



# Insect Resistant Cowpea Event 709A

Supporting Dossier for the Permit Application for Introduction into the  
Environment and Placing on the Market of a Genetically Modified Organism  
in Ghana

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**SUBMITTED TO**

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**REGULATORY DOSSIER NUMBER**

AAT/DPS-20VUIR-GH

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## Summary

Cowpea is a very important crop in the semiarid farming systems of West African countries and is unique in providing food, cash, and fodder. Cowpea has a high potential to increase farmers' and traders' incomes, thereby contributing to poverty reduction and food security. As a food crop, cowpea is a primary source of cheap protein for the ever-growing population of both rural and urban dwellers. The fodder and husks from cowpea form an important source of protein, fibre, and energy for livestock.

West Africa accounted for over 80 percent of global cowpea production in 2016, and in northern Ghana it is the second most important crop after groundnut. The most critical threat to cowpea production in Africa is destruction by insect pests, which can cause total yield loss if uncontrolled. The legume pod borer, *Maruca vitrata*, is one of the most serious pests of cowpea in this region, with reported yield losses of 20–80 percent.

Cowpea pod borer has been controlled primarily by synthetic insecticides, although in some cases resistance to these has developed. Additionally, insecticide costs are often out of the reach of growers, and those that are applied can pose risks to growers and their families owing to lack of appropriate training and personal protective equipment. Low literacy also affects the ability of many farmers to read and follow application instructions. To provide an additional tool for control of *M. vitrata*, cowpea has been bioengineered to have resistance to lepidopteran insect pests via expression of the Cry1Ab insecticidal protein from the widely used biopesticide, *Bacillus thuringiensis* (Bt).

Transgenic cowpea event AAT-7Ø9AA-4 (hereafter 709A cowpea) was produced by *Agrobacterium tumefaciens*-mediated transformation of embryogenic seed explants with plasmid pMB4 resulting in the introduction of the *cry1Ab* gene from *B. thuringiensis* subsp. *kurstaki* strain HD-1 and the neomycin phosphotransferase II encoding gene (*nptII*) from the Tn5 transposon of *Escherichia coli* strain K12 as a selectable marker. The Cry1Ab and neomycin phosphotransferase II (NPTII) proteins have a history of safe use in approved genetically engineered crops in many countries worldwide.

Molecular characterization of the introduced DNA within 709A cowpea confirmed the presence at a single insertion site of the transfer-DNA (T-DNA) region derived from plasmid pMB4 that was stably inherited over multiple generations as a single genetic locus according to Mendelian rules of inheritance. Nucleotide sequencing of the entire inserted DNA, including portions of the 5' and 3' flanking cowpea genomic sequence, was performed to confirm its organization and the potential for creating any new novel open reading frames. The inserted DNA was comprised of one complete copy of the plasmid pMB4 T-DNA, except for 10-bp and 29-bp truncations at the 5' and 3' termini, respectively, and a 735-bp inverted repeat derived from the 3' end of the T-DNA. The site of insertion of the T-DNA was mapped to a region on chromosome 2 of the cowpea genome. There were no new novel open reading frames created as a consequence of the DNA insertion that would have the potential to encode proteins with any significant amino acid sequence similarity to known or suspected toxins or allergens.

Expression of both Cry1Ab and NPTII is controlled by constitutive promoters and accumulation of some amount of each protein would be expected in all plant tissues. In samples obtained from field-grown plants of event 709A introgressed into cowpea variety IT97K-499-35, the highest concentrations of Cry1Ab were measured

in flower tissue (5.6–22.8  $\mu\text{g/g}$ ) and in pods (5.7–17.9  $\mu\text{g/g}$ ), while average Cry1Ab concentrations in leaves, green cotyledons, and dry seed ranged between 2.6–4.1  $\mu\text{g/g}$ . Tissue samples of leaves, flowers, pods, green cotyledons, and dry seed obtained from greenhouse-grown 709A plants contained similar amounts of Cry1Ab protein, with average concentrations ranging from *ca.* 2–5  $\mu\text{g/g}$ . Measurable amounts of Cry1Ab were also found in samples of roots, pollen, and anthers taken from 709A plants grown in the greenhouse.

The average concentrations of NPTII protein in samples of leaves, flowers, pods, green cotyledons, pollen, anthers, and roots obtained from 709A plants grown in the greenhouse were similar, ranging from 0.16–0.35  $\mu\text{g/g}$ . The highest amounts of NPTII protein were found in dry seed samples, with an average concentration of 1.5  $\mu\text{g/g}$ .

Cowpea consumption is the highest of all pulse crops in sub-Saharan Africa, accounting for *ca.* 42 percent of total pulse consumption in 2006–08. Within the different sub-regions, the highest rates of cowpea consumption are in West Africa, with Nigeria having the greatest *per capita* availability of *ca.* 18 kg per year. Using this rate of consumption (*ca.* 0.8 g/kg body weight per day), the maximum potential human daily dietary exposures to Cry1Ab and NPTII proteins from 709A cowpea were estimated to be *ca.* 2.4 and 1.3  $\mu\text{g/kg}$  body weight, respectively, based on the highest concentrations of these proteins measured in grain (seed).

Cowpea forage (bot, vines, and leaves) fresh, or dried as hay or silage, is typically used for livestock animal feed. Cowpea hay, which is comprised of leaves and stems (stover), plays a particularly critical role in feeding livestock during the dry season in many parts of West Africa. Estimates of maximum daily dietary exposure of cattle, sheep, and poultry to Cry1Ab and NPTII were derived for forage (fodder), hay, and seed derived from 709A cowpea. The highest exposures would be for lambs consuming diets containing up to 100 percent 709A cowpea fodder, where daily dietary intakes for Cry1Ab and NPTII were estimated at *ca.* 1.76 and 0.06 mg/kg body weight, respectively.

Both Cry1Ab and NPTII have received positive affirmations of safety during regulatory reviews of more than 90 and 120 transgenic events, respectively, in numerous crop species. The US Environmental Protection Agency has issued exemptions from the requirement of a tolerance for both the Cry1Ab and NPTII proteins expressed in event 709A cowpea, meaning that these proteins are considered safe at any conceivable level of dietary exposure.

The safety assessment of Cry1Ab and NPTII followed a “weight-of-evidence” approach that considered amino acid sequence similarity to known toxins or allergens, digestibility under standardized *in vitro* conditions, and appropriate oral toxicity studies. Bioinformatic analyses of both Cry1Ab and NPTII found no significant sequence similarities with known mammalian toxins and allergens. The Cry1Ab and NPTII proteins were rapidly degraded in simulated gastric fluid containing pepsin and neither protein was acutely toxic in mice when tested at dosages up to 4,000 mg/kg body weight and 5,000 mg/kg body weight, respectively. Relative to the highest estimates of potential human dietary exposure, the concentrations of Cry1Ab and NPTII proteins used to demonstrate lack of acute oral toxicity represented safety margins of 1.6 million-fold and 3.8 million-fold, respectively. There are no conceivable exposure scenarios, whether for infants consuming weaning foods containing 709A cowpea or

any other population sub-group consuming products derived from 709A cowpea, that would result in less than acceptable safety margins for Cry1Ab and NPTII.

The experience with genetically engineered crop plants with introduced traits conferring insect-resistance and/or herbicide-tolerance has indicated that the incorporation of these traits has not had a biologically meaningful impact on the composition of key nutrients and anti-nutrients. As part of the weight-of-evidence approach for evaluating whether there were any unanticipated consequences of the genetic modification, compositional analyses were performed to compare the concentrations of major nutrient components in samples of whole grain, leaves, and fodder collected from event 709A and control cowpea grown at four different locations representing typical cowpea growing conditions in West Africa. In addition, concentrations of key minerals and phytic acid were determined in samples of whole grain.

From the combined-sites analysis across the four locations, there were no statistically significant differences in concentrations of proximates, moisture, calories, minerals, or phytic acid between grain samples collected from event 709A and control cowpea. Similarly, there were no significant differences in concentrations of proximates, moisture, and calories between 709A and control cowpea leaf and fodder samples when analyzed using the combined-sites model. Overall, no consistent patterns emerged to suggest that biologically meaningful changes in composition or nutritive value of the grain, leaves, or fodder had occurred as a consequence of the genetic modification or expression of the Cry1Ab and NPTII proteins in 709A cowpea. Thus, food products derived from 709A cowpea are compositionally equivalent to their conventional counterparts.

The genetic modification resulting in cowpea event 709A was not intended to affect a specific agronomic or phenotypic characteristic, except to confer resistance to lepidopteran pests, such as the cowpea pod borer. To confirm that 709A cowpea was otherwise agronomically equivalent to its conventional counterpart, yield and other agronomic (phenotypic) measurements were collected from multi-location confined field trials conducted during 2016 at three different locations in northern Ghana, the main cowpea producing area. These data were supplemented with agronomic data collected from similar trials conducted during 2014 and 2015 and three locations in Nigeria.

Across the three locations where field trials were conducted in Ghana during 2016, significant differences were noted between 709A and control cowpea in pods per plant, *Maruca* damaged pods per plant, and *Maruca* damaged seed per plant. When data from the different locations were analyzed individually, there were significant reductions in the number of *Maruca*-damaged pods per plant on 709A plants compared to control cowpea at each trial site, while significant differences in pods per plant, *Maruca* damaged seed per plant, and total and healthy seed per plant were observed only at the Nyankpala location. There were no differences observed in percent germination (emergence), days to first flowering, plant height, total seed weight, sucking insect damaged seed weight, or healthy seed weight between 709A and control cowpea across locations.

From the field trials in Nigeria during 2014 and 2015, there were no significant differences observed between 709A and control cowpea when data from 2014 were

analyzed across locations, and in 2015, the only statistically significant difference between 709A and control plants from the combined-sites analysis was in the percentage of *Maruca* damaged pods per plant, which were near-zero for 709A plants and ca. 13 percent (range: 5.9–20.9) for control plants ( $p = 0.024$ ).

Overall assessment of the agronomic data from multiple years of testing at locations in Ghana and Nigeria did not identify trends of significant differences between 709A and control cowpea except for resistance to *Maruca* damage. Collectively, the comparative agronomic and phenotypic data support the conclusion that the genetic modification resulting in event 709A did not have an unintended, unexpected, effect on plant growth habit and general morphology, vegetative vigour, or grain yield. From the data and observations, there were no indications that 709A cowpea would be more invasive or persistent in the environment, or have altered susceptibility to pests and diseases, compared to conventional cowpea. Except for the intended resistance to *Maruca* pod borer, 709A cowpea is agronomically and phenotypically equivalent to conventional cowpea.

The data presented in this submission have not identified potential environmental hazards or health and safety concerns. Thus cowpea varieties containing event 709A will not pose a risk to the environment relative to conventional cowpea, and food and livestock feed derived from 709A cowpea are as safe as food and feed derived from conventional cowpea varieties.

## Study Catalogue

A listing of submitted study reports and their correlation with relevant sections of the application dossier is shown in the following table.

| Study ID     | Dossier Section | Citation.   |
|--------------|-----------------|---|
| DD2018-03014 | 4.1             | Moore, A. and Higgins, T. (2018b). Southern characterization of the inserted DNA within cowpea event AAT-7Ø9AA-4. Technical report, DD2018-03014 (unpublished), CSIRO Agriculture and Food., Canberra, ACT 2610, Australia.   |
| DD2018-03010 | 4.2             | Upadhyaya, N. and Higgins, T. (2018). Nucleotide sequence analysis of the inserted DNA and host genomic flanking regions in cowpea event AAT-7Ø9AA-4. Technical report, DD2018-03010 (unpublished), CSIRO Agriculture and Food, Canberra ACT, Australia.  |
| DD2018-03015 | 4.3             | Moore, A. and Higgins, T. J. (2018c). Stability of the inserted DNA across multiple generations of cowpea event AAT-7Ø9AA-4. Technical report, DD2018-03015 (unpublished), CSIRO Agriculture and Food, Canberra, ACT 2610, Australia.   |
| DD2018-05002 | 4.4             | Mohammed, B., Ishiyaku, M. F., Abdullahi, U., and Katung, M. (2018). Segregation of the introduced Cry1Ab insect resistance trait within multiple generations of cowpea event AAT-7Ø9AA-4. Technical report, DD2018-05002 (unpublished), African Agricultural Technology Foundation, Jabi-Abuja, Nigeria.   |
| DD2018-03011 | 5.1             | Moore, A. and Higgins, T. (2018a). Concentrations of Cry1Ab and NPTII in different plant tissues derived from cowpea event AAT-7Ø9AA-4. Technical report, DD2018-03011 (Unpublished), CSIRO Agriculture and Food, Canberra ACT, Australia.  |
| DD2018-05004 | 5.2             | Bogdanova, N. and MacKenzie, D. J. (2018a). Estimated daily dietary exposure to Cry1Ab and NPTII proteins expressed in cowpea event AAT-7Ø9AA-4. Technical report, DD2018-05004 (unpublished), Donald Danforth Plant Science Center, St. Louis, MO.   |
| DD2018-03025 | 5.3             | Bogdanova, N. and MacKenzie, D. J. (2018b). Estimated Livestock Animal Dietary Exposure to Cry1Ab and NPTII Proteins Expressed in Cowpea Event AAT-7Ø9AA-4. Technical report, DD2018-03025 (unpublished), Donald Danforth Plant Science Center, St. Louis, MO.  |
| DD2018-03002 | 6.1.2           | MacKenzie, D. (2018b). Amino acid sequence similarity search between Bacillus thuringiensis subsp. kurstaki strain HD-1 Cry1Ab and known and putative protein toxins. Technical report, DD2018-03002 (unpublished) Donald Danforth Plant Science Center, St. Louis, MO.   |
| DD2018-03001 | 6.1.3           | MacKenzie, D. (2018a). Amino acid sequence similarity search between Bacillus thuringiensis subsp. kurstaki strain HD-1 Cry1Ab and known and putative protein allergens. Technical report, DD2018-03001 (unpublished), Donald Danforth Plant Science Center, St. Louis, MO.   |
| DD2018-03005 | 6.2.2           | MacKenzie, D. (2018d). Amino acid sequence similarity search between Escherichia coli neomycin phosphotransferase II and known and putative protein toxins. Technical report, DD2018-03005 (unpublished), Donald Danforth Plant Science Center, St. Louis, MO.  |
| DD2018-03004 | 6.2.3           | MacKenzie, D. (2018c). Amino acid sequence similarity search between Escherichia coli neomycin phosphotransferase II and known and putative protein allergens. Technical report, DD2018-03004 (unpublished) Donald Danforth Plant Science Center, St. Louis, MO.  |
| DD2018-03003 | 7               | Abdourhamane, I., Addae, P., Nwankwo, F., Nangayo, F., Ishiyaku, M., Umar, M., Abudulai, M., Adazebra, G., and MacKenzie, D. (2018b). Compositional analysis of grain, leaves, and fodder from event AAT-7Ø9AA-4 and control cowpea grown during 2015 in West Africa. Technical report, DD2018-03003 (unpublished), African Agricultural Technology Foundation, Nairobi, Kenya.   |
| DD2018-05005 | 8.1             | Utono, I., Mohammed, B., Adamu, R., Abdourhamane, I., Addae, P., Nwankwo, F., Nangayo, F., Ishiyaku, M., Umar, M., Abudulai, M., and Adazebra, G. (2018). Agronomic and phenotypic characterization of event AAT-7Ø9AA-4 and non-transgenic control cowpea grown at multi-location confined field trials during 2014 in Nigeria. Technical report, DD2018-05005 (unpublished), African Agricultural Technology Foundation, Jabi-Abuja, Nigeria. |
| DD2018-05001 | 8.2             | Abdourhamane, I., Addae, P., Nwankwo, F., Nangayo, F., Ishiyaku, M., Umar, M., Abudulai, M., and Adazebra, G. (2018a). Agronomic and phenotypic characterization of event AAT-7Ø9AA-4 and non-transgenic control cowpea grown at multi-location confined field trials during 2015 in Nigeria. Technical report, DD2018-05001 (unpublished), African Agricultural Technology Foundation, Jabi-Abuja, Nigeria.                                    |
| DD2019-08001 | 8.3             | Abudulai, M., Adazebra, G., Seidu, A., and Atokple, I. (2019). Agronomic and phenotypic characterization of event AAT-7Ø9AA-4 and non-transgenic control cowpea grown at multi-location confined field trials during 2016 in Ghana. Technical report, DD2019-08001 (unpublished), the African Agricultural Technology Foundation and the Council for Scientific and Industrial Research, Tamale, Ghana.   |



## Abbreviations

|                |  |
|----------------|--|
| <b>AATF</b>    | African Agricultural Technology Foundation                   |
| <b>AIRS</b>    | aminoimidazole ribonucleotide synthetase                     |
| <b>ARMG</b>    | antibiotic resistance marker gene                            |
| <b>BLAST</b>   | basic local alignment search tool                            |
| <b>BLOSUM</b>  | BLOcks SUBstitution Matrix                                   |
| <b>BSA</b>     | bovine serum albumin   |
| <b>Bt</b>      | <i>Bacillus thuringiensis</i>                                |
| <b>CAT</b>     | castor bean catalase   |
| <b>CCM</b>     | cowpea co-cultivation medium                                 |
| <b>CFT</b>     | confined field trial   |
| <b>CI</b>      | confidence interval  |
| <b>CSIR</b>    | Council for Scientific and Industrial Research               |
| <b>CSIRO</b>   | Commonwealth Scientific and Industrial Research Organisation |
| <b>DDE</b>     | daily dietary exposure                                       |
| <b>DREAM</b>   | Dynamic Research Evaluation for Management                   |
| <b>DUS</b>     | distinctness, uniformity, and stability                      |
| <b>DWT</b>     | dry weight   |
| <b>EC</b>      | effective concentration                                      |
| <b>EEC</b>     | estimated environmental concentration                        |
| <b>EFSA</b>    | European Food Safety Authority                               |
| <b>ELISA</b>   | enzyme-linked immunosorbent assay                            |
| <b>EM</b>      | estimated marginal   |
| <b>EPA</b>     | Environmental Protection Agency                              |
| <b>ERA</b>     | environmental risk assessment                                |
| <b>FAO</b>     | Food and Agriculture Organization of the United Nations      |
| <b>FARRP</b>   | Food Allergy Research and Resource Program                   |
| <b>FASTA</b>   | FAST All sequence alignment tool                             |
| <b>FDA</b>     | Food and Drug Administration                                 |
| <b>FWT</b>     | fresh weight tissue  |
| <b>GENEEC</b>  | generic estimated environmental concentration                |
| <b>GM</b>      | genetically modified   |
| <b>HPT</b>     | hygromycin phosphotransferase                                |
| <b>IAR-ABU</b> | Institute for Agricultural Research, Ahmadu Bello University |
| <b>IFPRI</b>   | International Food Policy Research Institute                 |
| <b>IITA</b>    | International Institute of Tropical Agriculture              |
| <b>ILSI</b>    | International Life Sciences Institute                        |
| <b>INERA</b>   | Institut de l'Environnement et de Recherches Agricoles       |
| <b>IRM</b>     | insect resistance management                                 |
| <b>LB</b>      | Left Border  |
| <b>LC</b>      | lethal concentration   |
| <b>LS</b>      | least squares  |
| <b>MOE</b>     | margin of exposure   |
| <b>MRID</b>    | master record identification number                          |
| <b>NARS</b>    | National Agricultural Research System                        |
| <b>NBA</b>     | National Biosafety Authority                                 |



