Weevil Resistant Sweetpotato through Biotechnology

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Abstract

African sweetpotato weevils (SPW), *Cylas puncticollis* and *C. brunneus*, pose a major threat to sweetpotato, which plays a vital role in food security and income generation for both the urban and rural poor in Sub-Saharan Africa (SSA). SPW can limit sweetpotato production causing total crop loss. Control methods such as integrated pest management and breeding have not succeeded satisfactorily in curbing out these pests thus opening the door for using biotechnology and genetic engineering to make transgenic sweetpotato that are resistant to weevil infestation. At least three protein samples from *Bacillus thuringiensis* (Bt) have been found to be toxic to both SPW species at less than 1 ppm (Cry7Aa1, CryET33/CryET34, and Cry3Ca1). Corresponding gene constructs were developed using sporamin and β amylase regulatory sequences to express and accumulate high Cry protein levels in the storage root. Approximately 100 transformed events from sweetpotato cultivars (including one African cultivar), were produced by *Agrobacterium tumefaciens* transformation of petioles and somatic embryos. Gene expression from leaf tissues using qRT-PCR revealed up to 20X difference among events. Protein accumulation using DAS-ELISA and storage roots exhibited even larger variation between events. However, so far only 18 of the 90 events have produced storage roots which could be bio-assayed. Most events accumulated Cry proteins below the LC_{50} level, two events accumulated Cry protein at the LC_{50} level and only one event accumulated Cry protein above the LC_{50} level (3 times the LC_{50} level). Bioassays using transgenic tissues infested with SPW larvae are on-going but preliminary results reflect low toxicity as expected based on Cry protein content in the storage roots. Future steps include the screening of additional events, characterization of competitive binding of these Cry proteins and confined field trials of SPW-resistant events.

INTRODUCTION

In Sub-Saharan Africa (SSA) sweetpotato is an important staple food for both the urban and rural poor, and also a source of income from local market sales. Weevils are the most important threat in areas with significant dry spells to sweetpotato productivity, marketability, and sustainability. A recent survey on the socio-economic impact of weevils in Uganda, reports an average yield loss between wet and dry seasons of over 28% between both seasons (Kiiza et al., 2009).

The principal weevil species that cause severe damage to sweetpotato in SSA are *Cylas puncticollis*, and *C. brunneus*, both of which are widespread in SSA. Larvae create feeding tunnels that rapidly rot making the storage root unsuitable for human consumption due to production of bitter tasting and toxic sesquiterpenes. Integrated pest management to control weevil has been successful in Cuba for the control of *C. formicarius* (Lagnaoui et al., 2000). However, these practices are not likely to be

applicable in SSA because field sanitation, the most effective way of controlling weevils, is very labor intensive for small-scale farms practicing piecemeal harvesting. In addition, farmers in SSA are not willing to pay for pheromones should these be available.

Reported variation in susceptibility to weevils of African sweetpotato cultivars was found to be related mostly to escape mechanisms rather than genetic factors providing antibiosis type of resistance (Stathers et al., 2003a,b). However, the local cultivar 'New Kawogo' has been found with useful level of resistance under low infestation. The mechanism has been partially elucidated (Stevenson et al., 2009). This research in progress may provide a useful resistance component to be combined with the transgenic approach proposed here. However, no high level of resistance to weevils from the crop's germplasm is yet available for use in breeding.

Insect resistance, specifically to *Lepidopteran* and *Coleopteran* pests, has been successfully achieved in several crops using genetically engineered plants that express insecticidal proteins similar to the crystal (Cry) proteins from *Bacillus thuringiensis* (Bt). Use of these Bt crops has resulted in a significant reduction in insecticide use, yield increase, increased grower profitability, and increased diversity of non-target insects (Cattaneo et al., 2006). This makes exploring the Bt option particularly attractive given the lack of progress with conventional breeding.

