cultivars; Tanzania, New Kawogo and NASPOT 1. Cultivars Tanzania and New Kawogo are ranked among the five superior and farmer preferred cultivars grown in Uganda because of their wide adaptability and high dry matter content (Mwanga et al., 2001), whereas cv. NASPOT 1 dominates 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). Cultivar New Kawogo is rated as one of the most tolerant cultivars to weevil infestation while Tanzania and NASPOT 1 have been ranked as very susceptible to sweetpotato weevils.

Progeny generation and management

Crossing the sweetpotato was done in a screenhouse at National Crops Resources Research Institute (NaCRRI). The transgenic Jewel was used as female parent and the Ugandan cultivars 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. The plants were fertilized and irrigated according to standard regimes for screenhouse sweetpotato propagation. Pollen of the male parents was collected from a crossing block outside the facility. 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 followed by soaking in concentrated sulfuric acid (98% H_2SO_4) for 45 min to break dormancy. This was followed by several rinsings with water for 5 min and subsequently sown in nursery boxes.

PCR analysis of *cry7Aa1* gene

Genomic DNA was isolated from all sweetpotato leaf samples using the CTAB method (Stacey et al., 2000). Polymerase chain reaction (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'-ACAACATCATCACCATACCAAAC-3' and 5'-AAGAGCAAGATGCAAGTTTG-3' as forward and reverse primers, respectively. All the PCR experiments took place in a total volume of 25 μ l containing 50 ng of total plant DNA made as follows 12.5 μ l of Ready mix Taq® DNA polymerase (Sigma-Aldrich, Poole, UK)



Figure 2. Sweetpotato breeding process. (A) sweetpotato scion grafted on *I. setosa* root stock; (B) sweetpotato fully bloomed flower (C); seed capsules of sweetpotato produced from the crosses; (D) sweetpotato seed.

and primer F (0.5 μ l) and primer R (0.5 μ l) and adjusted to 25 μ l with nuclease free water. PCR conditions were as follows: initial denaturation of DNA at 93°C for 2 min and then amplified through 35 thermal cycles of 93°C for 15 s, 55°C for 30 s, 72°C for two minutes and ending by a final extension step at 72°C for 7 min. The PCR amplicons were separated by electrophoresis on 1% agarose gel in Tris-EDTA (TE) buffer stained with ethidium bromide and visualized under UV light. The presence or absence of the transgenes in the hybrids was used to confirm the transgenic and non transgenic progeny. The presence or absence of the transgenes in the F1 progeny was used to confirm the segregation in transgenic lines. The segregation data were analysed by the χ^2 test at $P < 0.05$ to determine if the observed segregation ratios of *cry7Aa1* fit the expected Mendelian 1:1 phenotypic ratio.

RESULTS AND DISCUSSION

Sweetpotato progeny development

In this study, all sweetpotato cultivars failed to flower under greenhouse conditions five months after planting. The failure to flower could be due to the uncontrolled environmental conditions in which the plants were exposed to in the greenhouse. 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 h day length or less, temperatures of 20 to 25°C and relative humidity of 75% usually promote flowering in sweetpotato. Flowering in the transgenic event was only achieved after grafting sweetpotato to its wild relative *I. setosa* (Figure 2A). Similarly, the Ugandan cultivars failed to flower under greenhouse conditions even after grafting; pollen was obtained from a field crossing block. Pollination was done on flowers of the female parent which were fully bloomed (Figure 2B). After pollination the initial fruit grew and produced round shaped green capsules with a diameter of 1 to 2cm (Figure 2C). Seeds that were removed from the capsules were dark brown, small with a diameter of 3 to 4mm (Figure 2D).

Not all sweetpotato flowers which were pollinated produced capsules that could be harvested. When ovaries of the transgenic event were crossed with the pollen from the Ugandan cultivars, the flowers had a tendency to abort. Probably, various degenerative processes 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 developed seed upon fertilization despite previous reports of complex incompatibilities and sterility mechanisms (Murata and Matsuda, 2003).