## ABBREVIATIONS

|                |  |
|----------------|--|
| <b>NOEC</b>    | no observed effect concentration   |
| <b>NOEL</b>    | no observed effect level   |
| <b>NPTII</b>   | neomycin phosphotransferase II   |
| <b>NPV</b>     | net present value  |
| <b>NTO</b>     | non-target organism  |
| <b>OECD</b>    | Organisation for Economic Cooperation and Development                        |
| <b>OGTR</b>    | Office of the Gene Technology Regulator                                      |
| <b>ORF</b>     | open reading frame   |
| <b>PAGE</b>    | polyacrylamide gel electrophoresis   |
| <b>PCR</b>     | polymerase chain reaction  |
| <b>RB</b>      | Right Border   |
| <b>RH</b>      | relative humidity  |
| <b>RO</b>      | reverse osmosis  |
| <b>RUBISCO</b> | ribulose-1,5-bisphosphate carboxylase  |
| <b>SARI</b>    | Savanna Agricultural Research Institute                                      |
| <b>SCSV</b>    | <i>Subterranean clover stunt virus</i>                                       |
| <b>SDS</b>     | sodium dodecylsulfate  |
| <b>SEM</b>     | shoot elongation medium  |
| <b>SGF</b>     | simulated gastric fluid  |
| <b>SIF</b>     | simulated intestinal fluid   |
| <b>SIM</b>     | shoot induction medium   |
| <b>SSA</b>     | sub-Saharan Africa   |
| <b>SSU</b>     | small sub-unit   |
| <b>T-DNA</b>   | transfer-DNA   |
| <b>TrEMBL</b>  | translated sequences from the European Molecular Biology Laboratory database |
| <b>UTR</b>     | untranslated region  |
| <b>VCU</b>     | value for cultivation and use  |
| <b>WHO</b>     | World Health Organization  |

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## GENERAL INFORMATION

### 1. General Information

#### 1.1. Applicant Details

- |  |   |
|--|---|
| (a) Applicant                          | Savanna Agricultural Research Institute (SARI)-Council for Scientific and Industrial Research (CSIR)  |
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| (f) Nature of the applicant's business | SARI is one of the 13 research institutes of CSIR and has a mandate is to provide farmers in the Northern, Upper East, and Upper West Regions of Ghana with appropriate technologies to increase their food and fibre crop production based on a sustainable production system which maintains and/or increases soil fertility. |

#### 1.2. Brief Identification of the Genetically Engineered Product

- |                            |  |
|----------------------------|--|
| Designation:               | 709A (OECD Unique Identifier AAT-7Ø9AA-4)  |
| Plant species:             | <i>Vigna unguiculata</i> L. Walp. (cowpea)   |
| Novel trait(s):            | Resistance to <i>Maruca</i> pod borer ( <i>Maruca vitrata</i> )  |
| Trait introduction method: | <i>Agrobacterium</i> -mediated transformation of embryogenic cowpea explants   |
| Introduced gene(s):        | Cry1Ab encoding gene from <i>Bacillus thuringiensis</i> var. <i>kurstaki</i> strain HD-1 ( <i>cry1Ab</i> ) and neomycin phosphotransferase II encoding gene ( <i>nptII</i> ) from the Tn5 transposon of <i>Escherichia coli</i> strain K12 |
| Proposed use:              | Production of cowpea for human consumption (e.g., leaves and dried beans, including derived products) and for use in livestock feeding (e.g., fodder)  |

#### 1.3. Purpose of the Application

This application is being submitted to the National Biosafety Authority (NBA) for the purposes of securing a permit for the introduction into the environment and placing on the market of 709A cowpea and its progeny pursuant to the Biosafety Act 2011 (Act 831) and the Biosafety (Management of Biotechnology) Regulations, 2019.

#### 1.4. Regulatory Status in other Jurisdictions

As of the date of this submission, the Institute for Agricultural Research, Zaria, Nigeria, has been granted a permit for the commercial release of event 709A cowpea in Nigeria effective 22 January 2019 until 31 December 2022.

#### 1.5. Rationale for the Development of 709A Cowpea

##### 1.5.1. Importance of Cowpea as a Crop and Commodity

Cowpea (*Vigna unguiculata* L. Walp.) is an important staple in the diet of more than 200 million households in SSA. Unlike many other legumes, the tender foliage and

## GENERAL INFORMATION

green pods are used as vegetable, harvested before crop maturity, while mature grains are harvested and stored for future food usage. The plant is very rich in protein (23–30 percent) and is the cheapest source of protein for lower income people, often called the meat of the poor. Cowpea also provides essential vitamins, including thiamine, folic acid, and niacin, and is also a good source of iron and fibre.

Cowpea has always been a dual purpose crop, being the main forage crop in the Sahel with its fodder harvested and dried, stored, and fed during the dry season to the small ruminants (sheep and goats) that belong mostly to women. Cowpea feed is an important commodity and can contribute up to 25 percent of farmers' income. Cowpea grains are highly consumed in humid and sub-humid areas where the crop cannot be grown because of high disease and insect pressure, and the diet is starchy (cassava, yam, and recently maize). The addition of cowpea gives a more balanced diet. This high demand for cowpea in the south has created a regional trade between the drier areas (Niger, Burkina Faso, northern Ghana and northern Nigeria) where the crop is produced and the southern highly populated and urbanized areas of West Africa (e.g., Abuja, Lagos, Ibadan, Accra, Abidjan, and Cotonou). Kano has the largest market for cowpea in West Africa and serves as a collection hub for shipments to the urban centres in southern Nigeria. Nigeria is both the largest producer and the largest importer of cowpea. It is estimated that 40 percent of cowpea consumed in Nigeria is imported and supply has not been able to keep up with demand, resulting in significant price increases (e.g., 48 percent in 2017).

### 1.5.2. Impact of Maruca Pod Borer

Cowpea is drought tolerant legume, and as with any legume, it fixes nitrogen and can be used to improve soil fertility. Despite many decades of breeding, cowpea yields are still very low in Africa, with an average of 200–400 kg/ha. Insect pests constitute one of the main constraints to cowpea production and many studies have shown that pesticide application at flowering and podding can increase yields up to two-fold.

The *Maruca* pod borer (*Maruca vitrata*) is considered the most devastating insect of cowpea, accounting for yield losses ranging from 20–80 percent (IITA, 1985). No source of resistance to *M. vitrata* has been identified after intensive screening of global cowpea germplasm. A few wild cowpea accessions with low level of pod borer resistance have been identified; however, the levels of resistance were either too low to have any breeding value, or reproductive barriers between the wild and cultivated cowpea thwarted efforts to transfer the resistance to domesticated cowpea. The control of *Maruca* relies on repeated application of expensive and toxic chemical insecticides, with 5–10 applications per season required. Traditional insecticides are typically not very effective against the pod borer because the chemical cannot reach the borer. And, the evolution of *Maruca* resistance to the synthetic pyrethroids, organophosphates, and organochlorines typically used (e.g., cypermethrin, dimethoate, and endosulfan) has made control even more difficult (Ekesi, 1999). The environment is also affected by the repeated application of chemical insecticides as beneficial insect populations are significantly reduced, thus aggravating infestations by the pod-sucking insect complex and promoting the emergence of new pests.

## HISTORY OF USE

### 1.5.3. Development of 709A Cowpea

African Agricultural Technology Foundation (AATF) in collaboration with three National Agricultural Research System (NARS) institutes, Institute for Agricultural Research, Ahmadu Bello University (IAR-ABU), Institut de l'Environnement et de Recherches Agricoles (INERA) in Burkina Faso, and SARI in Ghana, together with Commonwealth Scientific and Industrial Research Organisation (CSIRO) Agriculture and Food in Australia, have successfully developed *Maruca*-resistant cowpea lines carrying the *cry1Ab* gene derived from *Bacillus thuringiensis*, which was obtained on a royalty-free basis for humanitarian purposes. Genetic transformation of the cowpea variety IT86D-1010 was carried out at CSIRO and among the transgenic events produced, event 709A (OECD unique identifier AAT-7Ø9AA-4) was selected as the best with regards to resistance to *M. vitrata*, uncomplicated molecular-genetic characterization, and retention of all agronomic performance characteristics of the original parental variety.

Pod borer resistant 709A cowpea is intended to reduce pre-harvest losses and over-reliance on expensive, potentially damaging, and increasingly less effective chemical insecticides that are in routine use. Following general release authorization of event 709A the Cry1Ab insect resistance trait will be introgressed into additional farmer-preferred varieties suitable for variety evaluation and release.

## 2. History of Use of the Host and Donor Organisms

### 2.1. Host Organism

Cowpea (*Vigna unguiculata* L. Walp.) is a drought and stress-tolerant leguminous crop that is one of the most important staple food crops in SSA, especially in West Africa. With its high protein content of approximately 23–30 percent, cowpea is one of the most affordable sources of protein in the diet for most Ghanaians who consume approximately 5 kg per year *per capita*. The crop grows well in areas with erratic rainfall and poor soils, and is cultivated by both women and men for its leaves, green pods and grain for food in humans as well as haulm (stalk or stem) for livestock feed. Cowpea is particularly favoured by many small holder farmers because of its ability to fix nitrogen into the soil and compatibility as an intercrop in mixed cropping systems,

Cowpea production in Ghana is largely concentrated in the Guinea Savannah and forest transition zones (mostly in Upper West, Upper East, Northern Regions and some districts in the Brong Ahafo Region). Despite its importance, cowpea yields on farmers' fields are low, averaging <500 kg per hectare (Abudulai et al., 2017). This is mainly due to insect pests associated with the different growth stages of the crop, especially those that occur during flowering and pod formation stages.

The biology of cowpea has been extensively studied and is described in detail in a recent consensus document published by the Organisation for Economic Cooperation and Development (OECD) (OECD, 2015). Only key points related to its reproductive biology and use in food and feed will be highlighted in this document.

## HISTORY OF USE

### 2.1.1. Taxonomy

Cowpea belongs to the family Fabaceae (Leguminosae is also used as the family name with Papilionoideae as the subfamily), genus *Vigna*, section *Catiang*, species *unguiculata*. It is an annual plant and has two botanical varieties, the cultivated *Vigna unguiculata* var. *unguiculata* and the wild form *V. unguiculata* var. *spontanea*.

### 2.1.2. Centres of Origin and Genetic Diversity

The most likely centre of origin and domestication of cowpea is in West Africa about 2000 BC, and the progenitor of cultivated cowpea is the wild cowpea *V. unguiculata* var. *spontanea* (OECD, 2015). Based on the range of variation and number of varieties found in wild cowpea, as well as their primitive characteristics, such as perenniality, hairiness, small size of the pods and seeds, pod shattering, with pronounced exine (outer coat) on the surface of pollen, out-breeding, and bearded stigma, the highest genetic diversity and most primitive of the wild *V. unguiculata* occur in southern Africa in the region encompassing Namibia from the west, across Botswana, Zambia, Zimbabwe, and Mozambique to the east, and the Republic of South Africa and Swaziland to the south (Singh et al., 1997). The South-African Transvaal may have been the centre of speciation of *Vigna unguiculata* due to the presence there of the most primitive subspecies (OECD, 2015).

### 2.1.3. Reproductive Biology

Cowpea is cleistogamous, producing viable pollens and receptive stigma before anthesis, meaning that cowpea reproduction is entirely via self-pollination. There is no mechanical dispersion of pollen from the flowers of cultivated cowpeas because the anthers release pollen during the first half of the night when the flowers are still closed, and the pollen is sticky and heavy. Some out-crossing mediated by insects can occur naturally in the field, and while different insect species visit cowpea flowers, not all are responsible for pollen movement associated with out-crossing. As reported by Asiwe (2009), only honeybees and bumblebees are responsible for insect-vectored pollen movement because only such heavy insects could depress the wings of cowpea flowers and expose their stamens and stigmas for pollination. Out-crossing rates between cultivated cowpea varieties are low, ranging between 0.5–0.85 percent when cowpea was planted in alternate rows one meter apart, and between 0.01–0.13 percent when planted in concentric circles around a pollen source (Asiwe, 2009). There are no reports of hybridization between *V. unguiculata* and other *Vigna* species (Huesing et al., 2011).

### 2.1.4. Potential for Weediness

Although the establishment of feral populations of domesticated cowpea is theoretically possible, as reported in Japan (Berville et al., 2005), this has rarely been observed in Africa. A few small transient feral populations have been reported in coastal Kenya, which were not seen in consecutive years (OECD, 2015). Cowpea cultivars are generally not capable of creating long-lived seed banks in the soil because their seeds are permeable to water and lack dormancy (Lush et al., 1980). The domestication of cowpea has resulted in loss of many primitive traits typically associated with weediness, such as perenniality, hairiness, small size of seeds and pods, hard seeds, pod shattering, and significant out-crossing (OECD, 2015).

## HISTORY OF USE

### 2.1.5. Consumption Patterns

Cowpea is one of the most important tropical dual-purpose legumes, with grain, leaves, and pods being used for human food. Cowpea is a staple food and provides a major source of protein and nutrients to many people in SSA. Typically, cowpea is consumed after beans are soaked in water and cooked. Cowpeas are also consumed as roasted dried seed, flour, seedlings, leaves and green beans.

There is a wide range of local varieties of cowpea that are adapted to different environments. The nutritional composition of cowpea can be impacted by genetic characteristics, agro-climatic conditions, biotic stresses, and post-harvest management (Gonçalves et al., 2016; Murdock et al., 2003; Oluwatosin, 1998; Silveira et al., 2001). Processing can also affect the nutrient composition. For example, dehulling removes vitamins, cooking can reduce the levels of protein, and fermentation can increase levels of thiamine, niacin, oils, and protein (Adebooye and Singh, 2007; Akinyele and Akinlosotu, 1991; Akpapunam and Achinewhu, 1985).

For human consumption, cowpea is mainly grown for grain (dry and fresh) and sometimes for fresh pods. Cowpea can be used at all stages of growth. In some areas of Africa, cowpeas are cooked as green pods and the beans are consumed. Green leaves are an important food source in Africa and are prepared as potherb, like spinach. Immature snapped pods are used in the same way as snap beans often mixed with other food. Green cowpea seeds are boiled as a fresh vegetable. Dry mature seeds are also suitable for boiling and canning. The consumption of cowpea as a dietary staple in West Africa over millennia has produced an extensive and varied culinary practice and many individual foods and dishes.

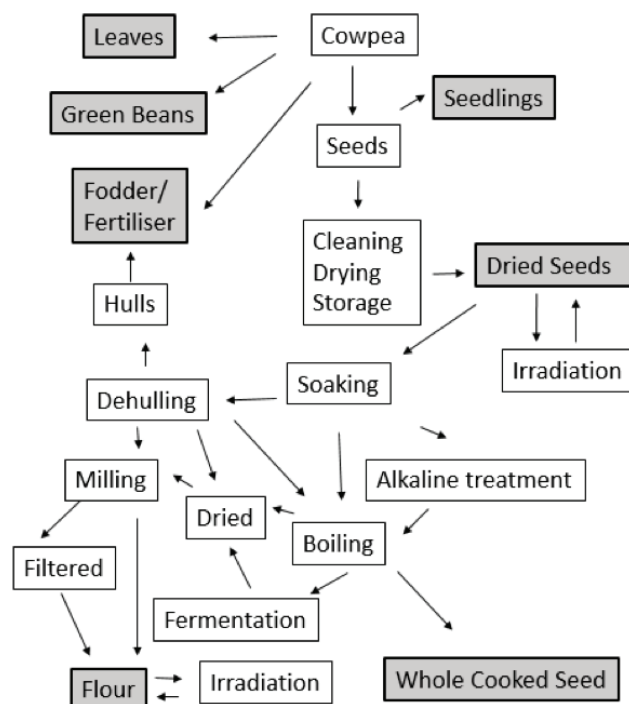
Popular cowpea-based recipes include stews and soups consumed with other staples like rice, gari (processed cassava), ripe plantains, and yams. Others are *koose*, *waakye*, *apprepensa* and *Tubani/Gablee*. *Koose* is a cowpea-based product prepared by adding water to cowpea flour, whipping, shaping into balls and deep frying. *Waakye* is a popular nutritious street food prepared from rice and whole cowpea. *Apprepensa* is prepared from roasted maize meal and cowpea flour while *Tubani/Gablee* is prepared from cowpea flour. Cereals are fortified with cowpea in weaning food formulations. Foods involving cowpea leaves have also been identified, and include *Nyombeica* (a mixture of cowpea leaves and whole maize or cowpea flour steam-cooked), and *Goara* (boiled cowpea leaves usually eaten with *koose*). Major constraints associated with cowpea utilization are its delayed cooking time that increases opportunity cost for women's time, flatulence factors, and storage chemical residues.

### 2.1.6. Processing

Processing of cowpeas and legumes in general is essential to make them nutritious, nontoxic, palatable and acceptable. Cowpea is utilized either as a whole grain or as decorticated or dehulled grain. It is decorticated by soaking in water (at room temperature) for about 30–60 min, and the seed coat (testa) is removed by squeezing the soaked grains between the palms or by gentle abrasion using grinding stones. The seed coat is separated by subsequent filtration (Adebooye and Singh, 2007).

Various processing technologies can be utilized to produce value-added cowpea food products (Figure 1), including dehulling, grinding, soaking, germination, fermentation,

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**Figure 1.** Methods of processing for cowpea value-added products. Shaded boxes represent end-use products. Source: Adapted from Madodé et al. (2013) and Prinyawiwatkul et al. (1996).

addition of salts, wet and dry heat treatments, cooking and roasting (Uzogara et al., 1988; Adebooye and Singh, 2007).

Soaking and boiling of cowpeas is required to improve texture and reduce the concentrations of indigestible oligosaccharides, which can also be reduced using fermentation processes.

### 2.1.7. Key Nutrients and Anti-Nutrients

High protein, high fibre, low glycemic index and antioxidant properties are considered the main nutritional values of cowpeas. The storage proteins in cowpea seeds are rich in the amino acids lysine and tryptophan when compared to cereal grains, but low in methionine and cysteine when compared to animal proteins.

Cowpeas are a rich source of the essential minerals, calcium, magnesium, potassium, iron, zinc, and phosphorus. The range of minerals can vary as the content is often correlated to the soil environment, and concentrations are generally higher in leaves than in seeds, where substantial mineral content is lost with dehulling of the seeds (Adebooye and Singh, 2007).

Cowpeas are an excellent source of thiamine and niacin, with lower amounts of other vitamins such as riboflavin and vitamin C. Vitamin levels are higher in vegetative tissue (e.g., leaves and germinated grain) than in seed as dehulling and cooking result in significant losses. As reported by Nnanna and Phillips (1989), seed coat removal resulted in up to 30 percent loss in niacin while thiamine content was reduced 41 percent by cooking.

## HISTORY OF USE

Like other grain legumes, cowpeas contain a range of anti-nutritional factors such as phytic acid, hemagglutinin, tannin, trypsin inhibitors, oxalate, phytate, polyphenols and oligosaccharides.

### 2.1.8. Use in Livestock Feed

Cowpea can be used in animal feed rations at all stages of growth. Forage (both, vines and leaves) fresh or dried as hay or silage are used as animal feed. Cowpea hay plays a particularly critical role in feeding animals during the dry season in many parts of West Africa. Cowpea leaves and stems (stover) are an important source of high-quality hay for livestock feed. Additional information on cowpea feed uses is provided in section 5.3, page 42.

### 2.1.9. Allergy

Although allergic reactions to legumes, including peanuts and soybeans, are relatively common, allergy to cowpeas is rare. There is a single documented case where the analyses of sera from six individual patients exhibiting allergic reaction to cowpeas identified two proteins of 41 kDa and 55 kDa, respectively, as the major allergens of cowpea (Rao, 2000).

## 2.2. Donor Organisms

### 2.2.1. *Bacillus thuringiensis*

*Bacillus thuringiensis* (order: Bacillales; family: Bacillaceae) subspecies *kurstaki* strain HD-1, which serves as the primary reference standard for all commercial insecticidal formulations of *B. thuringiensis* manufactured around the world (Day et al., 2014), was the source of the *cry1Ab* gene used in the development of event AAT-7Ø9AA-4 (709A) cowpea.

*Bacillus thuringiensis* (Bt) is a facultative anaerobic, gram-positive spore-forming bacterium that produces characteristic insecticidal proteins, as parasporal crystals, during the sporulation phase (Sanahuja et al., 2011). These crystals are predominantly comprised of one or more Crystal (Cry) and Cytolytic (Cyt)  $\delta$ -endotoxins that are highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo et al., 2007). Over 60 subspecies of *B. thuringiensis* have been described that collectively synthesize various types of Cry proteins with specific activity against the insect orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, and also to nematodes. The Cyt proteins are mostly found in *B. thuringiensis* strains that are active against Diptera.