The general objectives of this research are: (i) to demonstrate resistance to weevils (*Cylas puncticollis* and *C. brunneus*) in genetically engineered sweetpotato plants with genes expressing proteins active against the pest (ii) to develop local biotechnology capacity in Uganda to generate new transformed plants. We have previously established that three different Bt protein samples (Cry7Aa1, cryET33/cryET34, Cry3Ca1) were the most effective against both African weevils with an LC_{50} value (mg/ml diet) against 1st instar *C. puncticollis* and 2nd instar *C. brunneus* below 1 ppm (Ekobu et al., 2010). This value is low enough for insecticidal activity required for economic control and to achieve a "high dose" associated with insect-resistance management with the Bt crop approach (Moar, pers. commun.). We report here the progress achieved since the identification of the three most active Cry proteins against the African weevils.

MATERIALS AND METHODS

Five gene constructs were developed in Peru taking into account sweetpotato codon usage, promoters with storage root expression and wound response, and one selectable marker gene. Each of the gene elements were acquired free of property rights through either agreements or our own isolation. The coding sequence for each *cry* gene was synthesized chemically by Entelechon (Germany) and codon optimized with Leto 1.0 software. The regulatory elements were those of the sporamin (NCBI X13509) and β-amylase (NCBI D12882) genes from sweetpotato. For we chose the *nptII* gene because it is routinely used and deregulated in many crops available commercially worldwide.

Genetic transformation of sweetpotato was achieved through *Agrobacterium tumefaciens* strain EHA105 bearing one of the cry gene constructs applied to freshly cut petioles plus leaves which then regenerate directly to shoots, or to embryogenic callus followed by shoot regeneration. This first route, referred to as organogenesis, has been used for the US cultivar 'Jewel' and 31 African cultivars following Luo et al. (2006). The second route uses lateral or apical meristems, referred to as somatic embryogenesis, and was used for the same 31 African cultivars, 'Jonathasn', and a Peruvian cultivar, 'Huachano' using a modified protocol derived from Kreuze et al. (2008).

Gene expression was assayed using real-time quantitative PCR (RTqPCR) of the relative expression of the cry genes compared to a housekeeping gene which in our case was the cytochrome oxidase (cox) gene. Standard procedures were used starting with an extraction of RNA from leaves followed by cDNA synthesis to serve as template for the RTqPCR. Primers specific for each cry gene were designed using AlleleID 7.6 software. The expression level was calculated using the REST-Version 2009 software.

Cry protein accumulation was estimated from leaves, skin, and flesh of the storage roots using total soluble protein extracts obtained from standard protocols. First,

polyclonal antibodies from rabbits were produced using purified Cry proteins following standard protocols. Their specificity and titer were verified against the three Cry protein samples. Quantification of Cry protein in biological samples was done by double antibody sandwich ELISA techniques using standard protocols and staining with alkaline phosphatase. A standard curve was drawn using the stock of purified Cry proteins. Total soluble proteins were quantified using the Bradford standard protocol.

Bio-assays were developed using *Cylas puncticollis* as the insects are easier to handle. Four bio-assays were used: (i) an artificial diet assay as described in Ekobu et al. (2010); (ii) a whole root assay where each root was infested with 10 female adults for 24 h for ovoposition; (iii) a root chip assay in which two plugs of non-transgenic root each containing one egg were inserted; (iv) a small root assay in which two plugs from a non-transgenic flesh each containing one egg was inserted. Formation of pupae and adult emergence were calculated and compared to the non-transgenic cultivar.

RESULTS AND DISCUSSION

Cry Gene Constructs

We did not use the usual promoter driving constitutive expression (CaMV35s) for two reasons: (i) we only need weevil protection in the storage root where eggs are laid; (ii) the *cry* genes will be as similar as possible to sweetpotato gene which may be more appealing to regulators or/and consumers. Instead, we used two promoters reputed to have high storage root expression and be wound-inducible (Hattori et al., 1990; Wang et al., 2002; Yoshida et al., 1992; Ohto et al., 1992). Combinations of two weevil-resistant (WR) genes were also included as a component of the insect-resistance management.

