Transfer of sweetpotato-like genes expressing Cry proteins into sweetpotato varieties

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Abstract

Seven proteins from *Bacillus thuringiensis* (*Bt*) were previously tested for activity against the two African weevil species, *Cylas puncticollis* and *C. brunneus*. Three of them (Cry7Aa1, CryET33/CryET34, Cry3Ca1) were effective against both African weevils with an LC_{50} below 1 ppm. Therefore, *cry7Aa1*, *cry3Ca1*, and *cryET33/cryET34* gene constructs were developed taking into account sweetpotato optimized codon usage, promoters with storage root expression and wound response, and two selectable marker genes. Combinations of two weevil resistant (WR) genes were also included as an insect-resistance management component. A total of 10 WR gene constructs have been developed and 5 of them are currently used for direct gene transfer into relevant African sweetpotato varieties. Regeneration and genetic transformation protocols were applied to two African cultivars ("Tanzania" and "Wagabolige") selected as the most prominent cultivars in Uganda and neighboring countries. After almost two years of testing different regeneration and transformation protocols for the African varieties, scientists at CIP have obtained only one transformed shoot (from "Wagabolige") with one of the single-WR gene constructs. Non-African varieties "Jewel" and "Huachano" were genetically transformed with three single-WR and two double-WR gene constructs producing 18 and 12 putative transformed regenerants respectively. These plants, when confirmed to be genetically transformed with the WR gene constructs, will be used to produce leaves and storage roots in the biosafety greenhouse to be forwarded to the US and African partners for testing activity against weevils.

Keywords: Sweetpotato, weevil, *Bt*, genetic transformation.

Introduction

Sweetpotato (*Ipomoea batatas*) is an important crop in sub-Saharan Africa (SSA), especially during seasons when other staples are scarce. The African weevils *Cylas puncticollis* and *C. brunneus* are the main biological constraints, accounting to losses between 50 and 100%. Sweetpotato-weevil larvae attack the tuberous root, causing the most economic damage, even during storage, which is important for marketability and in dry seasons. Piecemeal harvesting is commonly practiced in SSA, which in turn favors weevil infestation, due to the exposure of the plants in the field for long periods (Stathers *et al.*, 2005).

Resistance to sweetpotato African weevils has not been achieved through conventional breeding due to the lack of the necessary genes in the sweetpotato gene pool. A potential source of resistance that could be used in conventional breeding is found in the latex (Stevenson *et al.*, 2009), however, varieties with this resistance have not been adopted widely in SSA. A biotechnological approach to attain this resistance could be used.

Bacillus thuringiensis (*Bt*) toxin-encoding genes have been used extensively in modern agriculture, through modification of the genome (James, 2008). *Bt* toxins active against weevils have been reported (Bravo *et al.*, 1998; Donovan *et al.*, 1992; Génissel *et al.*, 2003; Guzov *et al.*, 2007; Höfte *et al.*, 1987; Lambert *et al.*, 1992; Mettus & Baum, 2006; Peferoen *et al.*, 1998; Sekar *et al.*, 1987), some of which are being used commercially (Agbios, 2009). Based on the evidence of successful application of *Bt* technology to control insect pests, we have devised a strategy to introduce *Bt* genes into sweetpotato.

Previous experiments developed essentially at NARO in Uganda under the direction of Prof. Moar revealed that seven *Bt* toxins (CryET70, CryET33/CryET34, Cry3Aa3, Cry3Ba2, Cry3Bb3, Cry3Ca1 and Cry7Aa1) displayed activity against the two African sweetpotato weevil species. Three proteins had a LC_{50} below 1 ppm: CryET33/CryET34,

Cry3Ca1, Cry7Aa1 which made these good candidates for engineering resistance to weevils. We report here the progress in transferring the corresponding genes into sweetpotato.

Materials and methods

Transformation plasmids

All three *Bt*-gene coding sequences were optimized for sweetpotato codon usage and synthesized. The sporamin promoter and 3' regions were attached to both *cryET33/cryET34* and *cry3Ca1* coding sequences. The gene *cry7Aa1* was attached to the ß-amylase promoter and 3' region. All genes were inserted into pCAMBIA1305.1 (CAMBIA, Canberra, Australia) or into pCIP100, a modification of pCAMBIA1305.1 conferring kanamycin resistance to plants. Six binary vectors bearing single Bt genes were developed, three on pCAMBIA1305.1 and three on pCIP100. Another four binary vectors were constructed bearing either the *cry7Aa1* and the *cryET33/cryET34* genes or *cry7Aa1* and *cry3Ca1*, two of these vectors on pCAMBIA1305.1 and two on pCIP100.