Microbial formulations of Bt were first used in France in 1938 as a commercial insecticide (CERA, 2011). The first commercial *Bt* materials were available for field-testing in the United States in 1958 (Faust, 1974) and formulations containing combinations of several groups of Cry proteins have been registered in the United States since 1961 (Schnepf et al., 1998; Betz et al., 2000). In their history of widespread and continuous use over 50 years, Bt microbial pesticides have caused no known adverse human or environmental effects (EPA, 1998; Betz et al., 2000). In a comparative review of the toxicity studies submitted to the United States Environmental Protection Agency (EPA) to support the registration of Bt products, McClintock et al. (1995) found no

## THE GENETIC MODIFICATION

evidence of any significant adverse effects in body weight gain, clinical observations, or gross lesions upon necropsy, and that the existing data supported the lack of mammalian toxicity or pathogenicity. Similarly, the World Health Organization (WHO) International Program on Chemical Safety report on environmental health criteria for Bt concluded that: “Owing to their specific mode of action, Bt products are unlikely to pose any hazard to humans or other vertebrates...” and “Bt has not been documented to cause any adverse effects on human health when present in drinking water or food” (IPCS, 1999).

### 2.2.2. *Escherichia coli*

The Tn5 transposon from *Escherichia coli* (family *Enterobacteriaceae*) strain K12, a non-pathogenic strain, was the source of the neomycin phosphotransferase II (NPTII) encoding *nptII* gene (Beck et al., 1982). *Escherichia coli* is a gram-negative, motile, facultatively anaerobic rod-shaped bacterium. Certain serotypes are enteropathogenic and are known to cause diarrhoea in infants. Some strains also cause diarrhoea in adults. *Escherichia coli* is a normal inhabitant of the intestinal flora of humans and animals, where it generally does not cause disease.

## 3. The Genetic Modification

### 3.1. Transformation Method

The *Agrobacterium*-mediated transformation of the cowpea cultivar IT86D-1010 using plasmid pMB4 was essentially as described by Popelka et al. (2006) and modified by Higgins et al. (2012).

Dry cowpea seed (30 g) was sterilized in a 250 ml Schott bottle by adding 50 ml of 70 percent ethanol for 1 minute. The mixture was shaken vigorously for 30 seconds and the ethanol was then replaced with 50 ml of 20 percent commercial bleach. After a 30 min incubation at room temperature, the seeds were rinsed five times in sterile reverse osmosis (RO) purified water and then allowed to imbibe in 50 ml of sterile RO water overnight.

The imbibed seeds were drained and the seed coats removed aseptically. Each seed was split in two by separating the cotyledons. Using the cotyledon with the attached embryonic axis (referred to as the explant), the lower two-thirds of the radicle was excised. Approximately 50 explants were placed in sterile 250 ml flasks containing 10 ml of cowpea co-cultivation medium (CCM), to keep the explants hydrated until all seeds were prepared. For transformation, the CCM was replaced with approximately 25 ml of the *Agrobacterium* suspension to submerge the explants. The explants were then sonicated (Branson Model 1510E-DTH; output 70W) for 30 seconds and incubated for one hour on a rotary shaker at 28°C and 200 rpm. The explants were subsequently placed onto sterile filter papers to blot the excess medium. Co-cultivation plates were prepared by placing new sterile filter paper on CCM solidified with 0.8 percent granulated agar. The explants were transferred (30–40 per plate) facing down and co-cultivated for three days at 28°C with a 16 hour photoperiod.

Following co-cultivation, the explants were placed with the growing shoot facing down onto shoot induction medium (SIM), pH 5.6 containing kanamycin (100 mg/l) for selection. The explants were incubated for 12–14 days in a growth chamber at 28°C

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with a 16 hour photoperiod. At the second transfer, the cotyledon, primary shoots and any regrown radicle were removed, and the remaining portion of the explants were transferred to SIM with increased kanamycin (150 mg/l) for 12–14 days. At the third transfer, any brown callus was removed and the remaining tissue supporting multiple small shoots was transferred to SIM with kanamycin (150 mg/l). At the fourth transfer, multiple shoots were transferred sequentially up to seven times to SIM with more stringent selection using geneticin (30 mg/l). At each transfer, large clumps consisting of many shoots were separated into smaller clumps or selected as single shoots depending on their vigour and dead shoots were removed. By the seventh transfer, multiple shoots were separated and at the eighth transfer, single shoots were placed onto shoot elongation medium (SEM) pH 5.6 with geneticin (30 mg/l) and 25 mg/l Merrem (Ranbaxy Australia Pty) for elongation and rooting. Depending on their height, shoots were either placed in Petri plates or 250 ml containers with lids. Shoots that developed healthy roots were transferred into small pots (50 cm diameter x 10 cm height) containing a light sandy soil mix [25% perlite, 25% vermiculite, 30% coarse sand and 20% commercial potting mix (Bunnings, Canberra ACT Australia)], and subsequently acclimatized in the culture room for up to four weeks prior to transfer to the greenhouse.

### 3.2. Description of the Potentially Introduced Genetic Material

*Agrobacterium*-mediated transformation was performed using plasmid pMB4 (Figure 2), which contains two gene expression cassettes within the T-DNA (Figure 3). These gene expression cassettes are briefly described below, and summarized in Table 1.

The first cassette contains a copy of the NPTII encoding gene from the Tn5 transposon of *Escherichia coli* strain K12. To eliminate leaky expression of NPTII in *Agrobacterium* and prevent selection of false-positive kanamycin-resistant plants, the *nptII* gene was interrupted with a modified catalase-1 (CAT-1) intron from castor bean (*Ricinus communis*) (Ohta et al., 1990; Schünmann et al., 2003b). Transcription of the *nptII* gene is controlled by the S1 promoter from subterranean clover stunt virus (SCSV) and termination sequences are provided by the SCSV DNA segment 3. The *nptII* gene functions as a selectable marker immediately following transformation of plant cells during *in vitro* tissue culture (De Block et al., 1984; Horsch et al., 1984).

The second cassette contains a truncated version of the Cry1Ab encoding gene from *Bacillus thuringiensis* subs. *kurstaki* strain HD-1. The nucleotide sequence of the *cry1Ab* gene was codon-optimized for plant expression and encodes a 615-amino acid protein (68.9 kDa) corresponding to the trypsin-resistant insecticidally active core protein following cleavage of the 1155-amino acid native Cry1Ab protoxin (GenBank accession no. M60856.1) (Perlak et al., 1991). The *cry1Ab* gene is under the control of the promoter and 5' UTR of the RUBISCO SSU from *Arabidopsis thaliana* (Krebbers et al., 1988) with transcription termination sequences derived from the tobacco (*Nicotiana tabacum*) RUBISCO SSU (Mazur and Chui, 1985). Expression of Cry1Ab confers resistance to the lepidopteran pest *Maruca vitrata*, also known as cowpea pod borer.

### 3.3. Breeding of 709A Cowpea

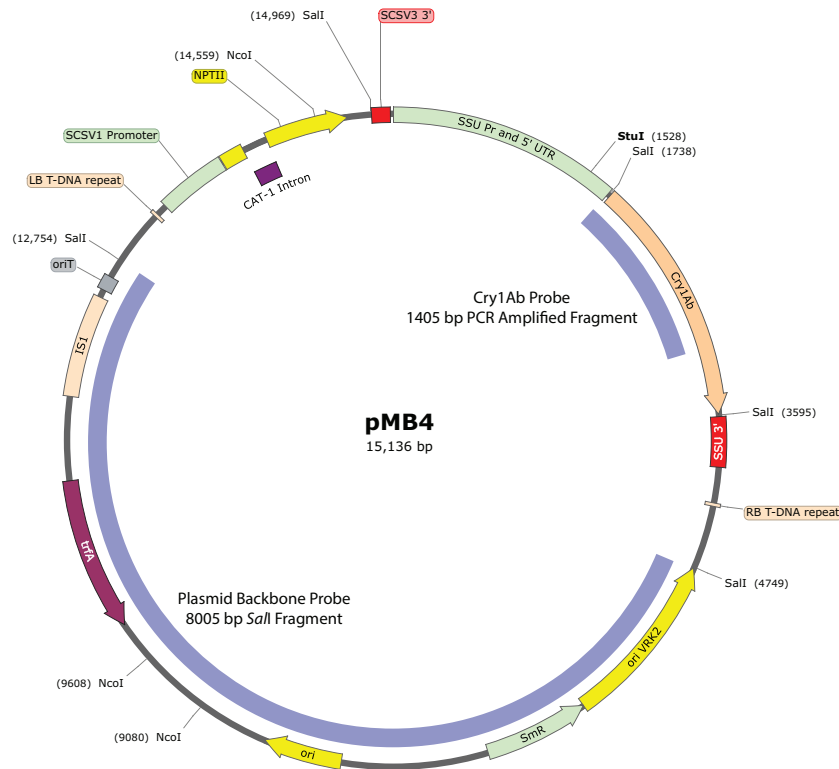
The breeding of 709A cowpea proceeded as indicated in Figure 4 to produce specific generations for the characterization and assessments conducted. Event 709A in

## THE GENETIC MODIFICATION

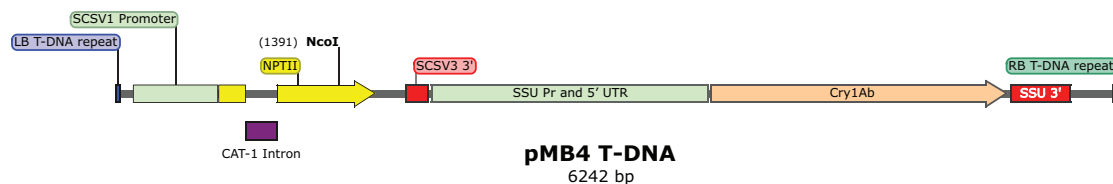
**Table 1.** Description of the genetic elements within the pMB4 T-DNA

| Gene Expression Cassette               | Location on pMB4 T-DNA (bp) | Genetic Element                           | Size (bp)   | Description  |
|--|-----------------------------|---|-------------|--|
|  | 1–25                        | Left border                               | 25          | Left border region of the T-DNA from <i>A. tumefaciens</i> nopaline Ti plasmid (Zambryski et al., 1982); GenBank accession no. J01825. Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the genome of the host plant (Yadav et al., 1982).                 |
|  | 26–76                       | Ti plasmid region                         | 51          | LB proximal sequence from Ti plasmid of <i>A. tumefaciens</i> contained on cloning vector plasmid pPLEX-506. GenBank accession no. AY159021 (Schünmann et al., 2003a).   |
|  | 77–108                      | Intervening sequence                      | 32          | DNA sequence used for cloning.   |
| <i>nptII</i> gene expression cassette  | 109–631                     | SCSV1 promoter                            | 523         | S1 promoter derived from subterranean clover stunt virus DNA segment 1 (SCSV1) (Boevink et al., 1995). GenBank accession no. AY159021 (Schünmann et al., 2003a).   |
|  | 632–640                     | Intervening sequence                      | 9           | DNA sequence used for cloning.   |
|  | 641–817 and 1008–1625       | <i>nptII</i> Coding sequence (2 segments) | 177 and 618 | Coding sequence for neomycin phosphotransferase II (NPTII) from <i>Escherichia coli</i> strain K12 (Beck et al., 1982). Contains the modified CAT-1 intron. GenBank accession no. AY159021 as described for plasmid pPLEX-506 (Schünmann et al., 2003a).   |
|  | 818–1007                    | CAT-1 Intron                              | 190         | The first intron of castor bean catalase gene (CAT-1) (Ohta et al., 1990). GenBank accession no. AY159021 (Schünmann et al., 2003a).   |
|  | 1626–1806                   | Intervening sequence                      | 181         | Sequence derived from plasmid cloning vector pPLEX-506. GenBank accession no. AY159021 (Schünmann et al., 2003b).  |
|  | 1807–1945                   | SCSV3 3'                                  | 139         | Termination sequences derived from subterranean clover stunt virus DNA segment 3 (SCSV3) (Boevink et al., 1995) contained on plasmid pPLEX-506. GenBank accession no. AY159021 (Schünmann et al., 2003a).  |
|  | 1946–1968                   | Intervening sequence                      | 23          | DNA sequence used for cloning.   |
| <i>cry1Ab</i> gene expression cassette | 1969–3694                   | SSU Promoter and 5' UTR                   | 1726        | Promoter and 5' untranslated region (UTR) of the ribulose-1,5-bisphosphate carboxylase small subunit polypeptide of <i>Arabidopsis thaliana</i> . GenBank accession no. X13611.1 (Krebbers et al., 1988).  |
|  | 3695–3714                   | Intervening sequence                      | 20          | DNA sequence used for cloning.   |
|  | 3715–5562                   | <i>cry1Ab</i> Gene                        | 1848        | Coding region of the <i>cry1Ab</i> gene derived from <i>Bacillus thuringiensis</i> subsp. <i>kurstaki</i> (Geiser et al., 1986), truncated and modified for plant expression (Perlak et al., 1991). GenBank accession no. Genbank: M60856.1  |
|  | 5563–5578                   | Intervening sequence                      | 16          | DNA sequence used for cloning.   |
|  | 5579–5951                   | SSU 3'                                    | 373         | Termination sequences of the small subunit (SSU) of ribulose bis-phosphate carboxylase-oxygenase from tobacco ( <i>Nicotiana tabacum</i> ). GenBank accession no. X02353.1 (Mazur and Chui, 1985).   |
|  | 5952–6100                   | Intervening sequence                      | 149         | DNA sequence used for cloning.   |
|  | 6101–6217                   | Ti plasmid region                         | 117         | RB proximal sequence from Ti plasmid of <i>A. tumefaciens</i> contained on cloning vector plasmid pPLEX-506. GenBank accession no. AY159021 (Schünmann et al., 2003a).   |
|  | 6218–6242                   | Right border                              | 25          | Right border region of T-DNA from <i>Agrobacterium tumefaciens</i> nopaline Ti plasmid (GenBank accession no. J01826). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the genome of the host plant (Yadav et al., 1982). Contained on plasmid pPLEX-506. |

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**Figure 2.** Schematic map of pMB4 indicating the organization of genetic elements. The Right Border (RB) and Left Border (LB) regions flank the T-DNA (Figure 3) that is intended for integration with the host plant genome during *Agrobacterium*-mediated transformation. Locations of hybridization probes specific for a portion of the *cry1Ab* gene and the entire plasmid backbone are shown.



**Figure 3.** Schematic diagram of the pMB4 T-DNA indicating the *cry1Ab* and *ntpII* genes along with their respective regulatory elements, including the *Subterranean clover stunt virus* (SCSV)1 promoter, SCSV3 termination sequences, the *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase (RUBISCO) small sub-unit (SSU) promoter and 5' untranslated region (UTR), and tobacco SSU 3' non-coding region. The size of the T-DNA is 6,242 bp.

background IT86D-1010 (T3 generation in direct line of descent from the original T0 transformant) was crossed into three different backgrounds, including: IT97K-499-35, an improved *Striga*-resistant variety (Kamara et al., 2008) released by International Institute of Tropical Agriculture (IITA) in Nigeria (2006), and Niger and Ghana (2008); IT86D-1010, the original parental line used for transformation; and IT93K-693-2 (Table 2). During the breeding and development of 709A cowpea, seed was produced for multiple purposes, including product evaluation, research testing, and regulatory testing. Seed was sourced from multiple generations of 709A cowpea in different genetic backgrounds depending on the study, and where applicable, studies utilized the appropriate non-transgenic null segregant comparator.

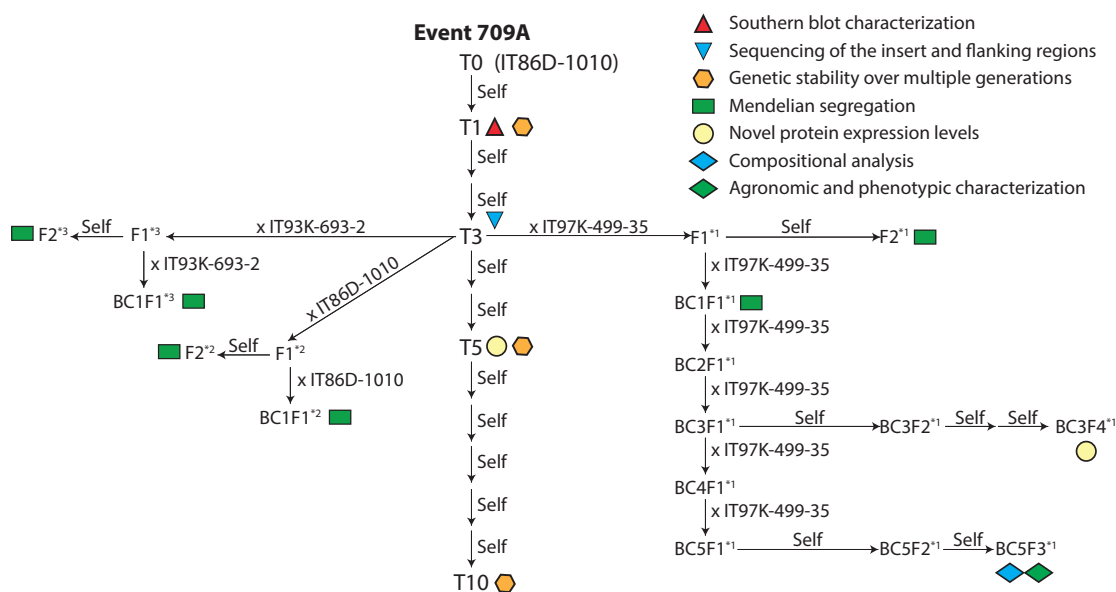
Biosafety reviews and approvals are “event-based” not “variety-based”. The biosafety review and issuance of a permit is for event 709A cowpea and its progeny (i.e., any other cowpea variety into which event 709A has been introgressed through breeding). The choice of Songotra variety (i.e., IT97K-499-35) for the purposes of biosafety evaluation was simply for convenience. Variety evaluation and registration comes after biosafety

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approval and it is at that point where new varieties containing event 709A will have to demonstrate value for cultivation and use (VCU) and distinctness, uniformity, and stability (DUS).

**Table 2.** Pedigree and description of parental plant materials

| Genotype     | Pedigree  | Description   |
|--------------|---|---|
| 709A         | Transformation event derived from IT86D-1010          | Sourced from Commonwealth Scientific and Industrial Research Organization laboratory, Canberra, Australia. Transgenic line resistant to <i>M. vitrata</i>   |
| IT97K-499-35 | Derived from a cross of IT93K-596-9-12 × IT93K-2046-1 | Its a medium maturing variety (about 75 d) with semi-erect growth habit. It is heat tolerant, photo-insensitive, and has large white seeds (about 18 g/100 seeds). In addition to being resistant to Striga and Alectra, it has combined resistance to major diseases and insect pests (Singh et al., 2006).  |
| IT86D-1010   | Derived from a cross between Tvx4659-03E × IT82E-60   | An advanced breeding line, medium maturity (71 d), photo-insensitive, with semi-erect growth habit. It has combined resistance to cowpea yellow mosaic, blackeye cowpea mosaic, and many strains of cowpea aphid-borne mosaic, Cercospora, smut, rust, Septoria, scab, Striga, Alectra, aphid, thrips, and bruchid (Van Boxtel et al., 2000; Lale and Kolo, 1998).  |
| IT93K-693-2  | IT88D-867-11 × IT89KD-374-57                          | An extra-early maturing (about 60 d) photo-insensitive and heat tolerant variety with semi-erect growth habit. It has green plants without purple pigmentation, with medium size seeds (about 14 g/100 seeds) with brown color and rough seed coat texture. It is resistant to Alectra as well as all five strains of Striga reported in West Africa (Singh, 2002) and has combined resistance to major diseases and insects (Singh et al., 2006). This variety has been used as a parent in crosses for identifying DNA markers for <i>S. gesnerioides</i> resistance (Boukar et al., 2004). |



**Figure 4.** Breeding diagram for 709A cowpea and generations used for analyses

## 4. Molecular Genetic Characterization of 709A Cowpea

Characterization of a recombinant-DNA plant at the molecular level is used to provide information about the number of copies of the inserted DNA and sites of insertion as well as the composition, integrity, and inheritance of the inserted DNA and introduced trait(s).