The cry gene for Cry7Aa1 is constituted by the first 1,351 of the β-amylase gene *AmyB* (NCBI D12882) with an extra 30 bp including a *Pst*I cloning site and 411 bp after the stop codon. The coding sequence for the cry7Aa1 gene was based on the amino acid sequence of the Cry7Aa1 toxin, residue 59 to 637 of NCBI AAA22351, starting with a methionine followed by a valine for appropriate initiation sequence context (Joshi et al., 1997). The cry genes for ET33-34 and Cry3Ca1 are constituted by the first 1,121 bp of the sporamin gene *gSPO-A1* (NCBI X13509) and 378 bp after the stop codon with small changes due to PCR cloning from local cultivar in Peru. The sample CryET33/CryET34 is an equimolar mix of two small Cry proteins. Therefore, we have used a fusion of both protein referred to as CryET33-34, and used as coding sequence the amino acid sequence described in patent US 2004/0023875 A1 (p.18-19). The coding sequence for cry3Ca1 gene was based on the amino acid sequence of the Cry3Ca1 toxin, residue 64 to 649 of NCBI CAA42469, starting with a methionine followed by a valine for the reason aforementioned. The small differences in the regulatory sequences are expected to be non-significant based on literature. Finally, the *cry* genes were inserted within the T-DNA region of the plant transformation vector pCAMBIA1305.1 which was deleted of its hygromycin resistance gene and replaced by a synthetic *nptII* gene producing 5 cry gene constructs (Table 1).

Genetic Transformation of Sweetpotato

We have pursued two strategies to genetically transform sweetpotato to introduce the *cry* genes. First, we used direct transformation of 'Jewel' via organogenesis and obtained 33 transgenic events with different *cry* genes (Table 2). All of them were selected on kanamycin, PCR positive, produced callus on highly selective media and were finally unambiguously demonstrated as transformed and independent event, by Southern blotting. This protocol was also used to screen 31 African cultivars available from the genebank in Peru. Using a threshold of 40%, 6 out of the 31 turned out to good regeneration efficiencies ('Mugande', 'Imby', 'Luapula', 'Kawogo', 'Zambezi' and 'Mafutha'). Secondly, we used in parallel the transformation protocol based on somatic embryogenesis using several competent cultivars including the same 31 African cultivars. After 8 to 12 months, the cultivar 'Imby' from Burundi produced 31 independent

transgenic events.

At the inception of the project, genetic transformation had been mostly successful with non-African cultivars ('Jewel', 'Huachano', 'Jonathan') of which the identification of one transgenic event with high accumulation of Cry proteins causing high mortality of weevils would offer the option for crossing with African germplasm (Transgenic breeding). Hence, we have made crosses with African cultivars and will study inheritance of the *cry* gene(s) with special attention to stability across genotypes of its accumulation in storage roots.

Gene Expression and Cry Protein Accumulation

Most of the transgenic events from 'Jewel' were analyzed for gene expression in leaves and accumulation of the Cry proteins in storage roots. Three transgenic events did not grow well and were therefore not studied. Using RNA extracted from leaves, we observed differences which were quantified relative to the event with the lowest expression for the cry gene tested. Hence, for *cry7Aa1* gene, 1 event out of 13 was found to have significantly higher expression; for *cry3Ca1* gene, 1 event out of 15 was found to have significantly higher expression; for *cryET33-34* gene, 2 events out of 10 were found to have significantly higher expression (Fig. 1). Transcription analysis using RNA extracted from few storage roots was conducted only to show that *cry* genes are expressed in storage roots.

Because production of storage roots was difficult in the environmental conditions in Peru, the small storage roots were reserved for the Cry protein quantification. We did not observe clear correlation between transcription in leaves and Cry protein accumulation in the leaf or storage roots (Fig. 2). However, this quantification assay will have to be conducted on storage roots produced under normal conditions. The level of accumulation of Cry proteins in the storage root flesh is relatively low with only two transgenic events around the LC_{50}.

Bio-Assays

After transferring the transgenic events from Peru to Africa, these produced normal storage roots which were challenged against weevils using four different assays. Each one had its pros and cons but none provided evidence that a transgenic event was conferring mortality against the weevils. The artificial diet assay may not have maintained the Cry protein active due to heating of the diet before pouring into petri dishes, the whole root assay infested with female adults required larger number of roots to be tested, root chip assays were too difficult to maintain free from fungal contamination, and the small root assay required also a larger number. Hence, in none of the transgenic events tested so far, was mortality achieved.