Plant transformation

Sweetpotato varieties Tanzania, Jewel, Wagabolige and Huachano were obtained from CIP's germplasm bank. The genetic transformation method used was that described in Medina-Bolivar *et al*. (2003). Regeneration was performed using a two-step protocol described in Ormachea (2008) for Wagabolige and Tanzania, Cruzado (2009) for Huachano and Luo *et al*. (2006) for Jewel.

Molecular analysis

Shoots were screened by callus testing, as described in Cruzado (2009), PCR amplification of the transgene, Southern blotting for transgene copy number and NCM-ELISA detection of the expressed protein. Polyclonal antibodies for NCM-ELISA detection were produced in rabbits by inoculating the purified *Bt* protein and collecting sera after 2 weeks. Transgene expression was quantified through real-time PCR based on SYBER Green I methodology. The results obtained were carried out using the software REST©. The housekeeping *cox* gene was used as reference of analysis.

Tuberous root and leaf production

Shoots confirmed to be positive were grown in the Biosafety greenhouse for tuberous root production. Plantlets were transferred from *in vitro* conditions directly into soil pellets. After one week they were planted on plastic pots and remained there until tuberous root formation.

Results and discusion

Transformed plants

Genetic transformation of the African variety Tanzania yielded no shoots, even after transforming more than 10,000 explants. Using approximately the same amount fo explants, we obtained one regenerating shoot for Wagabolige. This shoot has been confirmed to bear the transgenes and seems to have three copies of the *cry7Aa1* gene and two copies of the Kanamycin resistance *nptII* gene. This could be due to incomplete transfer of the T-DNA in one of the insertions.

Five Huachano shoots were obtained bearing the double gene construct *cry7Aa1* and *cryET33/cryET34*. These shoots were positive by callus and PCR screening.

Jewel transformation yielded one shoot bearing the *cryET33/cryET34* gene, 8 shoots with the *cry7Aa1* gene and 9 shoots with the *cry3Ca1* gene, 2 shoots bearing the double gene construct *cry7Aa1* and *cryET33/cryET34*, and 6 shoots with the double gene construct *cry7Aa1* and *cry3Ca1*. All shoots were positive by PCR and callus

testing. NCM-ELISA was assayed for 6 different shoots bearing the *cry7Aa1* gene and 4 bearing the *cry3Ca1* gene. All shoots were positive by NCM-ELISA.

Transgene expression

Five transformed events bearing the *cry7Aa1* gene were analyzed for transgene expression in the leaf. The event with the lowest expression (LE) is used as reference. Two events were found to have statistically significantly higher expression than the LE reference with expression levels of 10.01 and 1.74 times higher (Figure 1), whereas the other two events did not have significantly higher expression levels.

The transgene expression ratios in four plants were not higher in comparison with the housekeeping *cox* gene. Because the promoters used to guide gene expression were taken from the genes of the two major proteins in the tuberous root (Hattori & Nakamura, 1988; Nakamura *et al.*, 1991), it is expected that gene expression in the tuberous roots will be higher. This will allow us to localize the expression, a strategy used for insect resistance management (Ferré *et al.*, 2005).

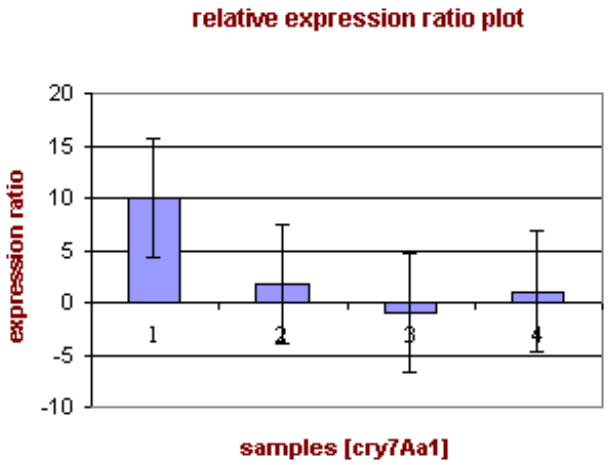

Figure 1. Quantitative determination of gene expression in transformed events of sweetpotato var. jewel with *cry7Aa1* inserted

Tuberous root and leaf assays

Tuberous roots and leaves produced in the Biosafety greenhouse will be used for toxin quantification by DAS-ELISA and gene expression by Real-time PCR.

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