Knowledge of the introduced, or modified, genes, their regulation, and the site of integration within the host genome in the case of transgenic plants, may provide

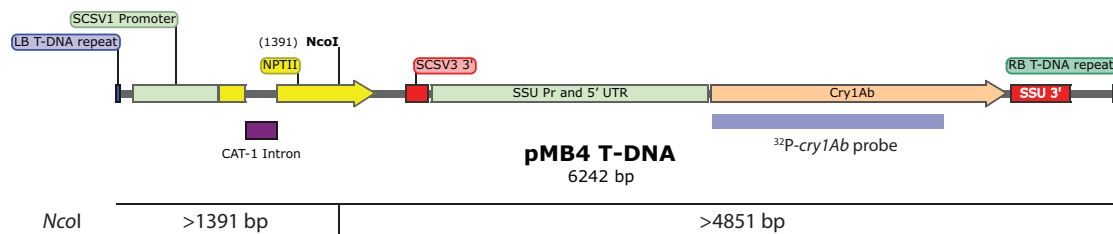
information on possible direct and indirect consequences of the genetic modification. For example, the potential for adverse effects resulting from insertional inactivation, or activation, can be minimized by characterizing the adjacent host DNA and avoiding those events containing transgenes in close proximity to genes known to be important to plant metabolism or to affect the production of potentially toxic or allergic compounds. On the other hand, while information on transgene copy number or the presence of associated plasmid backbone sequences may help to explain empirical observations of reduced transgene expression, there is no *per se* correlation between copy number and safety.

It is important to emphasize that, while necessary, the molecular characterization of the introduced (or modified) DNA is not a sufficient means of predicting possible unanticipated consequences nor is it a replacement for direct measurements of gene expression, agronomic and phenotypic characteristics, or changes in the levels of nutrients and anti-nutrients. As noted by OECD (2010), “It assists in the prediction of the phenotype and the phenotype will ultimately determine whether the recombinant-DNA plant poses any risk/safety concerns.”

#### 4.1. Southern Hybridization Characterization

Southern blot analyses were performed to investigate the number of sites of insertion of the pMB4 T-DNA in event 709A cowpea and the absence of plasmid backbone sequences (Moore and Higgins, 2018b). To determine the number of copies of the inserted DNA, samples of genomic DNA prepared from the T3 generation of event 709A in IT86D-1010 cowpea was subjected to restriction endonuclease digestion with *NcoI* enzyme followed by electrophoretic separation and transfer onto nylon membrane. Hybridization was performed with a DNA probe specific to the *cry1Ab* gene. The lack of integration of plasmid backbone sequences from pMB4 was confirmed using a hybridization probe corresponding to the entire backbone region (see Figure 2, page 27).

The *NcoI* restriction enzyme has a unique recognition site at position 1,391 within the pMB4 T-DNA and was used to provide information about the number of copies of the introduced DNA within the 709A cowpea genome. The predicted fragment sizes following *NcoI* digestion are >1,391 bp, corresponding to the 5’ proximal region spanning the junction of the pMB4 T-DNA insert and the cowpea genome, and >4,851 bp, corresponding to the 3’ proximal region spanning the junction of the T-DNA insert and the cowpea genome (Figure 5).

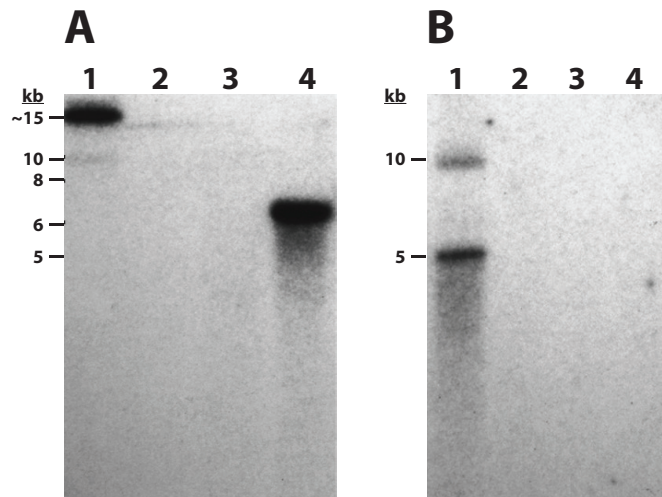


**Figure 5.** Schematic diagram of the T-DNA region from plasmid pMB4 showing expected fragment sizes following digestion of 709A cowpea DNA with *NcoI* restriction endonuclease. As illustrated, only the fragment >4,851 bp will be detected using the <sup>32</sup>P-labelled *cry1Ab* probe.

Thus, the insertion of a single copy of the pMB4 T-DNA in 709A cowpea should result in a single detectable hybridization fragment of >4,851 bp following *NcoI* digestion

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and hybridization with the *cry1Ab* gene probe, which was consistent with the observed hybridization pattern showing detection of a single *ca.* 7 kb fragment (Figure 6, panel A, lane 4).



**Figure 6.** Samples of genomic DNA (*ca.* 10 µg) isolated from control IT86D-1010 (lane 3, panels A and B) and event 709A cowpea (lane 4, panels A and B) were subjected to restriction endonuclease digestion using *NcoI* followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with <sup>32</sup>P-labelled probes specific for *cry1Ab* (panel A) or the pMB4 plasmid backbone region (panel B). Following washing, hybridized probes were visualized using autoradiography. In each blot, lane 1 contained either linearized pMB4 plasmid DNA (panel A) or *NcoI* digested plasmid pMB4 (panel B) as a positive control. Lane 2 was blank in each blot and molecular size markers are indicated.

Southern blot analysis of *NcoI*-digested genomic DNA obtained from event 709A cowpea was performed to demonstrate the lack of integration of any sequences derived from the pMB4 plasmid backbone. For this analysis, a <sup>32</sup>P-labelled probe corresponding to the entire backbone region was utilized.

Hybridizing fragments were not detected when the backbone probe was tested against *NcoI*-digested 709A cowpea genomic DNA (Figure 6, lane 4, panel B), confirming the lack of integration of any plasmid backbone sequences. A positive control sample containing *NcoI*-digested pMB4 plasmid DNA did result in the detection of two fragments of *ca.* 10 kb and 5 kb, corresponding to the expected sizes of 9,657 bp and 4,951 bp, respectively, that hybridize to the backbone probe (Figure 6, panel B, lane 1).

Based on the results of Southern hybridization analysis, event 709A cowpea contained a single copy of the pMB4 T-DNA integrated at a single site within the cowpea genome. The introduced DNA in 709A cowpea did not contain any sequences derived from the pMB4 plasmid backbone region.

### 4.2. Nucleotide Sequence Analysis of the Inserted DNA and Flanking Regions

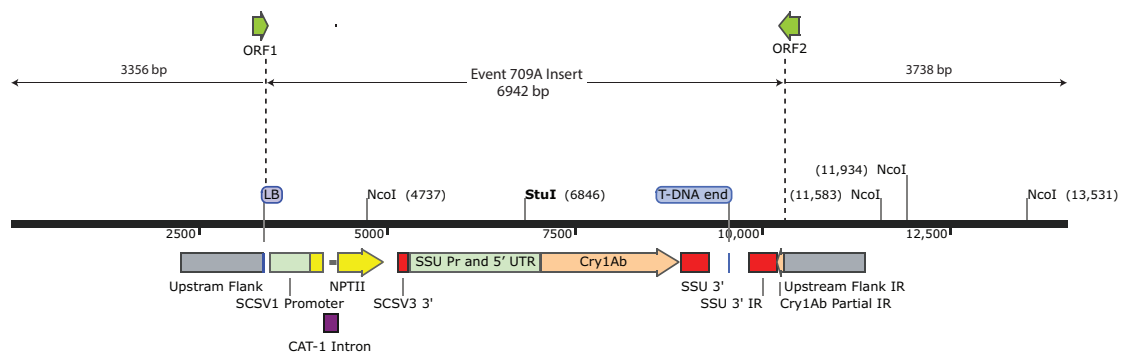
The nucleotide sequence of the entire inserted DNA within 709A cowpea, including a portion of the 5' and 3' flanking host genomic region, was determined in order to confirm the organization of the insert, contiguity of the functional elements, and to identify any sequence changes or rearrangements (Upadhyaya and Higgins, 2018). In addition, an open reading frame (ORF) analysis was conducted to investigate the possibility of

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creating any new start-to-stop ORFs spanning the 5' or 3' junctional regions that could potentially encode sequences homologous to known allergens or toxins.

Using inverse polymerase chain reaction (PCR) for the LB region, 554 bp of sequence was recovered, of which 438 bp was derived from flanking cowpea genomic DNA sequence. However, inverse PCR for the RB failed to yield a product and thus genomic sequencing of both 709A cowpea and its parental line, IT86D-1010, was required to elucidate the sequences surrounding the insertion site (Spriggs et al., 2018).

A total of 14,036 bp of 709A cowpea genomic sequence, comprising 3,356 bp of the 5' genomic border sequence, 3,738 bp of the 3' genomic border sequence, and 6,942 bp of the inserted T-DNA is illustrated in Figure 7. The inserted DNA was comprised of one copy of the pMB4 T-DNA (6207 bp), containing a 10-bp deletion on the left border end and a 29-bp deletion from the 3' terminus that included the entire 25-bp right border repeat plus an additional four upstream nucleotides. Adjacent to the 3' end of the inserted T-DNA was a 735-bp inverted repeat containing a duplicated tobacco ribulose 1, 5-bisphosphate carboxylase SSU 3' region (373 bp) and 85 bp derived from the 3' terminus of the *cry1Ab* coding sequence. Additionally, a 1081-bp region of the left border proximal flanking genomic sequence was duplicated as an inverted repeat downstream of the inserted DNA.



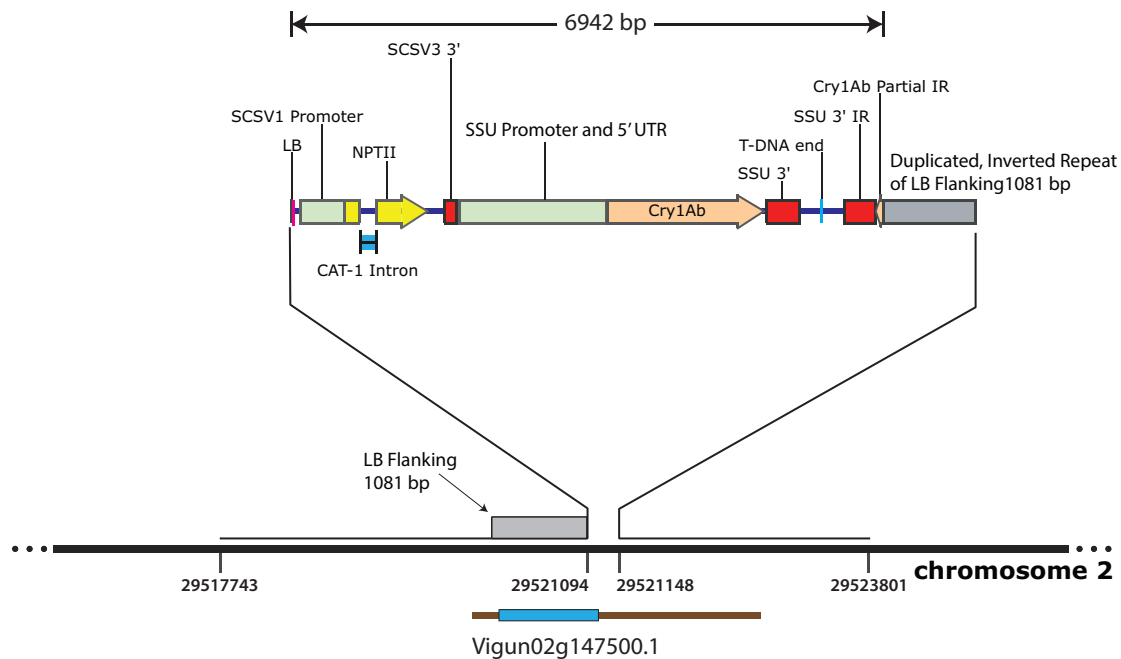
**Figure 7.** Schematic diagram of the 709A cowpea insert indicating the *cry1Ab* and *nptII* genes along with their respective regulatory elements. The location of two putative ORFs spanning the 5' and 3' junctions of the T-DNA insert and the cowpea genomic DNA are indicated as ORF1 and ORF2, respectively.

While the precise mechanism is unclear, recent work indicates that T-DNA integration into the plant genome is primarily mediated by endogenous plant DNA repair systems, as surveys of T-DNA integration sites show characteristics highly similar to those of double-strand repair sites such as deletions and inversions (Kleinboelting et al., 2015). Similar deletions, insertions, and rearrangements of genomic DNA at T-DNA integration sites have been found in other transgenic events without giving rise to safety concerns, e.g. EFSA (2014).

Basic local alignment search tool (BLAST) searches using the 5' and 3' flanking region sequences as queries identified that the site of insertion of the T-DNA was located on chromosome 2 (Figure 8), within the coding sequence for a hypothetical protein (Vigun02g147500.1).

To investigate the possibility of creating new novel ORFs as a consequence of the T-DNA insertion in event 709A cowpea, an open reading frame analysis was conducted to look for potential start-to-stop ORFs that spanned either the 5' or 3' junctional regions. This

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**Figure 8.** From the analysis of 5' and 3' flanking host genomic DNA, the site of T-DNA insertion within 709A cowpea was mapped to a region on chromosome 2, within the coding sequence for a hypothetical 79-amino acid protein (Vigun02g147500.1).

analysis examined each of three possible reading frames in both orientations (i.e., six possible reading frames in total) for potential ORFs capable of encoding sequences of 30 or more amino acids. Two ORFs were identified, one that spanned the 5' T-DNA insert–genomic DNA border (Figure 7; ORF1, 219 bp, 72 amino acids), and one in the reverse orientation that spanned the 3' T-DNA insert–genomic DNA border (Figure 7; ORF2, 264 bp, 87 amino acids). The coding sequence of ORF1 was comprised of 201 bp derived from the endogenous Vigun02g147500.1 coding sequence, corresponding to the N-terminal 67 amino acids, and junction-spanning sequences encoding five additional amino acids.

**Table 3.** Location and identity of the ORFs spanning the 5' and 3' T-DNA–genomic DNA junctions and present in the inverted repeat region of flanking cowpea genomic sequence

| ID   | Nucleotide Location | Strand | Length (amino acids) | Deduced Amino Acid Sequence   | No. of Allergen Hits | No. of FASTA Hits <sup>a</sup> |
|------|---------------------|--------|----------------------|---|----------------------|--------------------------------|
| ORF1 | 5855–6073           | +      | 72 (8.0 kDa)         | MRLSISLMAV LLLLLVTMPT<br>LVNSRVLAPQ QNLTHHKPIK<br>NQPQFTLSVT KAKAIRTRLL<br>IESQVNTISI VV                | None                 | None                           |
| ORF2 | 12938–13201         | -      | 87 (9.5 kDa)         | MRLSISLMAV LLLLLVTMPT<br>LVNSRVLAPQ QNLTHHKPIK<br>NQPQFTLSVT KAKAIRTRLL<br>IESQVNTTCS TAATRCTSTG SLSQLR | None                 | None                           |

<sup>a</sup> Sequences were queried against a protein toxin database using FASTA with an *E*-value cutoff of  $1 \times 10^{-5}$ .

To search for potential similarity to known toxins, the amino acid sequence of each ORF was queried against a toxin database using the FAST All sequence alignment tool (FASTA) to identify possible significant sequence similarity with known or potential toxins. The toxin database was created from a subset of sequences derived from the UniProt Knowledgebase, comprised of 556,825 manually annotated and reviewed sequences from Swiss-Prot and 108,857,716 automatically annotated, un-reviewed sequences from translated sequences from the European Molecular Biology Laboratory

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database (TrEMBL) (The UniProt Consortium, 2014)<sup>1</sup>, that were selected using a keyword search on toxins (KW800). The collection contained a total of 35,359 sequences as of April 6, 2018, comprising 6,574 reviewed sequences from Swiss-Prot and 28,785 un-reviewed sequences from TrEMBL. The BLOcks SUBstitution Matrix (BLOSUM) 50 similarity scoring matrix was used for FASTA36 alignments (Pearson, 2013).

An *E*-score<sup>2</sup> acceptance criteria of  $1 \times 10^{-5}$  was used to identify sequences from the toxin database with potential for significant sequence similarity to the query sequences of each ORF. The FASTA36 search resulted in no significant hits returned (Table 3).

To assess the potential for allergen cross-reactivity, the amino acid sequence of each ORF was compared to a peer-reviewed database of 2089 known and putative allergen and celiac protein sequences residing in the Food Allergy Research and Resource Program (FARRP) dataset at the University of Nebraska.<sup>3</sup> Potential identities between each of the query sequences and proteins in the allergen database were evaluated with the FASTA sequence alignment tool using an *E*-value threshold  $1 \times 10^{-5}$ . No significant alignments were returned. The standard greater than 35 percent identity threshold over any 80 amino acid length sequence alignment between a query sequence and an allergen was used to indicate the potential for cross-reactivity. No identity matches of greater than 35 percent over 80 residues were observed for either ORF1 or ORF2. Each query sequence was also evaluated for any eight contiguous identical amino acid matches to the allergens contained in the FARRP database. This was done using an algorithm that generates all possible eight-word peptides from both the query and dataset proteins and evaluates each query “word” against all dataset “words” for perfect matches. There were no eight contiguous identical amino acid matches observed for either ORF1 or ORF2 (Table 3).

In conclusion, nucleotide sequence analysis of the entire T-DNA insert within 709A cowpea confirmed the organization of the inserted DNA. The inserted DNA was comprised of one complete copy of the plasmid pMB4 T-DNA, except for 10-bp and 29-bp truncations at the 5' and 3' termini, respectively, and a 735-bp inverted repeat derived from the 3' end of the T-DNA. The site of insertion of the T-DNA was mapped to a region on chromosome 2 of the cowpea genome. There were no new novel open reading frames created as a consequence of the DNA insertion that would have the potential to encode proteins with any significant amino acid sequence similarity to known or suspected toxins or allergens.

### 4.3. Stability of the Introduced DNA across Multiple Generations

The stability (inheritance) of the inserted DNA within event 709A cowpea was investigated using event-specific PCR analysis of genomic DNA prepared from progeny

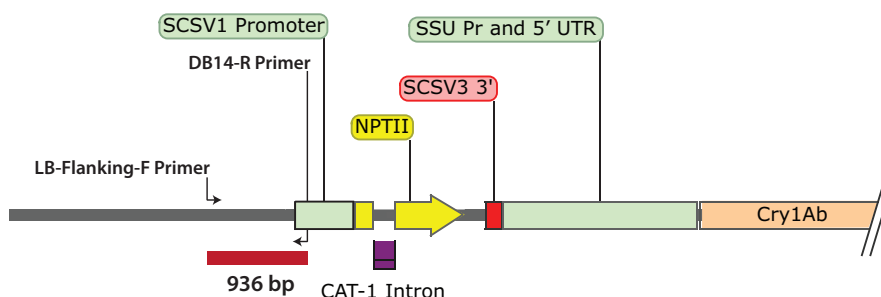
<sup>1</sup> TrEMBL is a computer-annotated protein sequence database supplementing the SWISS-PROT Protein Sequence Data Bank. TrEMBL contains the translations of all coding sequences (CDS) present in the EMBL Nucleotide Sequence Database not yet integrated in SWISS-PROT. TrEMBL can be considered as a preliminary section of SWISS-PROT. For all TrEMBL entries which should finally be upgraded to the standard SWISS-PROT quality, SWISS-PROT accession numbers have been assigned.