CONCLUSION

Three *cry* genes were developed as sweetpotato-like genes using gene regulatory sequences of sweetpotato genes to accumulate Cry proteins in the storage root where the efficacy is primarily needed. These were assembled into 5 *cry* gene constructs. These were carefully developed to be free of any contractual or intellectual property rights restrictions on use in African countries.

Genetic transformation of the sweetpotato crop remains a tedious process. When regeneration of African cultivars was tested by organogenesis, approximately 20% of them presented workable regeneration efficiency (above 40%). Somatic embryogenesis was a far slower protocol but yielded more independent transgenic events. One African cultivar was found to be amenable to genetic transformation through somatic embryogenesis but more will be needed to broaden the genetic base of such new trait. Alternatively or complementarily, transgenic breeding is feasible in sweetpotato but will require most likely at least two cycles of crossing and progeny selection.

Bio-assays conducted on 29 transgenic events from 'Jewel' did not result in revealing efficacy against weevils. These assays were done on normal storage roots using

Cylas puncticollis. The quantification of Cry protein in storage roots produced in Peru gives us a clue towards a possible explanation for lack of efficacy. Indeed, low levels were observed with only two transgenic events accumulating Cry proteins around 1 µg/g of fresh weight of flesh. This value is the one conferring mortality in the artificial diet using purified Cry proteins. However, the artificial diet has 3 times less dry matter which may cause the larvae to feed more than in a normal storage root. The artificial diet is prepared using purified pro-toxin whereas in the plant we have the toxin fragment leading to possible difference in stability and activity. Finally, the larvae suffer more stress in the artificial diet due to direct mechanical handling and are therefore more susceptible to any additional stress than when hatching in the root flesh.

In conclusion, the absence of mortality is likely to be explained by low accumulation of Cry protein in the storage roots. Therefore, more transgenic events will be tested to identify one with higher levels of Cry protein accumulation, new *cry* genes will be constructed with translation enhancers and pro-toxins, and bio-assays will be conducted to detect sub-lethal activity against the sweetpotato weevils. Despite this setback, we will persist in our efforts to produce sweetpotato weevil resistant cultivars because weevils are responsible for significant crop losses annually in sub Saharan Africa affecting the poorest when there is food shortage.

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Tables

Table 1. The five *cry* gene constructs developed using the β-amylase and sporamin genes from sweetpotato where their original coding sequence was substituted by a synthetic sequence to express the toxin fragment of Cry7Aa1 and Cry3Ca1 and a fusion protein of CryET33 and CryET34.WR stands for weevil-resistant.

Weevil-resistant genes and constructs		
WR gene 1	WR gene 2	*cry* gene construct
β-Amy:cry7Aa1	none	pCIP78
gSPOA1:cry3Ca1	none	pCIP79
gSPOA1:ET33-34	none	pCIP82
β-Amy:cry7Aa1	gSPOA1:cry3Ca1	pCIP84
gSPOA1:ET33-34	β-Amy:cry7Aa1	pCIP85

Table 2. Transgenic events with *cry* genes from sweetpotato cultivars: 'Jewel' was through organogenesis while all others were through somatic embryogenesis.

cry gene construct	Transgenic events from sweetpotato cultivars					
	Jewel	Wagabolige*	Huachano	Imby	Mugande*	Jonathan
pCIP78 (*cry7Aa1*)	8	1	nd[+]	nd	nd	nd
pCIP79 (*cry3Ca1*)	9	nd	nd	nd	nd	nd
pCIP82 (*cryET33-34*)	8	nd	nd	nd	nd	nd
pCIP84 (*cry7Aa1+cry3Ca1*)	6	nd	0	31	2	18
pCIP85 (*cry7Aa1+cryET33-34*)	2	nd	5	nd	nd	nd

* indicates not true-to-type.
[+] nd, not done.