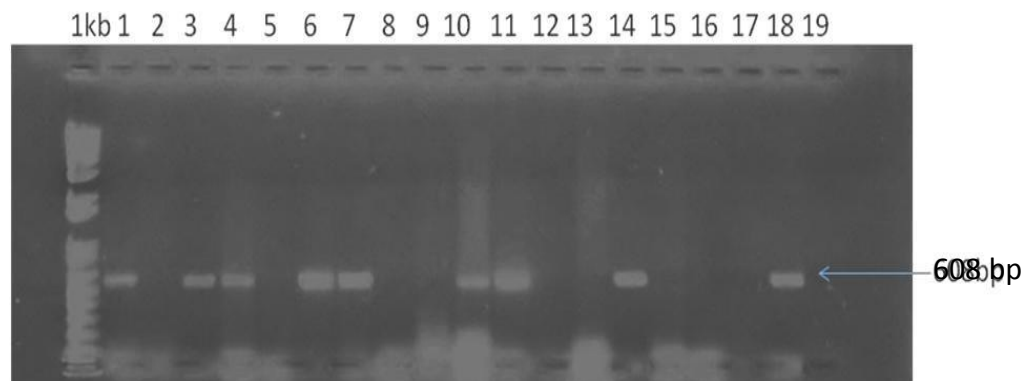
The polyploidy nature of sweetpotato ($2n = 6x = 90$) and numerous mating incompatibilities, make crossing this crop difficult (Murata and Matsuda, 2003). Crosses between event CIP410008.7 and Ugandan cultivars, however, managed to produce capsules and seeds in this study. The number of capsules produced 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 1). Some crosses were not possible and practically all crosses produced much less than the potential four seed per capsule. Each sweetpotato cross in this study produced 1 to 2 seeds per capsule. After scarification with sulfuric acid to break the seed dormancy and floating in water, all the seeds planted germinated. Seeds that were light or deformed were discarded.

Segregation and integration of *cry* gene

Due to the heterozygous nature of sweetpotato, crosses among the sweetpotato cultivars resulted in high levels of segregation at F1 generation that enable evaluation or selection. The F1 progenies produced in this study were

Table 1. Sweetpotato seed capsule and seed production from crosses between CIP410008.7 and three Ugandan cultivars.

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

**Figure 3.** PCR analysis for the F1 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. 1kb ladder, 1-17 plant numbers, Positive control (18) and negative control (19).

evaluated using PCR to check the integration of the weevil resistance gene. PCR analysis of *cry7Aa1* using the gene-specific primers in some samples of progenies from crosses between CIP410008.7 and Ugandan cultivars are shown in Figure 3. In some sweetpotato samples evaluated, the amplification produced a DNA band sized 608 bp and some did not show any amplification. This suggests that some of the evaluated progenies positively contained the *cry* gene that was integrated from transgenic CIP410008.7 which is the weevil resistant parent in the crosses.

Sweetpotato is considered an autohexaploid with hexasomic inheritance (Kumagai et al., 1990; Anwar et al., 2009). 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 a 4:1 ratio if present as a double copy (duplex). Southern blot analysis has shown that the *cry* gene was integrated with one copy in the genome of transgenic event CIP410008.7 (Ghislain M, Pers comm). 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 were susceptible to the weevil had an allelic composition at the locus as rrrrrr which is recessive for weevil resistance. The presence of the *cry*

gene in this study followed a simplex hexasomic model of inheritance in all the three families.

In all the 86 plants tested, dominance effects of the *cry* gene were observed because at least 50% of all progenies from the crosses carried *cry7Aa1* gene. Crosses of CIP410008.7 x New Kawogo, CIP410008.7 x Tanzania and CIP410008.7 x NASPOT 1 produced 25, 13 and 8 progenies carrying the *cry* gene derived from the transgenic parent respectively (Table 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). 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.

Crosses between commercial sweetpotato cultivars would theoretically produce a high diversity for many characters. Each F1 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.

Table 2. Segregation of cry7Aa1 gene by PCR analysis on the F1 progenies of sweetpotato.

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

In practice, a 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.

Conclusion

Our data illustrates the feasibility of crossing transgenic and non transgenic sweetpotato to obtain transgenic progeny following the established segregation ratios. The F1 sweetpotato progenies that produced DNA bands sized 608 bp positively contained cry gene derived from transgenic event CIP410008.7. The sweetpotato lines that contained the cry gene need to be tested further for their resistance to the sweetpotato weevil in the laboratory and selected further for their farmer preferred morphological characteristics. The purpose of this work is to provide plant breeders with some direction for future research in transgenic breeding.