<sup>2</sup> The *E*-score is a parameter that describes the number of matches one can “expect” to see by chance when searching a database of a particular size. It decreases exponentially as the score of the match increases. The lower the *E*-score, the closer it is to zero, the more “significant” the match. Typically, alignments between two sequences require an *E*-score of  $1 \times 10^{-5}$  or less to be considered to have sufficient sequence similarity to infer homology.

<sup>3</sup> The FARRP allergen protein database resides at AllergenOnline (<http://www.allergenonline.org>). Version 18B was released on JMarch 23, 2018, and contains 2089 peer-reviewed sequences representing 831 taxonomic-protein groups.

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plants spanning ten selfing generations in direct line of descent from the original transformant (Moore and Higgins, 2018c).



**Figure 9.** Schematic diagram showing the LB proximal region of the 709A insert junction with the cowpea genome. Positions of the event-specific primers are illustrated as well as the 936-bp amplified fragment used as a diagnostic for the presence of the inserted DNA.

For event-specific detection, PCR reactions were performed using the LB-Flanking-Forward and DB-14-Reverse primers (Table 4), which should result in amplification of at 936-bp DNA fragment (Figure 9) that was diagnostic for the presence of the inserted DNA in event 709A cowpea.

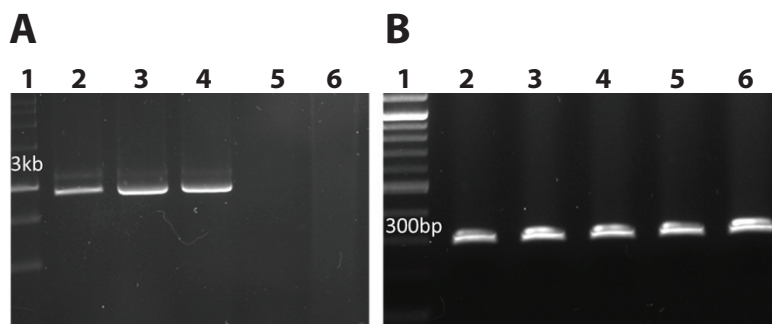
**Table 4.** Primers used for event-specific and endogenous PCR amplifications

| Name                | Primers (5' to 3')       | Amplicon Size (bp) |
|---------------------|--------------------------|--------------------|
| LB-Flanking-Forward | GGATTGTAAGATAAAAATTACTCC | 936                |
| DB14-Reverse        | GCAATTTCACTAACCGGCCA     |                    |
| AIRS-Forward        | CACTGGTGGTGGTTTCACAG     | 300                |
| AIRS-Reverse        | TCCGTCTCATCTCGGAGTCT     |                    |

*Vigna unguiculata* aminoimidazole ribonucleotide synthetase (AIRS), GenBank reference U30895.1, is a single copy endogenous gene that can be used as a reference for assessing the quality of cowpea genomic DNA for PCR analysis, as well as calculation of gene of interest copy number in quantitative PCR applications. Primers (Table 4) were designed to amplify a short 300-bp section of the AIRS gene by PCR, as a method of assessing genomic DNA quality and providing a positive control for PCR reactions.

Event-specific PCR analysis of genomic DNA prepared from the T1, T5, and T10 generations of 709A cowpea resulted in positive detection of the 936-bp amplified fragment (Figure 10, lanes 2, 3, and 4, panel A). Samples containing non-transgenic negative control DNA from IT86D-1010 cowpea (Figure 10, lane 5, panel A) or genomic DNA from event 701 (Figure 10, lane 6, panel A), produced using the same construct as used in the production of event 709A, did not result in specific amplification. PCR reactions performed using primers specific for the endogenous cowpea AIRS encoding gene resulted in amplification of the predicted *ca.* 300-bp fragment for all genomic DNA samples (Figure 10, lanes 2–6, panel B).

Based on the results of event-specific PCR analysis, the inserted DNA derived from plasmid pMB4 T-DNA was stably inherited within progeny plants spanning ten generations 709A cowpea in direct line of descent from the original transformation event.



**Figure 10.** Samples of genomic DNA prepared from the T1, T5, and T10 generations of event 709A cowpea in IT86D-1010 genetic background (lanes 2, 3, and 4, respectively, in panels A and B), control DNA from non-transgenic IT86D-1010 (lane 5 in panels A and B), and DNA from event 701, a sister event to 709A (lane 6, panels A and B), were analyzed by PCR using event 709A-specific primers (panel A) or primers designed to amplify the endogenous cowpea AIRS encoding gene. Following amplification, samples were subjected to electrophoresis on 0.8% agarose and amplified fragments were visualized by ethidium bromide fluorescence and photomicrography.

#### 4.4. Mendelian Inheritance of the Inserted DNA

The inheritance pattern of the expressed Cry1Ab protein within multiple generations of 709A cowpea was analyzed by lateral flow immunoassay testing (Mohammed et al., 2018). Analyses were performed on individual plants from two segregating generations ( $F_2^{*1,*2,*3}$  and  $BC_1F_1^{*1,*2,*3}$ ) in each of three genetic backgrounds (Figure 4, page 28). Within each generation, numbers of plants testing positive for the presence of Cry1Ab protein were compared with expected frequencies based on Mendelian rules of inheritance, and the significance of any differences was evaluated by Chi-square analysis.

Stable inheritance and expression of transgenes in transgenic crop plants is an essential component of successful employment of genetic engineering in traditional breeding programs (Wu et al., 2002). When a foreign gene is inserted into the host genome, it often leads to the expected 3:1 segregation ratio in  $F_2$  populations and 1:1 in the back-cross progenies (Wang et al., 2012). Deviations from expected Mendelian ratios have been observed (Datta et al., 1990; Goto et al., 1993; Peng et al., 1995) and possible mechanisms have been proposed, including lower viability and fertilization ability of transgenic pollen (Wu et al., 2002) and low fertility of inter-species crosses (Wang et al., 2012).

For event 709A, no deviations from expected segregation ratios were observed. Chi-square ( $X^2$ ) analysis of the data found no statistically significant differences between the observed and expected segregation ratios for the two segregating generations of 709A cowpea in IT97K-499-35, IT86D-1010, and IT93K-693-2 genetic backgrounds (Table 5).

Artificial infestation methods with *Maruca* larvae were used to follow the inheritance of the insect resistance trait in progeny derived from crosses of 709A (IT86D-1010) × IT97K-499-35 (Table 6). While not amenable to statistical analysis, data on pod damage following *Maruca* infestation were consistent with the stable integration and predictable inheritance of the insect resistance trait conferred by expression of Cry1Ab protein.

Analysis of two segregating generations ( $F_2^{*1,*2,*3}$  and  $BC_1F_1^{*1,*2,*3}$ ) that represented different crosses and back-crosses between event 709A (IT86D-1010) and

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**Table 5.** Mendelian inheritance of the Cry1Ab encoding gene within multiple generations of 709A cowpea

| Generation <sup>a</sup>   | Total | Expected <sup>b</sup> |        | Observed <sup>c</sup> |        | Chi Square <sup>d</sup> | p-Value |
|---------------------------|-------|-----------------------|--------|-----------------------|--------|-------------------------|---------|
|                           |       | Present               | Absent | Present               | Absent |                         |         |
| F2 <sup>*1</sup> (3:1)    | 89    | 67                    | 22     | 65                    | 24     | 0.117                   | 0.732   |
| F2 <sup>*2</sup> (3:1)    | 105   | 79                    | 26     | 81                    | 24     | 0.105                   | 0.746   |
| F2 <sup>*3</sup> (3:1)    | 131   | 98                    | 33     | 101                   | 30     | 0.188                   | 0.665   |
| BC1F1 <sup>*1</sup> (1:1) | 32    | 16                    | 16     | 13                    | 19     | 0.568                   | 0.451   |
| BC1F1 <sup>*2</sup> (1:1) | 19    | 10                    | 9      | 10                    | 9      | 0.000                   | >0.999  |
| BC1F1 <sup>*3</sup> (1:1) | 28    | 14                    | 14     | 17                    | 11     | 0.650                   | 0.420   |

<sup>a</sup> Generations identified with the superscript “\*1”, “\*2”, and “\*3” designations represented different 709A breeding lines where the recurring non-transgenic parent was IT97K-499-35, IT86D-1010, and IT93K-693-2, respectively.

<sup>b</sup> Expected values of Cry1Ab presence are rounded up to the nearest integer value and expected values for Cry1Ab absence are rounded down to the nearest integer value.

<sup>c</sup> Cry1Ab QuickStix™ lateral flow immunoassay testing was performed on each plant from each generation.

<sup>d</sup> The analysis tested the hypothesis that the introduced gene was segregating in a Mendelian fashion. The critical value to reject the hypothesis at the five percent level is 3.84. Since the Chi-square ( $X^2$ ) square value was less than 3.84 within each generation, the observed differences were not statistically significant.

**Table 6.** Evaluation of event 709A and IT97K-499-35 parental lines and their progeny following artificial infestation with *Maruca pod* borer

| Generations              | Total Number of Pods | No. of Damaged Pods | No. of Undamaged Pods | Percent Damaged Pods (%) | Percent Undamaged Pods (%) |
|--------------------------|----------------------|---------------------|-----------------------|--------------------------|----------------------------|
| 709A (IT86D-1010)        | 1226                 | 0                   | 1226                  | 0                        | 100                        |
| IT97K-499-35             | 666                  | 153                 | 513                   | 23                       | 77                         |
| F1 (709A × IT97K-499-35) | 1816                 | 0                   | 1816                  | 0                        | 100                        |
| F2                       | 2257                 | 136                 | 2121                  | 6                        | 94                         |
| BC1F1 <sup>a</sup>       | 1093                 | 102                 | 991                   | 9                        | 91                         |

<sup>a</sup> The recurrent parent for back-crossing was IT97K-499-35.

non-transgenic cowpea varieties confirmed that the introduced Cry1Ab encoding gene segregated as a single genetic locus according to Mendelian rules of inheritance.

### 4.5. Antibiotic Resistance Marker Genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the presence in the food of the antibiotic resistance gene DNA *per se* (WHO, 1993). There have been concerns expressed however that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present the human digestive tract, and that this could compromise the therapeutic use of some antibiotics. This section will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes, such as the *nptII* gene, from a genetically modified (GM) food to microorganisms present in the human digestive tract, including relevant regulatory policy related to the use of antibiotic resistance marker genes (ARMGs).

The first issue that must be considered in relation the presence of the *nptII* gene in a GM food is the probability that this gene would be successfully transferred to and expressed in microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

- (i) A fragment of DNA containing the coding region of the *nptII* gene would have to be released as a linear fragment from the DNA of the GM food;
- (ii) The DNA fragment would then have to survive exposure to various nucleases excreted by the salivary glands, the pancreas, and the intestine;

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- (iii) The DNA fragment would have to compete for uptake with dietary DNA and would have to be available at a time and place in which competent bacteria develop or reside;
- (iv) The recipient bacteria would have to be competent for transformation, which is rare (Davison, 1999);
- (v) The DNA fragment would have to be stably integrated into the bacterium, either as a self-replicating plasmid or through a rare recombination event with the bacterial chromosome;
- (vi) The *nptII* gene would have to be expressed, that is, would have to be integrated into the bacterial chromosome in close association with a promoter or would need to already be associated with a promoter that will function in the recipient bacterium; and
- (vii) The *nptII* gene would have to be stably maintained by the bacterial population.

The transfer of the *nptII* gene to microorganisms in the human digestive tract is therefore considered to be highly unlikely because of the number and complexity of the steps that would need to take place consecutively (EFSA, 2007).

Of particular note, the introduction of an intron within the selectable marker gene contained on some transformation plasmids renders the gene (e.g., *nptII*, *hpt*) unable to be expressed in bacteria (Libiakova et al., 2001; Wang et al., 1997) as bacteria lack the RNA splicing machinery necessary to remove introns from the precursor messenger RNA. Thus, even if the marker gene were to be transferred from the transgenic plant DNA to bacteria in the human digestive tract, it would not be expressed and therefore would not render recipient bacteria antibiotic resistant. This is the case for transgenic cowpea event AAT-7Ø9AA-4, where the *nptII* gene present in the introduced T-DNA from plasmid pMB4 is interrupted with the castor bean catalase (CAT)-1 intron (see Figure 3 on page ??).

The second and most important issue that must be considered is the potential impact on human health in the unlikely event successful transfer of a functional antibiotic resistance gene to microorganisms in the human digestive tract did occur.

In the case of the *nptII* gene, the human health impacts are considered to be negligible. While kanamycin (an antibiotic against which the *nptII* encoded enzyme confers resistance) is used in clinical medicine as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis, the *nptII* gene has not been implicated in such resistance (EFSA, 2009).

The GMO Panel of the European Food Safety Authority (EFSA) has examined the use of ARMGs and has concluded for Group-1 ARMGs, which includes *nptII* and *hpt* (hygromycin phosphotransferase (HPT)), that with regard to safety there is no rationale for inhibiting or restricting the use of genes in this category, either for field experimentation or for the purpose of placing on the market (EFSA, 2004). EFSA concluded that the use of *nptII* and *hpt* genes as selectable markers in GM plants and derived food and feed does not pose a risk to human or animal health or to the environment (EFSA, 2009).

In a similar review, the Office of the Gene Technology Regulator (OGTR) in Australia agreed that the evidence strongly suggests horizontal gene transfer from plants to

bacteria is extremely rare (Keese, 2008), with most genetic material (DNA) being degraded in the stomach and intestines. In addition, these genes were originally isolated from bacteria which are widespread in the environment, including in the digestive tract of people and animals. Transfer of ARMGs between bacteria is far more likely than transfer from GM plants to bacteria. According to OGTR, no feasible pathway links a plant possessing either the *nptII* or *hpt* gene and environmental damage (OGTR, 2012).

The risks posed by the possibility of horizontal gene transfer from transgenic plants containing the *nptII* gene to bacteria in the human digestive tract are negligible. The probability of such transfer is extremely remote, the relative impact if it were to occur is insignificant considering that the *nptII* gene is already widespread within the environment and microbial communities.

Finally, in the case of transgenic plant events, such as AAT-709AA-4 cowpea, where the *nptII* gene is interrupted with the CAT-1 intron, expression of this gene in bacteria is not possible thus removing any residual concerns related to the remote possibility of horizontal gene transfer.

#### 4.6. Conclusions from Molecular Characterization

Molecular characterization of the introduced DNA within 709A cowpea confirmed the presence at a single insertion site of the T-DNA region derived from plasmid pMB4 that was stably inherited over multiple generations as a single genetic locus according to Mendelian rules of inheritance. Nucleotide sequencing of the entire inserted DNA, including portions of the 5' and 3' flanking cowpea genomic sequence, was performed to confirm its organization and the potential for creating any new novel open reading frames. The inserted DNA was comprised of one complete copy of the plasmid pMB4 T-DNA, except for 10-bp and 29-bp truncations at the 5' and 3' termini, respectively, and a 735-bp inverted repeat derived from the 3' end of the T-DNA. The site of insertion of the T-DNA was mapped to a region on chromosome 2 of the cowpea genome. There were no new novel open reading frames created as a consequence of the DNA insertion that would have the potential to encode proteins with any significant amino acid sequence similarity to known or suspected toxins or allergens.

## 5. Novel Protein Expression and Dietary Exposure Analysis

### 5.1. Concentrations of Cry1Ab and NPTII Protein in Various Plant Tissues

In order to estimate potential human, animal, and environmental exposure to the Cry1Ab and NPTII proteins expressed in 709A cowpea, the concentration of these proteins in plant tissues representing likely exposure pathways was determined by quantitative enzyme-linked immunosorbent assay (ELISA) (Moore and Higgins, 2018a). Samples of leaf, flower, pod, green cotyledon, and dry seed were collected from 709A (introgressed into IT97K-499-35) and control IT97K-499-35 plants grown under confined field trial conditions in Nigeria in 2014. These tissues as well as samples of root, pollen, and anther wall tissue were also harvested from greenhouse grown 709A (IT86D-1010 background) and control non-transgenic IT86D-1010 plants.

Expression of both Cry1Ab and NPTII is controlled by constitutive promoters and accumulation of some amount of each protein would be expected in all plant tissues.

In samples obtained from T<sub>5</sub> generation IT86D-1010 plants containing event 709A, the highest concentrations of Cry1Ab were measured in leaf tissue (1.9–8.1 µg/g), while the concentrations in dry seed, green cotyledons, pods, and flowers were somewhat lower, ranging between 1.95–5.2 µg/g (Table 7). Measurable amounts of Cry1Ab were also found in samples of roots, pollen, and anthers taken from 709A plants.

The average concentrations of NPTII protein in samples of leaves, flowers, pods, green cotyledons, pollen, anthers, and roots obtained from 709A plants grown in the greenhouse were similar, ranging from 0.16–0.35 µg/g. The highest amounts of NPTII protein were found in dry seed samples, with an average concentration of 1.5 µg/g (Table 7).

As expected, no quantifiable amounts of Cry1Ab and NPTII protein were found in any tissue samples derived from control, non-transgenic, plants.

**Table 7.** Concentrations of Cry1Ab and NPTII in tissue samples derived from 709A cowpea

| Tissue Samples  | Cry1Ab (µg/g FWT) <sup>a</sup> | NPTII (µg/g FWT) |
|-----------------|--------------------------------|------------------|
| Leaf            | 5.1 (1.9–8.1)                  | 0.33 (0.21–0.45) |
| Flower          | 4.5 (3.2–5.2)                  | 0.21 (0.21–0.21) |
| Pods            | 3.7 (2.9–4.0)                  | 0.35 (0.31–0.41) |
| Green Cotyledon | 2.2 (1.95–3.0)                 | 0.16 (0.15–0.16) |
| Dry Seed        | 2.5 (2.3–2.8)                  | 1.5 (1.3–1.6)    |
| Pollen          | 0.12 (0.06–0.18)               | 0.22 (0.21–0.23) |
| Anther Wall     | 1.1 (0.98–1.2)                 | 0.30 (0.29–0.32) |
| Roots           | 0.25 (0.18–0.33)               | 0.18 (0.06–0.30) |

<sup>a</sup> Values represent the mean of replicate samples with the lowest and highest individual values shown in parentheses. Concentrations are uncorrected for extraction efficiency and expressed in µg protein per gram fresh weight tissue (FWT).

Keeping in mind variability due to differences in promoters used to drive gene expression and in sampling and testing methodologies, the highest concentrations of Cry1Ab measured in 709A plant tissues (leaf, pod, and flower tissue) were not inconsistent with what has been observed for different transgenic maize lines expressing Cry1Ab protein (Table 8).

### 5.2. Estimated Human Daily Dietary Exposure to Cry1Ab and NPTII Proteins

The assessment of potential dietary exposure to new or novel proteins in the diet is an essential component of food and feed safety assessment. The assessment is conducted in a step-wise process where initially a conservative “worst-case” scenario is considered

**Table 8.** Highest reported Cry1Ab concentrations in plant tissues derived from different transgenic events

| Crop                                | Transformation Event | Plant Tissue | Cry1Ab (ng/g FWT) <sup>a</sup> |
|-------------------------------------|----------------------|--------------|--------------------------------|
| Cowpea ( <i>Vigna unguiculata</i> ) | 709A                 | Leaf         | 5100 <sup>b</sup>              |
|                                     |                      | Pod          | 3800                           |
|                                     |                      | Flower       | 9400                           |
| Maize ( <i>Zea mays</i> )           | BT 176               | Leaf         | 16400                          |
|                                     | MON80100             | Whole Plant  | 3029                           |
|                                     | Bt-11                | Leaf         | 1770                           |
|                                     | MON 809              | Leaf         | 5300                           |
|                                     | MON 810              | Leaf         | 1630                           |
|                                     | MON 802              | Leaf         | 10340 <sup>c</sup>             |
|                                     |                      | Leaf         | 9550                           |

<sup>a</sup> Values are reported as mean concentrations determined for samples derived from field-grown plants, unless otherwise noted. Rigorous comparisons are limited due to differences in promoters used to drive gene expression, and in sample collection and processing methodology. Data for maize transformation events was sourced from ILSI-RF (2016).