Figures

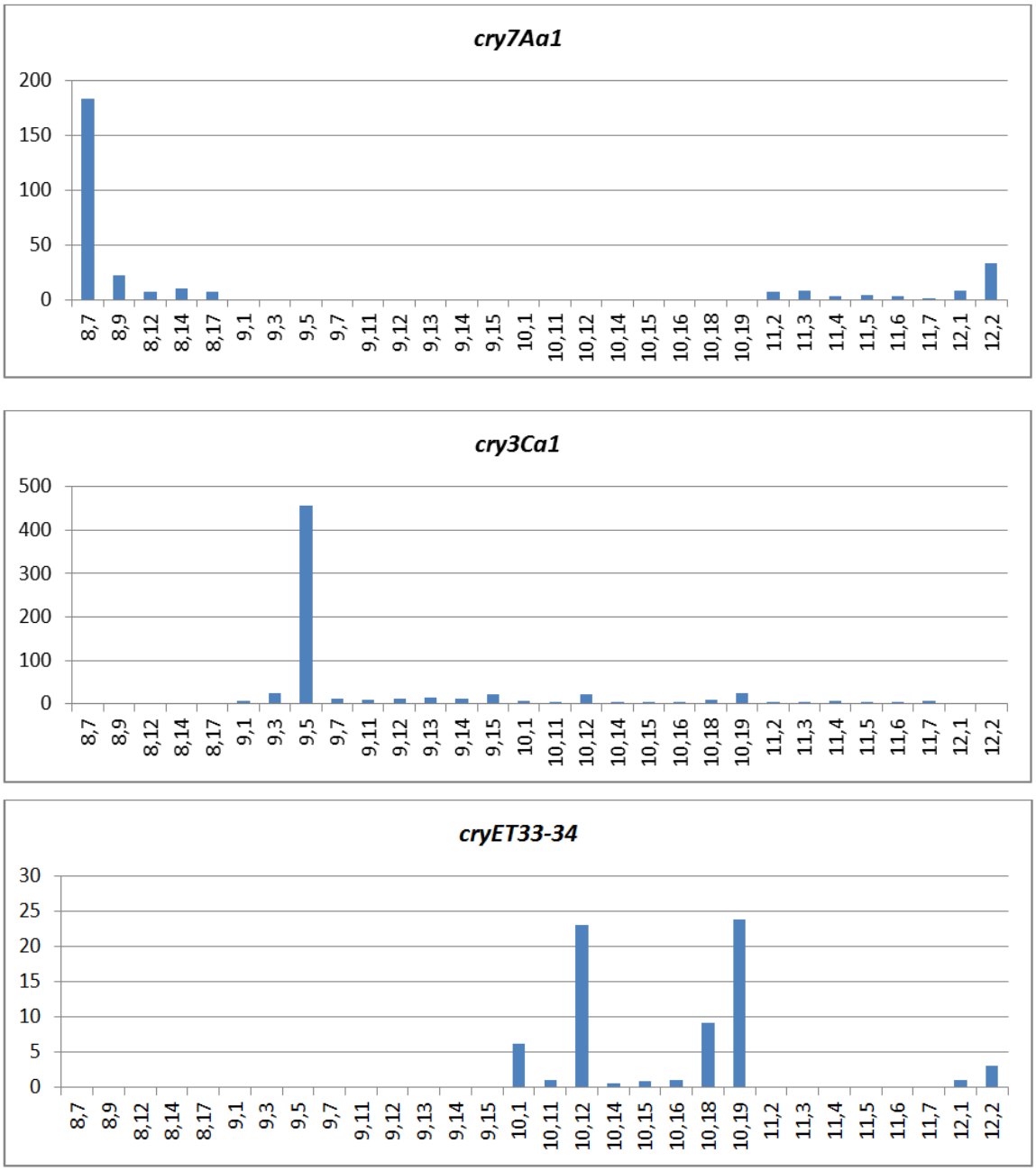

Fig. 1. Relative expression plot of *cry* genes in leaves of transformed events from the cultivar 'Jewel'. The Y axis represents the relative amount with respect to the lowest expresser event. The X axis represents the transgenic events (*.x have *cry7Aa1*; 9.x have *cry3Ca1*; 10.x have *cryET33-34*; 11.x have *cry7Aa1* + *cry3Ca1*; and 12.x have *cry7Aa1* + *cryET33-34*).