ACKNOWLEDGEMENTS

We acknowledge Mr Moses Mwendha for taking care of transgenic plants in the greenhouse, and the International Potato Center and the Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) for funding this work.

REFERENCES

- Andrade M, Barker I, Cole D, Dapaah H, Elliott H, Fuentes S, Grüneberg W, Kapinga R, Kroschel J, Labarta R, Lemaga B, Loechl C, Low J, Lynam J, Mwanga R, Ortiz O, Oswald A, Thiele G (2009). Unleashing the potential of sweetpotato in Sub-Saharan Africa: Current challenges and way forward. Working Paper 2009. Lima, Peru: International Potato Center (CIP).
- Anwar N, Kikuchi A, Kamugai T, Watanabe KN (2009). Nucleotide sequence variation associated with β -amylase in the sweetpotato *Ipomoea batatas* (L.) Lam. *Breed. Sci.* 59:209-216.
- Fuglie KO (2007). Priorities for sweetpotato research in developing Countries: Results of a survey. *Hort. Sci.* 42:1200-1206.
- Kreuze KF, Valkonen JPT, Ghislain M (2009). Genetic Engineering. In Loebenstein G, Thottappilly G (eds.), *The Sweetpotato*. Springer-Verlag New York Inc.
- Kumagai T, Umemura Y, Baba T, Iwanaga M (1990). The inheritance of beta-amylase null in storage roots of sweet potato, *Ipomoea batatas* (L.) Lam. *Theor. Appl. Genet.* 79(3):369-376.
- Luo HR, Santa Maria M, Benavides J, Zhang DP, Zhang YZ, Ghislain M (2006). Rapid genetic transformation of sweetpotato (*Ipomoea batatas* (L.) Lam) via organogenesis. *Afr. J. Biotechnol.* 5:1851-1857.
- Murata T, Matsuda Y (2003). Histological studies on the relationship between the process from fertilisation to embryogenesis and the low seed set of sweetpotato, *Ipomoea batatas* (L.) Lam. *Breed. Sci.* 53:41-49.
- Mwanga ROM, Odongo B, P'Obwoya CO, Gibson RW, Smit NEJM, Carey EE (2001). Release of five sweet potato cultivars in Uganda. *Hort. Sci.* 36:385-386.
- Mwanga, ROM, Odongo B, Turyamureeba GM, Alajo A, Yencho GC, Gibson RW, Smit NEJM, Carey E E (2003). Release of six Sweet potato cultivars (NASPOT1 to NASPOT 6) in Uganda. *Hort. Sci.* 38:475-476.
- Okada Y, Nishiguchi M, Saito A, Kimura T, Mori M, Hanada K, Sakai J, Matsuda Y, Murata T (2002). Inheritance and stability of the virus-resistant gene in the progeny of transgenic sweetpotato. *Plant Breed.* 121:249-253.
- Rossel G, Espinoza C, Javier M, Tay D (2008). Regeneration guidelines: sweet potato. In: Dulloo ME, Thormann I, Jorge MA, Hanson J (eds). *Crop specific regeneration guidelines CGIAR System-wide Genetic Resource Programme*, Rome, Italy.
- Sefasi A, Kreuze J, Ghislain M, Manrique S, Kiggundu A, Ssemakula G, Mukasa SB (2012). Induction of somatic embryogenesis in recalcitrant sweetpotato (*Ipomoea batatas* L.) cultivars. *Afr. J. Biotechnol* (In press).
- Sorensen KA (2009). Sweetpotato Insects: Identification, Biology and Management. In: Loebenstein G, Thottappilly G (eds.) *The Sweetpotato*. Springer-Verlag New York Inc.
- Stacey J, Isaac PG, Rapley R (2000). Isolation and Purification of DNA from Plants. *The Nucleic Acid Protocols Handbook*. Humana Press.
- Stathers TE, Rees D, Nyango A, Kiozya H, Mbilinyi L, Jeremiah S, Kabi S, Smit N (2003). Sweetpotato infestation by *Cylas* spp. in East Africa: II. Investigating the role of root characteristics. *Int. J. Pest Manag.* 49:141-146.
- Stevenson PC, Muyinza H, Hall DR, Porter EA, Farman D, Talwana H, Mwanga, ROM (2009). Chemical basis for resistance in sweetpotato *Ipomoea batatas* to the sweetpotato weevil *Cylas puncticollis*. *Pure Appl. Chem.* 81:141-151.