<sup>b</sup> Measured in leaf samples from greenhouse-grown plants.

<sup>c</sup> Value represents highest observed value from a sample of six, where the mean was 9350 ng/g FWT.

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that reflects much higher levels of exposure than would actually be expected in order to account for any realistically conceivable exposure scenario (Garcia-Alonso, 2013). Risk assessment conducted under conservative worst-case scenarios helps ensure a margin of safety under actual exposure conditions.

Expression studies conducted to determine the concentrations of the novel proteins in the genetically engineered plant provide data on the expected levels of the proteins in feedstuffs, and feed consumption tables provide estimates of expected dietary intake of the modified feed.

Conservative estimates of dietary exposure to Cry1Ab and NPTII proteins for consumers of 709A cowpea grain were developed by Bogdanova and MacKenzie (2018a).

Cowpea consumption is the highest of all pulse crops in Sub-Saharan Africa (SSA), accounting for *ca.* 42 percent of total pulse consumption in 2006–08 (Akibode, 2011). Within the different sub-regions of SSA, the highest rates of cowpea consumption are in West Africa (Table 9), with Nigeria having the greatest *per capita* availability of *ca.* 18 kg per year (Table 10).

**Table 9.** Total and *per capita* consumption of cowpea in SSA and sub-regions

|                        | Total Consumption (million tons) <sup>a</sup> |      |      |      |      | Consumption <i>per capita</i> (kg) |      |      |       |      |
|------------------------|---|------|------|------|------|------------------------------------|------|------|-------|------|
|                        | CA <sup>b</sup>                               | EA   | SA   | WA   | SSA  | CA                                 | EA   | SA   | WA    | SSA  |
| 1994–1996              | 0.06  | 0.20 | 0.01 | 2.39 | 2.65 | 0.71                               | 0.90 | 0.15 | 11.43 | 3.66 |
| 2006–2008              | 0.16  | 0.30 | 0.01 | 4.50 | 4.97 | 1.33                               | 0.98 | 0.14 | 15.84 | 5.14 |
| Annual Growth Rate (%) | 7.0   | 2.9  | 0.7  | 4.6  | 4.6  | 4.6                                | 0.6  | -0.5 | 2.4   | 2.5  |

<sup>a</sup> As actual consumption data are not available, Food and Agriculture Organization of the United Nations (FAO) data were used to estimate total availability and *per capita* availability by adding quantity produced to quantity imported and subtracting quantity exported. This estimate does not take into account stocks held by private traders or the public sector, wastages after harvest and before consumption, and quantity used in livestock feed. Thus consumption refers to quantity 'available' for consumption rather than quantity actually consumed. The *per capita* availability is obtained by dividing the total availability by the population. Values are from Table A18 in Akibode (2011).

<sup>b</sup> CA = Central Africa; EA = East Africa; SA = Southern Africa; WA = West Africa; SSA = Sub-Saharan Africa.

**Table 10.** Production and consumption of cowpea in West and Central Africa (1990–1999)

| Country       | Harvested Area (1000s ha) | Average Yield (t/ha) | Production (1000 t dry grain) | Consumption (kg <i>per capita per year</i> ) <sup>a</sup> |
|---------------|---------------------------|----------------------|-------------------------------|---|
| Nigeria       | 3425                      | 0.494                | 1691                          | 18  |
| Niger         | 3268                      | 0.110                | 359                           | 1.5   |
| Mali          | 322                       | 0.244                | 79                            | 1.5   |
| Burkina Faso  | 201                       | 0.777                | 156                           | 1.5   |
| Togo          | 135                       | 0.284                | 38                            | 9   |
| Benin         | 100                       | 0.635                | 64                            | 9   |
| Senegal       | 95                        | 0.341                | 32                            | 1.5   |
| Ghana         | 85                        | 0.663                | 57                            | 9   |
| Mauritania    | 52                        | 0.331                | 17                            | 2.5   |
| Côte d'Ivoire | 40                        | 0.500                | 20                            | 1.8   |
| Chad          | 44                        | 0.489                | 21                            | 1.5   |
| Cameroon      | 38                        | 0.827                | 31                            | 1.5   |

<sup>a</sup> See footnote 'a' from Table 9 regarding the derivation of *per capita* consumption. Values are from Table 4 in the FAO publication on Cowpea: Post-Harvest Operations (Gomez, 2004).

The consumption data presented in Tables 9 and 10 are only for cowpea dried grain, and only reflect amounts available for consumption since cowpea consumption data from household surveys are not available, as explained in footnote 'a' for Table 9. Other cowpea products are also used as food, such as green leaves, immature snapped pods, and green cowpea seeds; however, consumption data for these products are lacking.

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Using the highest *per capita* cowpea utilization in Nigeria of 18 kg/yr and an estimated average adult body weight of 60.7 kg in Africa (Walpole et al., 2012), the maximum daily cowpea intake was calculated as shown in equation 1.

$$\text{Daily Cowpea Grain Intake} = \frac{18 \text{ (kg/yr)}}{365 \text{ (day/yr)} \times 60.7 \text{ (kg BW)}} \times 1000 \text{ (g/kg)} = 0.812 \text{ (g/kg body weight)} \quad (1)$$

Considering the food uses of cowpea, likely routes of dietary exposure to Cry1Ab and NPTII would be via consumption of leaves, pods, and grain (dry seed). As estimates of consumption were not available for either leaves or pods, the exposure assessment focused on grain (seed) consumption and used the highest measured values in dry seed of 2.9  $\mu\text{g/g}$  and 1.6  $\mu\text{g/g}$  for Cry1Ab and NPTII, respectively.

In deriving estimates of maximum potential daily dietary exposure to the Cry1Ab and NPTII proteins expressed in 709A cowpea, the following assumptions were used:

- Mean daily dietary cowpea consumption of 0.812 g/kg body weight.
- One hundred percent of the dietary cowpea intake is from 709A cowpea and is comprised of cowpea grain.
- The grain concentrations of Cry1Ab and NPTII used for estimation are the highest values measured in samples of dried grain collected from any individual trial site location.

Using these assumptions and equation 2, estimated maximum DDEs to Cry1Ab and NPTII were derived (Table 11).

$$\text{DDE (g/kg)} = 0.812 \text{ (g/kg body weight)} \times \text{Protein Concentration (}\mu\text{g/g)} \quad (2)$$

**Table 11.** Estimated maximum potential daily dietary exposure to Cry1Ab and NPTII

| Protein | Concentration ( $\mu\text{g/g}$ FWT) | Daily Dietary Exposure ( $\mu\text{g/kg}$ body weight) |
|---------|--------------------------------------|--|
| Cry1Ab  | 2.9                                  | 2.35   |
| NPTII   | 1.6                                  | 1.30   |

<sup>a</sup> Daily dietary exposures ( $\mu\text{g/kg}$  body weight) to Cry1Ab and NPTII were calculated based on daily *per capita* cowpea consumption of 0.812 g/kg body weight.

The maximum potential human daily dietary exposures to Cry1Ab and NPTII proteins from 709A cowpea were estimated to be *ca.* 2.4 and 1.3  $\mu\text{g/kg}$  body weight, respectively, based on the highest concentrations of these proteins measured in grain (seed) and a maximum daily cowpea intake of 0.812 g/kg body weight.

More realistic estimates of exposure are likely to be significantly less considering that the estimated cowpea consumption is based on amounts available for consumption, which also include stocks held by private traders or the public sector, wastages after harvest and before consumption, and quantity used in livestock feed. Additionally, it is unlikely that 100 percent of daily cowpea consumption will be substituted with 709A cowpea.

5.3. Estimated Livestock Animal Daily Dietary Exposure to Cry1Ab and NPTII Proteins

Conservative estimates of dietary exposure to Cry1Ab and NPTII proteins for livestock animals consuming forage, hay, and seed derived from 709A cowpea were also developed (Bogdanova and MacKenzie, 2018b).

Cowpea forage (bot, vines, and leaves) fresh, or dried as hay or silage, is typically used for livestock animal feed. There have been attempts to use cowpea leaf meal in swine feeding (Heuzé et al., 2015). Cowpea hay, which is comprised of leaves and stems (stover), plays a particularly critical role in feeding livestock during the dry season in many parts of West Africa (OECD, 2015).

Fodder (haulms, crop residues of seed production) is an important component of cropping-livestock systems in semiarid regions of the tropics where its high nutritional quality allows it to be used as a supplement to livestock diets based on cereal stovers and other low-quality forages. The haulms contain about 45–65 percent stems, 35–50 percent leaves, and sometimes roots (Anele et al., 2010). Cowpea pod husks obtained after threshing are also used to feed livestock (Grings et al., 2012). Sheep fed 200–400 g cowpea haulms as a supplement to a basal diet of sorghum stover have shown an average daily live weight gain of about 80 g per animal, which was twice that obtained with sorghum fodder alone (Singh et al., 2003).

The OECD Table of Feedstuffs Derived from Field Crops (OECD, 2013) describes the use of cowpea parts fed to different livestock species (Table 12).

**Table 12.** Consumption of cowpea feedstuffs by different livestock species

|                      | Cattle                                |       | Sheep |      | Swine    |           | Poultry |       |        |
|----------------------|---------------------------------------|-------|-------|------|----------|-----------|---------|-------|--------|
|                      | Beef                                  | Dairy | Ewe   | Lamb | Breeding | Finishing | Broiler | Layer | Turkey |
| Body Weight (kg)     | 500                                   | 500   | 75    | 40   | 260      | 100       | 2       | 2     | 7      |
| Daily Intake (kg DM) | 20                                    | 20    | 2.5   | 1.7  | 6        | 3.1       | 0.16    | 0.15  | 0.5    |
|                      | Percent of Diet (as fed) <sup>a</sup> |       |       |      |          |           |         |       |        |
| Forage (30% DM)      | 100                                   | 60    | 100   | 100  | 20       | –         | –       | –     | –      |
| Hay (86% DM)         | 100                                   | 60    | 65    | 35   | 20       | 10        | –       | –     | –      |
| Seed (88% DM)        | 20                                    | 20    | 75    | 75   | 10       | 20        | 10      | 10    | 10     |

<sup>a</sup> Consumption data for cowpea forage, hay, and seed are the highest values for a country or region reported in the OECD Table of Feedstuffs Derived from Field Crops (OECD, 2013). DM = dry matter.

Cowpea forage (fodder), hay, and seed feed intake values for cattle, sheep, swine, and poultry were obtained from the OECD Table of Feedstuffs Derived from Field Crops (OECD, 2013). Where consumption data were provided for more than one country or region, the highest rate of consumption across regions was used in calculating DDEs to Cry1Ab and NPTII.

Exposure calculations for Cry1Ab and NPTII derived from 709A cowpea forage, hay, and seed used the highest concentrations of each protein measured at any single field location or in the greenhouse (NPTII). For forage and hay, it was assumed these could be composed of leaves and pods, up to 50 percent by weight of each, which implies concentrations of Cry1Ab and NPTII up to 12.7 µg/g and 0.43 µg/g, respectively. For dry seed, the Cry1Ab and NPTII concentrations used for exposure calculations were 2.9 µg/g and 1.6 µg/g, respectively. The calculation of DDE was performed according to equation 1, below. Calculated DDEs to Cry1Ab and NPTII for different livestock species consuming cowpea feedstuffs are shown in Table 12.

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$$\text{DDE (mg/kg body weight)} = \frac{\text{Protein Concentration (mg/kg)} \times \text{Diet (\%)} \times \text{Daily Feed Intake (kg)}}{\text{Dry Matter (\%)} \times \text{Animal Body Weight (kg)}} \quad (3)$$

**Table 13.** Summary of DDEs of Cry1Ab and NPTII for different livestock animal species

| Animal  | Type      | Daily Dietary Exposure (mg/kg body weight) <sup>a</sup> |       |        |       |        |       |
|---------|-----------|---|-------|--------|-------|--------|-------|
|         |           | Forage  |       | Hay    |       | Seed   |       |
|         |           | Cry1Ab  | NPTII | Cry1Ab | NPTII | Cry1Ab | NPTII |
| Cattle  | Beef      | 1.693   | 0.057 | 0.591  | 0.020 | 0.026  | 0.015 |
|         | Dairy     | 1.016   | 0.034 | 0.354  | 0.012 | 0.026  | 0.015 |
| Sheep   | Ewe       | 1.245   | 0.042 | 0.282  | 0.010 | 0.073  | 0.040 |
|         | Lamb      | 1.764   | 0.060 | 0.220  | 0.007 | 0.103  | 0.057 |
| Swine   | Breeding  | 0.195   | 0.007 | 0.068  | 0.002 | 0.008  | 0.004 |
|         | Finishing | NA <sup>b</sup>   | NA    | 0.037  | 0.001 | 0.020  | 0.011 |
| Poultry | Broiler   | NA  | NA    | NA     | NA    | 0.026  | 0.015 |
|         | Layer     | NA  | NA    | NA     | NA    | 0.023  | 0.012 |
|         | Turkey    | NA  | NA    | NA     | NA    | 0.021  | 0.011 |

<sup>a</sup> Calculations of daily dietary exposures utilized feed intake values for cattle, sheep, swine, and poultry from the OECD Table of Feedstuffs Derived from Field Crops (OECD, 2013) and concentrations of Cry1Ab and NPTII in cowpea forage and hay of 12.7 µg/g and 0.43 µg/g, respectively. For dry seed, the highest measured concentrations of Cry1Ab and NPTII were used, which were 2.9 µg/g and 1.6 µg/g, respectively (Table 7).

<sup>b</sup> NA = Not applicable. Cowpea hay and/or forage (fodder) are not feedstuffs for this animal species.

The highest exposures to newly-expressed proteins in 709A cowpea would be for lambs consuming diets containing up to 100 percent fodder, where daily dietary intakes for Cry1Ab and NPTII were estimated at *ca.* 1.76 and 0.06 mg/kg body weight, respectively. Because it is unlikely that all of the cowpea fodder in a feed ration would be substituted with 709A cowpea, more realistic dietary exposures are likely to be less.

## 6. Protein Safety

As a macronutrient, protein is an essential component of the human diet and, although individual proteins mediate a diverse range of biological functions, consumption of proteins as a class of dietary substances is not inherently associated with adverse effects (FAO/WHO, 1996). Only a small number of dietary proteins have the potential to exert anti-nutritional or toxic effects, or elicit allergic reaction in previously sensitized individuals.

Assessing the safety of newly-expressed proteins produced in the edible portions of a genetically engineered food crop is an integral component of the overall safety assessment. As there is currently no single criterion that is sufficiently predictive of potential toxicity or allergenicity, a “weight-of-evidence” approach is recommended for hazard identification that considers the history of use, amino acid sequence similarity to known toxins or allergens, function or mode of action, digestibility under standardized *in vitro* conditions, stability to heat or processing, and expression levels and potential dietary exposure (Codex, 2003; Delaney et al., 2008). Conventional toxicology studies are not considered necessary where the newly-expressed protein, or a closely related one, has been consumed safely in food at equivalent intakes or where the new substance is not present in the food (Codex, 2003; Health Canada, 2006). Only when a potential for hazard has been identified, or when the previous assessment does not permit a determination of safety, is further hazard characterization warranted (Delaney et al., 2008; Hammond et al., 2013). This tier-2 characterization may include appropriate

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oral toxicity studies or other hypothesis-based toxicology studies when the protein's biochemical function suggests it may be potentially toxic to non-target organisms.

A tiered “weight-of-evidence” approach was followed in assessing the safety of the Cry1Ab and NPTII proteins expressed in 709A cowpea, and is described in the following sections.

### 6.1. Cry1Ab Protein

#### 6.1.1. Familiarity of the Cry1Ab Protein

The structures of several different classes of Bt insecticidal crystal proteins have been determined by X-ray crystallography: Cry1Aa (Grochulski et al., 1995), Cry2Aa (Morse et al., 2001), Cry3Aa (Li et al., 1991), Cry3Bb (Galitsky et al., 2001), Cry4Ba (Boonserm et al., 2005), and Cry1Ac (Derbyshire et al., 2001). These proteins all share a similar three-domain structure, indicating similar modes of action (Kanintronkul et al., 2005).

The N-terminal domain I, comprised of a bundle of seven  $\alpha$ -helices in which the central hydrophobic helix- $\alpha$ 5 is encircled by six other amphipathic helices, is responsible for pore formation and membrane insertion. Domain II contains three anti-parallel  $\beta$ -sheets connected by loops, which vary in length and amino acid sequence depending on the Cry protein, that are implicated in receptor binding and hence in determining the specificity of the toxin for insect larvae. The C-terminal domain III, which consists of a  $\beta$ -sandwich containing two anti-parallel  $\beta$ -sheets in a jellyroll conformation, is believed to maintain stability of the active protein against further proteolytic cleavage and to promote target membrane permeabilization, and may also participate in receptor interaction (Lee et al., 1995). The structural similarity of the exposed loop regions of both domains II and III with several carbohydrate-binding proteins (de Maagd et al., 2003) has suggested that carbohydrate moieties could play an important role in the mode of action of three-domain Cry proteins (Bravo et al., 2007).

Within the midgut of susceptible insect species, Cry1Ab is processed at the N-terminus by serine protease-mediated cleavage at arginine-28 and at the C-terminus to yield an insecticidally active core protein (Hofte et al., 1986). The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (De Maagd et al., 2001) before inserting into the membrane. Toxin insertion leads to the formation of lytic pores in microvilli of apical membranes (Aronson and Shai, 2001) followed by cell lysis and disruption of the midgut epithelium resulting in the release of cell contents thereby providing spores a germinating medium leading to a severe septicemia and insect death (De Maagd et al., 2001).

Numerous Cry1A proteins within diverse, naturally occurring Bt strains have been isolated from North America, China (He et al., 2011), Iran (Salekjalali et al., 2012), Brazil (Monnerat et al., 2007), India (Patel and Ingle, 2012) and even Antarctica (Prabhakar and Bishop, 2011). As with Cry2A proteins, the Cry1A proteins are present in DiPel®, a commercial biological insecticide based on a naturally occurring *B. thuringiensis kurstaki* strain (Tabashnik et al., 1993) that contains Cry1Aa, Cry1Ab, and Cry1Ac proteins. Biological insecticides based on Bt, including DiPel®, are available in many countries in Africa (Kabaluk et al., 2010).

Data and information relevant to assessing the environmental, food, and feed safety of Cry1Ab have been the subject of recent reviews by the International Life Sciences

Institute (ILSI) Research Foundation (CERA, 2011; ILSI-RF, 2016). Cry1Ab safety assessments have supported positive regulatory decisions on at least 91 transgenic events and combined event products in crops such as maize (80), cotton (8), rice (2), and sugarcane (1).

6.1.2. Lack of Primary Sequence Structural Alerts for Potential Oral Toxicity

One component of the tier-1 safety assessment of proteins (Delaney et al., 2008) is a bioinformatic evaluation of the amino acid sequence similarity between the protein and known protein toxins (Codex, 2003), which is described in MacKenzie (2018b) for the Cry1Ab protein.

Potential structural similarities shared between the Cry1Ab protein and sequences in a protein toxin database were evaluated using version 36 the FASTA sequence alignment tool (Pearson and Lipman, 1988; Pearson, 1996, 2000). The FASTA36 program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous.

The target sequence for bioinformatic analysis was the truncated Cry1Ab protein corresponding to the N-terminal 615 amino acids of the 1155-amino acid native Cry1Ab protein (GenBank Accession No. M60856.1; Figure 11).

```

      10          20          30          40          50          60
MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
      70          80          90         100         110         120
VDIIWGFIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD
     130         140         150         160         170         180
PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
     190         200         210         220         230         240
RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV
     250         260         270         280         290         300
LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSGFRGSAQGIEGSIRSPHLM DIL
     310         320         330         340         350         360
NSITIIYTDHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR
     370         380         390         400         410         420
TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV
     430         440         450         460         470         480
PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST
     490         500         510         520         530         540
NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS
     550         560         570         580         590         600
IDGRPINQGNFSATMSSGNSLQSGSFRTVGFTTFFNFSNGSSVFTLSAHVFNNGNEVYID
     610
RIEFVPAEVTFEAEY

```

**Figure 11.** Deduced amino acid sequence of the Cry1Ab protein. The protein is 615 amino acids in length with a calculated molecular weight of ca. 68.9 kDa.