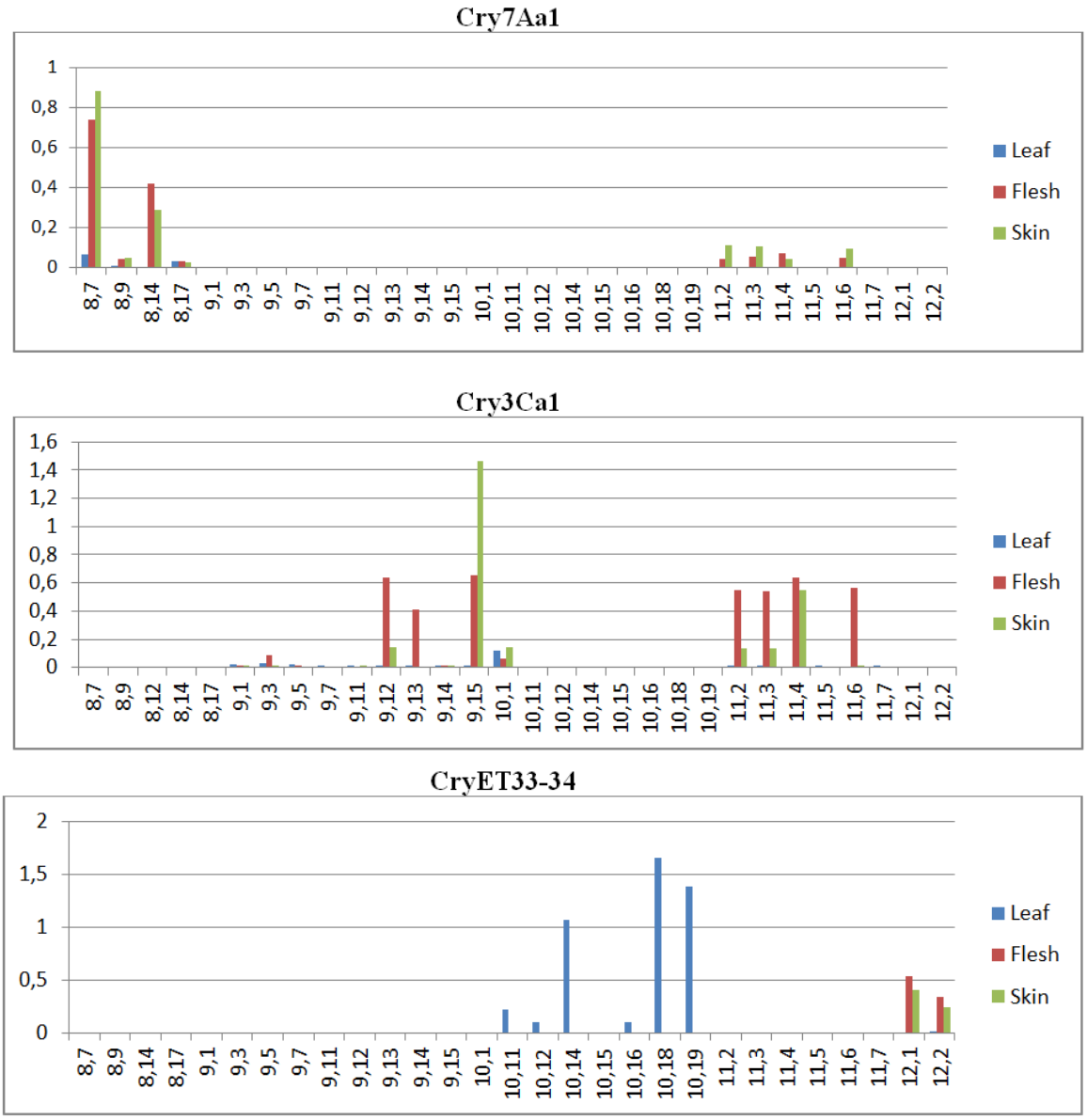

Fig. 2. Relative accumulation plot of Cry proteins in leaves of transformed events from the cultivar 'Jewel'. Each assay was conducted using antibodies against the protoxin and are represented here as µg per g fresh weight (Y axis). The X axis represents the transgenic events referred in Figure 1.