A FASTA36 bioinformatic alignment search using the Cry1Ab amino acid sequence as the query sequence was performed against a toxin database to identify possible significant sequence similarity with known or potential toxins. The toxin database was created from a subset of sequences derived from the UniProt Knowledgebase,

comprised of 556,825 manually annotated and reviewed sequences from Swiss-Prot and 108,857,716 automatically annotated, un-reviewed sequences from TrEMBL (The UniProt Consortium, 2014), that were selected using a keyword search on toxins (KW800). The collection contained a total of 35,049 sequences as of March 2, 2018, including 6,499 reviewed sequences from Swiss-Prot and 28,550 un-reviewed sequences from TrEMBL. The default BLOSUM50 similarity scoring matrix was used for FASTA36 alignments (Pearson, 2013).

An *E*-score acceptance criteria of  $1 \times 10^{-5}$  was used to identify sequences from the toxin database with potential for significant sequence similarity to the Cry1Ab protein. The *E*-score is a parameter that describes the number of matches one can “expect” to see by chance when searching a database of a particular size. It decreases exponentially as the score of the match increases. The lower the *E*-score, or the closer it is to zero, the more “significant” the match. Typically, alignments between two sequences require an *E*-score of  $1 \times 10^{-5}$  or less to be considered to have sufficient sequence similarity to infer homology.

A search using the Cry1Ab query sequence returned 996 protein accessions from the toxin database with an *E*-score less than  $1 \times 10^{-5}$ , including 805 accessions that were identified as either Cry proteins, parasporal crystal proteins, pesticidal or insecticidal proteins, or  $\delta$ -endotoxins derived from *Bacillus thuringiensis* (742), *B. cereus* (34), other *Bacillus* sp. (11), *Brevibacillus laterosporus* (4), *Paenibacillus lentimorbus* (4), *Lysinibacillus sphaericus* (3), *Paenibacillus popilliae* (2), *Paraclostridium bifermentans* (2), *B. wiedmannii* (1), *B. weihenstephanensis* (1), or *Clostridium puniceum* (1). These represent a class of highly specific insecticidal proteins with well characterized modes of action that are considered to pose a low risk for humans and animals (Bravo et al., 2007).

A further 189 of the returned accessions were annotated as un-named proteins having one or more of the Pfam<sup>4</sup>  $\delta$ -endotoxin domains associated with either the N-terminus (Endotoxin\_N, PF03945), the central region (Endotoxin\_M, PF00555), or the C-terminus (Endotoxin\_C, PF03944), and representing phylogenetically related Cry variants derived from *B. cereus* (75), *B. toyonensis* (57), *B. thuringiensis* (46), *B. mycoides* (4), or other *Bacillus* sp. (7).

Of the remaining two accessions, one was identified as a D-fructose-6-phosphate amidotransferase from *Bacillus thuringiensis* serovar *chinensis* CT-43, and the other a polyketide synthase module from *Paenibacillus popilliae*. Both of these accessions were annotated as belonging to the  $\delta$ -endotoxin family.

In summary, using conservative search criteria, it was concluded that the Cry1Ab query sequence showed no significant sequence similarity to any proteins known, or suspected, to be of mammalian toxicological concern. This conclusion is further supported by the numerous reviews of similar Cry1Ab bioinformatic analyses by regulatory authorities who have determined that Cry1Ab does not share structural similarities with proteins toxic to humans or livestock animals (ILSI-RF, 2016).

<sup>4</sup> The Pfam database is a large collection of protein families, each represented by multiple sequence alignments. Proteins are generally composed of one or more functional regions, commonly termed domains. Different combinations of domains give rise to the diverse range of proteins found in nature. The identification of domains that occur within proteins can therefore provide insights into their function. The Pfam database is accessible at: <http://pfam.sanger.ac.uk>.

### 6.1.3. Lack of Significant Amino Acid Sequence Similarity with Known Allergens

There is currently no definitive test that can be relied upon to predict allergic response in humans to a novel dietary protein, thus an integrated, stepwise, case-by-case approach is recommended. One component of this assessment includes an evaluation of whether significant amino acid sequence similarity exists between the newly-expressed protein and known allergens. The purpose of a sequence homology comparison is to assess the extent to which a new protein is similar in structure to a known allergen, and this information may suggest whether there is any potential for allergic cross-reaction between the newly expressed protein and a known allergen.

As recommended by Codex (2003), IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35 percent identity in a segment of 80 or more amino acids. The window size of 80 amino acids is meant to correspond with a typical domain size in a protein, and recognizes that a single domain may contain epitopes that mediate antibody binding.

It is also possible that proteins structurally unrelated to allergens, gliadins, and glutenins may contain smaller immunologically meaningful epitopes. A sliding window search was first recommended by Metcalfe et al. (1996) to determine if small regions of proteins that represented IgE-binding epitopes or T-cell epitopes shared identities with known allergens that would be missed in the global sequence alignment. Although considerable variation has been reported in the sizes of IgE binding epitopes, ranging from four amino acids in length to as much as 23 amino acids long, the vast majority consist of at least eight amino acids (Thomas et al., 2005; Bannon and Ogawa, 2006). Using a sliding window of less than eight amino acids can produce matches containing considerable uncertainty as to their significance depending on the length of the query sequence (Silvanovich et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005). In fact, the use of any short-alignment criteria for predicting the allergenic potential of proteins has also been criticized (Goodman et al., 2008; Cressman and Ladics, 2009). An *in silico* study by Herman et al. (2009) found that the allergen-sequence pairs only sharing 8-amino-acid identity, but not >35 percent identity over 80 amino acids, were unlikely to be cross-reactive allergens.

A negative outcome of a sequence homology evaluation indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence similarity should be considered along with the other data in assessing the allergenic potential of newly expressed proteins (Codex, 2003).

As part of assessing the potential allergenicity of the Cry1Ab protein, an evaluation of the significance of any amino acid sequence similarity with known allergens was conducted (MacKenzie, 2018a).

To assess the potential for allergenic cross-reactivity, the 615-amino acid N-terminal sequence encoded by the *cry1Ab* gene (Perlak et al., 1991) was compared to a peer-reviewed database of 2093 known and putative allergen and celiac protein sequences residing in the FARRP dataset at the University of Nebraska.<sup>5</sup>

<sup>5</sup> The Food Allergy Research and Resource Program (FARRP) allergen protein database resides at AllergenOnline (<http://www.allergenonline.org>). Version 18A was released on February 1, 2018, and contains 2093 peer-reviewed sequences representing 832 taxonomic-protein groups.

Potential identities between the Cry1Ab query sequence and proteins in the allergen database were evaluated with the FASTA35 sequence alignment tool using the default parameters.<sup>6</sup> The recommended greater than 35 percent identity threshold over any 80-amino acid length sequence alignment between the query sequence and an allergen was used to indicate the potential for cross-reactivity. No identity matches of > 35 percent over 80 residues were observed (Table 14).

The Cry1Ab query sequence was also evaluated for any eight contiguous identical amino acid matches to the allergens contained in the FARRP database. This was done using an algorithm that generates all possible eight-word peptides from both the query and dataset proteins and evaluates each query “word” against all dataset “words” for perfect matches. There were no eight contiguous identical amino acid matches observed (Table 14).

**Table 14.** Search results using Cry1Ab query sequence against the FARRP database

|  |  |
|--|--|
| <b>Database</b>                                  | AllergenOnline Database v18A (February 1, 2018) <sup>a</sup>   |
| <b>Input Query</b>                               | >Cry1Ab<br>MDNNPINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI<br>EQLINQRIEE FARNQAISRL EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV QNYQVPLLSV<br>YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTTLV<br>LDIVSLFPNY DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMIDL NSITIYTDH RGEYYWSGHQ<br>IMASPVGFSG PEFTFPLYGT MGNAAQQRI VAQLGQGVYR TLLSSTLYRRP FNIQINNQL SVLDGTEFAY GTSSNLPSAV<br>YRKSQTVDSL DEIPPQNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA PMFSWIHRSA EFNNIIPSSQ ITQIPLTKST<br>NLGSGTSVVK GPGFTGGDIL RRTSPGQIST LRVNITAPLS QRYRVIRIYA STTNLQFHTS IDGRPINQGN FSATMSSGSN<br>LQSGSFRTVG FTTPFNFSNG SSVFTLSAHV FNSGNEVYID RIEFVPAEVT FEAEY |
| <b>Length</b>                                    | 615  |
| <b>Number of 80mers</b>                          | 536  |
| <b>Number of Sequences with Hits</b>             | 0  |
| <b>Number of 8mers</b>                           | 608  |
| <b>Number of Sequences with Exact 8mer Match</b> | 0  |

<sup>a</sup> Database search performed on 2 March 2018.

Based on the results of this analysis, the lack of potentially significant sequence similarity between the Cry1Ab protein and known and putative allergens indicates that it is not a known allergen and is unlikely to be cross-reactive to known allergens.

#### 6.1.4. Rapid Digestibility of Cry1Ab Protein

Susceptibility to digestion has been routinely evaluated as part of the safety assessment of novel proteins introduced into genetically engineered plants. Proteins that are readily digestible are likely to behave like other dietary proteins upon ingestion and unlikely to result in allergic or toxic reactions. Astwood et al. (1996) were among the first to provide empirical evidence supporting a correlation between the pepsin resistance of a protein and its allergen status. Although a number of subsequent studies have indicated a weaker link between stability to digestion and allergenicity (Fu et al., 2002; Herman et al., 2007; Bogh and Madsen, 2015), the resistance of a novel food protein to pepsin

<sup>6</sup> The FASTA version 35.04 used on the AllergenOnline website uses the BLOSUM 50 scoring matrix (Henikoff and Henikoff, 1992), a “word size” of 2, and an expectation value score (*E*-score) cut-off of 1. The *E*-score is a parameter that describes the number of matches one can “expect” to see by chance when searching a database of a particular size. It decreases exponentially as the score of the match increases. The lower the *E*-score, or the closer it is to zero, the more “significant” the match.

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digestion under acidic conditions remains generally accepted as one factor to consider in a weight-of-evidence approach for assessing potential allergenicity and toxicity (Bannon et al., 2002; Codex, 2003; Goodman et al., 2005).

The pepsin digestibility assay is not meant to predict whether a given protein will always be digested in the stomach of the consumer, but it does provide a simple, standardized, *in vitro* correlative assay to evaluate relative protein digestibility (Thomas et al., 2004).

The susceptibility of Cry1Ab (trypsin-resistant core) protein to proteolytic degradation was evaluated in simulated gastric fluid (SGF) containing pepsin (Ream, 1994). Samples of *E. coli*-produced Cry1Ab protein were incubated in SGF containing pepsin and samples were removed at different time points and subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis followed by western immunoblot labelling, or insect bioassay. More than 90 percent of the initially added Cry1Ab protein degraded within two minutes incubation as detected by western blot analysis. Protein degradation also paralleled loss of insecticidal activity, with 74–90 percent of Cry1Ab activity lost within two minutes of incubation in SGF containing pepsin, the earliest time point measured.

To put the rapid degradation of the Cry1Ab protein in the SGF system into perspective, approximately 50 percent of solid food has been estimated to empty from the human stomach within two hours, while liquid empties in approximately 25 minutes (Sleisenger and Fordtran, 1989).

These data support the conclusion that Cry1Ab expressed in transgenic plants will be readily digested as a conventional dietary protein under typical mammalian gastric conditions.

### 6.1.5. Lack of Acute Toxicity of Cry1Ab Protein

Based on the well-known function and specificity of the Cry1Ab protein, lack of significant amino acid sequence similarity with known mammalian toxins, and its rapid pepsin digestibility, it can be predicted that Cry1Ab protein is unlikely to be acutely toxic to mammals by the oral route.

However, as a further assurance of safety, the potential for acute toxicity resulting from a single oral exposure to Cry1Ab protein was investigated in mice (Naylor, 1992). Groups of ten male and ten female CD-1 strain mice were dosed orally by gavage with: vehicle control (50 mM carbonate buffer pH 9.6); vehicle control containing bovine serum albumin (BSA) at a dose of 4,000 mg/kg body weight; or purified microbial-expressed Cry1Ab (trypsin-resistant core) protein at three dosing levels: 400, 1,000, or 4,000 mg/kg body weight. After gavage, mortality, moribundity, and clinical signs were monitored twice daily for signs of toxicity. Body weights were measured prior to dosing and at day-7. Food consumption was measured daily. On test day-8, all male mice were euthanized and necropsied. Internal cavities were opened, and organs examined *in situ* and approximately 40 tissues from each animal were saved. Hollow organs were opened and examined. The same was done for all female mice on test day-9.

There were no statistically significant differences in terminal body weights or food consumption (Table 15) and there were no mortalities during the in-life phase of the study. A few incidental pathological changes were observed at necropsy which were

randomly distributed among all groups and are commonly observed for the strain of mice used in laboratory testing. None of these findings were considered related to treatment. The only statistically significant difference was in the variances for the groups in total food consumption that was attributed to a single female mouse in the BSA control group. Elimination of this one outlying data point resulted in no statistically significant difference in any of the groups for any of the parameters analyzed.

**Table 15.** Summary of terminal body weights and food consumption of mice administered Cry1Ab or vehicle control

| Test Group <sup>a</sup>         | Sex    | Terminal Mean Body Weight (g ± SD <sup>b</sup> ) | Mean Food Consumption (g ± SD <sup>b</sup> ) |
|---------------------------------|--------|--|--|
| Vehicle control                 | Male   | 30.0 ± 1.72                                      | 5.3 ± 0.68 BT <sup>c</sup>                   |
| Vehicle control, 4000 mg/kg BSA | Male   | 31.0 ± 0.97                                      | 6.2 ± 1.60                                   |
| 400 mg/kg Cry1Ab                | Male   | 30.5 ± 1.18                                      | 5.3 ± 1.23                                   |
| 1000 mg/kg Cry1Ab               | Male   | 31.1 ± 1.64                                      | 5.3 ± 0.40                                   |
| 4000 mg/kg Cry1Ab               | Male   | 30.5 ± 1.87                                      | 5.5 ± 1.12                                   |
| Vehicle control                 | Female | 25.1 ± 1.53                                      | 6.4 ± 2.14                                   |
| Vehicle control, 4000 mg/kg BSA | Female | 24.7 ± 1.39                                      | 7.3 ± 2.34                                   |
| 400 mg/kg Cry1Ab                | Female | 25.2 ± 2.13                                      | 8.0 ± 3.69                                   |
| 1000 mg/kg Cry1Ab               | Female | 25.0 ± 1.36                                      | 8.0 ± 2.32                                   |
| 4000 mg/kg Cry1Ab               | Female | 25.5 ± 1.70                                      | 6.8 ± 2.65                                   |

<sup>a</sup> Each test group consisted of 10 randomized male or female CD-1 mice. The vehicle control consisted of 50 mM carbonate buffer pH 9.6 and the negative control protein was BSA. Source: Naylor (1992).

<sup>b</sup> SD = Standard deviation.

<sup>c</sup> BT = Bartlett's test indicates statistically significant difference among variances of the different groups (P<0.01).

### 6.1.6. Conclusions on the Safety of Cry1Ab Protein

The weight-of-evidence supporting the lack of identifiable hazards associated with Cry1Ab protein included:

- The known function and specificity of Cry1Ab in binding to specific receptors on the brush border membrane of midgut epithelial cells only in target lepidopteran insect species, thereby resulting in cell lysis and septicemia causing insect death. This activity does not occur in mammals, other vertebrates, or other orders.
- Bioinformatic analyses showed that the Cry1Ab protein does not display significant amino acid sequence similarity to known or putative protein mammalian toxins or allergens.
- The Cry1Ab protein is rapidly degraded in SGF containing pepsin, with more than 90 percent degradation within two minutes as assessed by SDS-PAGE and western immunoblot analysis.
- The Cry1Ab protein is not acutely toxic in mice at dosages up to 4,000 mg/kg body weight, the highest dosage tested.

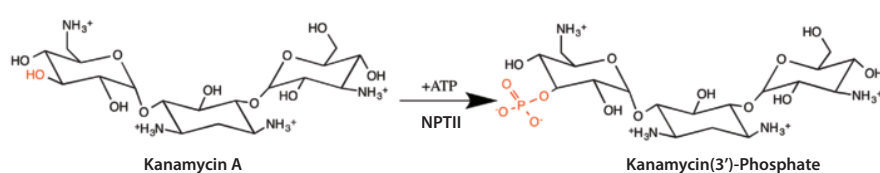
In combination, the data described above support the conclusion that Cry1Ab protein is unlikely to be toxic or allergenic to humans or animals.

## 6.2. NPTII Protein

### 6.2.1. Familiarity of the NPTII Protein

Bacterial aminoglycoside 3'-phosphotransferase II (APH [3] II, E.C 2.7.1.95), also known as neomycin phosphotransferase II (NPTII), was shown to be effective as a

selectable marker in mammalian and yeast cells, therefore it was the first to be tested in plants. Since that time it has become the most widely used selectable marker system in plants. NPTII catalyses the ATP-dependent phosphorylation of the 3'-hydroxyl group of the amino-hexose portion of certain aminoglycosides including neomycin, kanamycin, geneticin (G418), and paramomycin (Figure 12) thereby inactivating the antibiotics. The *nptII* gene (also known as *neo*) from *Escherichia coli* transposon Tn5 (Beck et al., 1982) was first used to construct chimeric genes for constitutive expression in plants by fusing it with the 5' and 3' regulatory sequences of the *A. tumefaciens* T-DNA gene nopaline synthase (*nos*). It was shown to be efficient in the selection of transformed petunia or tobacco cells on kanamycin or G418 Fraley et al. (1983); Bevan et al. (1983); Herrera-Estrella et al. (1983).



**Figure 12.** NPTII (aminoglycoside 3-phosphotransferase II [APH(3')]) catalyzes the phosphorylation of kanamycin A, a 4,6-disubstituted aminoglycoside, at the 3'-hydroxyl group, thus rendering it inactive as an inhibitor of protein synthesis.

The safety of the NPTII protein has been extensively studied and reported in the scientific literature (Fuchs et al., 1993; Flavell et al., 1992; Nap et al., 1992). There is also a long history of approval of GM crops containing the *nptII* marker gene and expressing the NPTII protein. The US Food and Drug Administration (FDA) was the first regulatory agency to consider the food safety of the *nptII* marker gene and NPTII protein, and approved its presence in food as an additive in 1994 (FDA, 1994). In guidance to the industry the FDA concluded that “The *kan*’ gene is safe to use as a selectable marker in the development of transgenic crops” (FDA, 1998). The US EPA has issued an exemption from the requirement of a tolerance for the NPTII protein (US-EPA, 1994), which means that NPTII protein is safe at any level expressed in GM crops.

### 6.2.2. Lack of Primary Sequence Structural Alerts for Potential Oral Toxicity

Potential structural similarities shared between the NPTII protein and sequences in a protein toxin database were evaluated using version 36 the FASTA sequence alignment tool (Pearson and Lipman, 1988; Pearson, 1996, 2000). The FASTA36 program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous.

The target sequence for bioinformatic analysis was the 264-amino acid NPTII protein encoded by the *nptII* gene derived from *Escherichia coli* strain K12 (Beck et al., 1982).

A FASTA36 bioinformatic alignment search using the NPTII amino acid sequence as the query sequence was performed against a toxin database to identify possible significant sequence similarity with known or potential toxins. The toxin database was created from a subset of sequences derived from the UniProt Knowledgebase, comprised of 556,825 manually annotated and reviewed sequences from Swiss-Prot

```

      10      20      30      40      50      60
MIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNEL
      70      80      90     100     110     120
QDEAARLSWLATTGVPCAAFLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAM
     130     140     150     160     170     180
RRLHTLDPATCPFDPHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELEFARLKARMPD
     190     200     210     220     230     240
GEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADRF
     250     260
LVLYGIAAPDSQRIAFYRLLDEFF

```

**Figure 13.** Deduced amino acid sequence of the NPTII protein. The protein is 264 amino acids in length with a calculated molecular weight of ca. 29.0 kDa.

and 108,857,716 automatically annotated, un-reviewed sequences from TrEMBL (The UniProt Consortium, 2014), that were selected using a keyword search on toxins (KW800). The collection contained a total of 35,049 sequences as of March 11, 2018, including 6,499 reviewed sequences from Swiss-Prot and 28,550 un-reviewed sequences from TrEMBL. The default BLOSUM50 similarity scoring matrix was used for FASTA36 alignments (Pearson, 2013).

An *E*-score acceptance criteria of  $1 \times 10^{-5}$  was used to identify sequences from the toxin database with potential for significant sequence similarity to the NPTII protein. The *E*-score is a parameter that describes the number of matches one can “expect” to see by chance when searching a database of a particular size. It decreases exponentially as the score of the match increases. The lower the *E*-score, or the closer it is to zero, the more “significant” the match. Typically, alignments between two sequences require an *E*-score of  $1 \times 10^{-5}$  or less to be considered to have sufficient sequence similarity to infer homology.

The toxin database search using the NPTII query sequence did not return any entries with an *E*-score less than  $1 \times 10^{-5}$  (MacKenzie, 2018d).

In summary, using conservative search criteria, it was concluded that the NPTII query sequence showed no significant sequence similarity to any proteins known, or suspected, to be of mammalian toxicological concern.

### 6.2.3. Lack of Significant Amino Acid Sequence Similarity with Known Allergens

As part of assessing the potential allergenicity of the NPTII protein, an evaluation of the significance of any amino acid sequence similarity with known allergens was conducted MacKenzie (2018c).

To assess the potential for allergenic cross-reactivity, the 264-amino acid sequence encoded by the *nptII* gene was compared to a peer-reviewed database of 2093 known and putative allergen and celiac protein sequences residing in the FARRP dataset at the University of Nebraska.<sup>7</sup>

Potential identities between the NPTII query sequence and proteins in the allergen database were evaluated with the FASTA35 sequence alignment tool using the default

<sup>7</sup> The Food Allergy Research and Resource Program (FARRP) allergen protein database resides at AllergenOnline (<http://www.allergenonline.org>). Version 18A was released on February 1, 2018, and contains 2093 peer-reviewed sequences representing 832 taxonomic-protein groups.

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parameters.<sup>8</sup> The recommended greater than 35 percent identity threshold over any 80-amino acid length sequence alignment between the query sequence and an allergen was used to indicate the potential for cross-reactivity. No identity matches of > 35 percent over 80 residues were observed (Table 16).

The NPTII query sequence was also evaluated for any eight contiguous identical amino acid matches to the allergens contained in the FARRP database. This was done using an algorithm that generates all possible eight-word peptides from both the query and dataset proteins and evaluates each query “word” against all dataset “words” for perfect matches. There were no eight contiguous identical amino acid matches observed (Table 16).

**Table 16.** Search results using NPTII query sequence against the FARRP database

|  |   |
|--|---|
| <b>Database</b>                                  | AllergenOnline Database v18A (February 1, 2018) <sup>a</sup>  |
| <b>Input Query</b>                               | >NPTII (264 aa)<br>MIEQDGLHAG SPAAWVERLF GYDWAQQTIG CSDAAVFRLS AQGRPVLFVK TDLGGALNEL QDEAARLSWL ATTVGPAAV<br>LDVVTEAGRD WLLGVEVPGQ DLLSSHLAPA EKVSIMADAM RRLHTLDPAT CPFDHQAKHR IERARTRMEA GLVDQDDLDE<br>EHQGLAPAEI FARLKARMPD GEDLVVTHGD ACLPNIMVEN GRFSGFDICG RLGVADRYQD IALATRDIAE ELGGEWADRF<br>LVLYGIAAPD SQRIAFYRLL DEFF |
| <b>Length</b>                                    | 264   |
| <b>Number of 80mers</b>                          | 185   |
| <b>Number of Sequences with Hits</b>             | 0   |
| <b>Number of 8mers</b>                           | 257   |
| <b>Number of Sequences with Exact 8mer Match</b> | 0   |

<sup>a</sup> Database search performed on 11 March 2018.

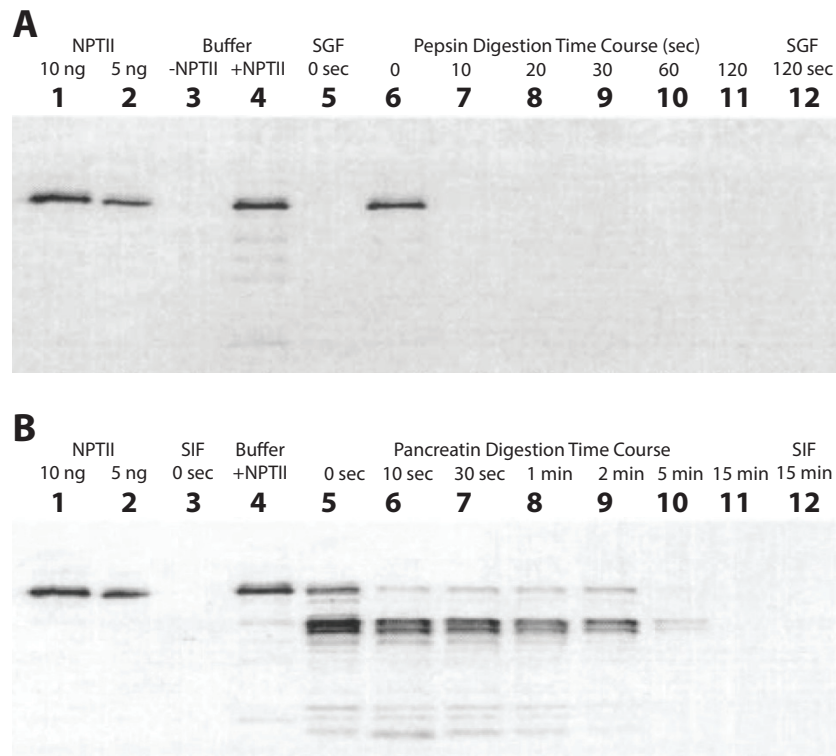
Based on the results of this analysis, the lack of potentially significant sequence similarity between the NPTII protein and known and putative allergens indicates that it is not a known allergen and is unlikely to be cross-reactive to known allergens.

### 6.2.4. Rapid Digestibility of NPTII Protein

The susceptibility of NPTII protein to proteolytic degradation was evaluated in SGF containing pepsin (Fuchs et al., 1993). Samples of microbial-produced NPTII protein were incubated for 0, 10, 20, 30, 60 and 120 seconds in SGF containing pepsin. Samples were removed at stated time points and subjected to SDS-PAGE analysis followed by western immunoblot labelling. No intact NPTII protein was detected at the earliest time point sampled (Figure 14, panel A).

Although not required for proteins that are rapidly and completely digested in the standardized pepsin digestion model, the rapid degradation of NPTII protein in the presence of SIF containing pancreatin was also confirmed (Fuchs et al., 1993). Samples of microbial-produced NPTII protein were incubated for 0, 10, and 30 seconds, and continued for 1, 2, 5 and 15 minutes, in SIF containing pancreatin. Samples were removed at stated time points and subjected to SDS-PAGE analysis followed by western

<sup>8</sup> The FASTA version 35.04 used on the AllergenOnline website uses the BLOSUM 50 scoring matrix (Henikoff and Henikoff, 1992), a “word size” of 2, and an expectation value score (*E*-score) cut-off of 1. The *E*-score is a parameter that describes the number of matches one can “expect” to see by chance when searching a database of a particular size. It decreases exponentially as the score of the match increases. The lower the *E*-score, or the closer it is to zero, the more “significant” the match.



**Figure 14.** Western immunoblot analyses of samples of microbial-produced NPTII protein incubated in the presence of SGF containing pepsin (Panel A) or simulated intestinal fluid (SIF) containing pancreatin (Panel B). Pepsin digestion reactions were sampled at time-zero and after 10, 20, 30, 60, and 120 seconds of incubation (lanes 6–11, panel A). Control samples for the SGF digestion included purified NPTII protein (10 ng and 5 ng; lanes 1 and 2, respectively, panel A), buffer without or with NPTII protein (lanes 3 and 4, respectively, panel A), and SGF without NPTII sampled at 0 and 120 seconds (lanes 5 and 12, respectively, panel A). Pancreatin digestion reactions were sampled at time-zero and after 10 sec, 30 sec, 1 min, 2 min, 5 min, and 15 min of incubation (lanes 5–11, panel B). Control samples for the SIF digestion included purified NPTII (10 and 5 ng; lanes 1 and 2, respectively, panel B), NPTII in sample buffer without pancreatin (lane 4, panel B), and SIF without NPTII sampled at 0 sec and 15 min (lanes 3 and 12, respectively, panel B). All samples from both sets of digestions were analyzed by SDS-PAGE followed by western immunoblotting using specific rabbit anti-NPTII IgG. Source: Fuchs et al. (1993).

immunoblot labelling. Approximately 50 percent of the NPTII protein was degraded after 2–5 min of incubation in SIF containing pancreatin and no intact NPTII was detectable after 11 min of incubation (Figure 14, panel B). Transit times through the intestine are known to be between 3–5 hours (Hall, 2016). Therefore, in the unlikely event that trace amounts of NPTII protein should enter the small intestine, these data support the conclusion that there is minimal, if any potential for intact NPTII protein to reach the intestinal mucosa and trigger an IgE mediated (i.e. allergic) response (Fuchs et al., 1993).

These data support the conclusion that NPTII expressed in transgenic plants will be readily digested as a conventional dietary protein under typical mammalian gastric conditions.

#### 6.2.5. Lack of Acute Toxicity of NPTII Protein

An analysis of potential toxicological hazards associated with the NPTII protein that considers the lack of primary sequence structural alerts for toxicity, the defined mode of action of NPTII and the rapid pepsin digestibility of NPTII, does not identify any

concerns. Based on these considerations, it can be predicted that NPTII protein is unlikely to be acutely toxic by the oral route.

However, as a further assurance of safety, the potential for acute toxicity resulting from a single oral exposure to NPTII protein was investigated in mice (Fuchs et al., 1993). Groups of ten male and ten female CD-1 strain mice were dosed orally by gavage with: vehicle control (50 mM carbonate buffer pH 9.6); or purified microbial-expressed NPTII protein at three dosing levels: 100, 1000, or 5000 mg/kg body weight. After gavage, mortality, moribundity, and clinical signs were monitored twice daily. Detailed observations for signs of toxicity were performed on day-7. Food intake was recorded on days 1 and 7, and body weight was measured on day-1 (the day of gavage administration), and day-7. On test day-8, all male mice were euthanized and necropsied. Internal cavities were opened, and organs examined *in situ* then removed. Hollow organs were opened and examined. The same was done for all female mice on test day-9.

All animals survived through day-7, until euthanized on day-8 (males) or day-9 (females). No mortality or moribundity was observed in any of the groups throughout the study. There were no differences in cage-side behaviour, nor in clinical observations. There were no significant differences in group mean terminal body weights (Table 17) and no gross lesions were observed at necropsy compared to the vehicle control group. No differences in food consumption attributable to the treatments were observed (Table 17).

**Table 17.** Summary of terminal body weights and food consumption of mice administered NPTII or vehicle control

| Test Group <sup>a</sup> | Sex    | Terminal Mean Body Weight (g ± SD <sup>b</sup> ) | Mean Food Consumption (g ± SD <sup>b</sup> ) |
|-------------------------|--------|--|--|
| Vehicle control         | Male   | 28.42 ± 1.60                                     | 4.9 ± 0.50 BT                                |
| 100 mg/kg NPTII         | Male   | 28.40 ± 1.49                                     | 4.7 ± 0.51 *                                 |
| 1000 mg/kg NPTII        | Male   | 28.31 ± 1.13                                     | 5.4 ± 1.15                                   |
| 5000 mg/kg NPTII        | Male   | 28.51 ± 1.73                                     | 5.2 ± 1.35                                   |
| Vehicle control         | Female | 22.93 ± 1.60                                     | 9.4 ± 3.10                                   |
| 100 mg/kg NPTII         | Female | 22.17 ± 1.02                                     | 5.8 ± 1.94 *                                 |
| 1000 mg/kg NPTII        | Female | 22.00 ± 0.73                                     | 7.5 ± 3.11                                   |
| 5000 mg/kg NPTII        | Female | 21.98 ± 1.55                                     | 6.1 ± 2.40 *                                 |

<sup>a</sup> Each test group consisted of 10 randomized male or female CD-1 mice. Because of excessive diet spillage, there were only nine diet consumption measurements for the male 100 mg/kg NPTII, and the female vehicle control and 5000 mg/kg NPTII treatments.

<sup>b</sup> SD = Standard deviation.

\* Denotes a statistically significant difference ( $p < 0.05$ ) from the vehicle control using Dunnett's two-tailed test. BT denotes statistically significant difference among variances of the different groups ( $p < 0.01$ ) using Bartlett's test. Source: Fuchs et al. (1993).

Under the conditions of this testing, oral administration of NPTII test substance at concentrations up to 5,000 mg/kg body weight produced no test substance-related clinical signs of toxicity, body weight losses, gross lesions, or mortality, further substantiating the predicted lack of acute oral toxicity of NPTII.

#### 6.2.6. Conclusions on the Safety of NPTII Protein

The weight-of-evidence supporting the lack of identifiable hazards associated with NPTII protein included:

- The known function of NPTII in catalyzing the ATP-dependent phosphorylation of the 3'-hydroxyl group of the amino-hexose portion of certain aminoglycosides is not similar to the activities of known protein toxins.

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- Bioinformatic analyses showed that the NPTII protein does not display significant amino acid sequence similarity to known or putative protein toxins or allergens.
- The NPTII protein is rapidly degraded in SGF containing pepsin and SIF containing pancreatin. No intact NPTII or NPTII-derived fragments were detected after exposure to pepsin-containing SGF or pancreatin-containing SIF as assessed by western immunoblot labelling following SDS-PAGE analysis.
- The NPTII protein is not acutely toxic in mice at dosages up to 5,000 mg/kg body weight, the highest dosage tested.

In combination, the data described above support the conclusion that NPTII protein is unlikely to be toxic or allergenic to humans or animals.

## 7. Compositional Analysis

For new varieties without purposefully altered nutritional properties, which includes the vast majority of currently authorized genetically modified crops, the compositional assessment is part of the weight-of-evidence approach for evaluating whether there were any unanticipated consequences of the genetic modification. The experience with genetically modified crop plants with introduced traits conferring insect-resistance and/or herbicide-tolerance has indicated that the incorporation of these traits has little biologically meaningful impact on the composition of key nutrients and anti-nutrients (Harrigan et al., 2010). As with products of conventional plant breeding, most compositional variation is due to environmental and agronomic factors, and the base genetics of the plant variety (Harrigan et al., 2007; Ricroch, 2013).

The compositional analysis compared the concentrations of major nutrient components in samples of whole grain, leaves, and fodder collected from event 709A and control cowpea grown at four different locations representing typical cowpea growing conditions in West Africa (Figure 15, Table 18). In addition, concentrations of key minerals and phytic acid were determined in samples of whole grain. At each site, planting and cultivation was done according to local agronomic practices. Four blocks (replicates) of each entry were established at each test site in a randomized complete block design. Further details on the field trial locations and site management, and the analytical procedures and statistical analyses are described in Abdourhamane et al. (2018b).

**Table 18.** Trial locations

| Trial Site Code | Site Name             | Institution                             | Country | Agroecological Zone | Latitude and Longitude Coordinates |
|-----------------|-----------------------|---|---------|---------------------|------------------------------------|
| ZA              | Zaria, Kaduna State   | Institute for Agricultural Research     | Nigeria | Northern Guinea     | 11°15' 15.89" N<br>7°36' 36.7" E   |
| MJ              | Minjibir, Kano State  | Institute for Agricultural Research     | Nigeria | Sudano-Sahelian     | 12°25' 29.08" N<br>8°53' 33.05" E  |
| BK              | Bakura, Zamfara State | IITA                                    | Nigeria | Sudan Savannah      | 12°41' 4.60" N<br>5°53' 25.3" E    |
| TA              | Tamale                | Savanna Agricultural Research Institute | Ghana   | Guinea Savannah     | 9°15' 0.86" N<br>0°49' 26.30" W    |

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**Figure 15.** Map showing field trial locations in Nigeria and Ghana during 2015.

Statistical analyses of the composition data were conducted using a mixed model analysis of variance for a combination of all sites. The combined-sites analysis used the model:

$$y_{ijk} = \mu_i + l_j + r_{k(j)} + (\mu l)_{ij} + \varepsilon_{ijk}$$

where  $\mu_i$  denotes the mean of the  $i^{th}$  entry (fixed effect),  $l_j$  denotes the effect of the  $j^{th}$  site (random effect),  $r_{k(j)}$  denotes the effect of the  $k^{th}$  block within the  $j^{th}$  site (random effect),  $(\mu l)_{ij}$  denotes the interaction between the entries and sites (random effect) and  $\varepsilon_{ijk}$  denotes the effect of the plot assigned the  $i^{th}$  entry in the  $k^{th}$  block of the  $j^{th}$  site (random effect or residual).

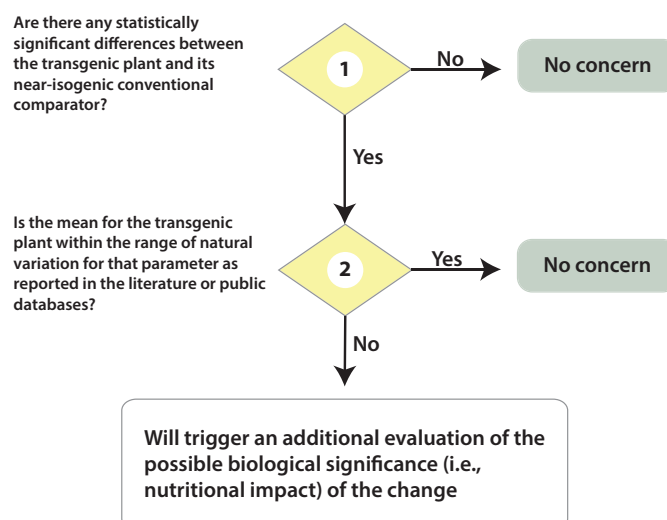
For each compositional parameter, the least squares (LS)-mean value across sites was derived from the corresponding statistical model for event 709A cowpea and the control IT97KN cowpea using the “emmeans” package (Lenth, 2013).

The first step in the evaluation was to test for differences in LS-mean values between the transgenic and control entries (Figure 16). Where a statistically significant difference ( $p$ -value < 0.05) was identified in the combined-sites, multi-year analysis, further context for interpreting the possible biological significance of the difference was gathered through comparisons with the range of values for each analyte reported in the OECD consensus document on new cowpea varieties (OECD, 2018a) and in the published literature (Belane and Dakora, 2012; Heuzé and Tran, 2015; Heuzé et al., 2015; Gonçalves et al., 2016; Muranaka et al., 2016). Analyte ranges for 709A cowpea that fell within the combined literature range for that analyte were considered to be within the range of normal variability of conventional cowpea.

### 7.1. Proximates

The major constituents, or proximates, of cowpea whole grain are protein, fat, ash, and carbohydrates. These components have been useful in characterizing the nutritive

## COMPOSITIONAL ANALYSIS



**Figure 16.** Comparative decision tree for evaluating differences measured in analyte concentrations between test and control samples.

and functional grain characteristics representing germplasm accessions, diverse cowpea genotypes, and cultivated varieties (cultivars) (Muranaka et al., 2016), breeding populations (Asare et al., 2013), and local market varieties in Ghana (Appiah et al., 2011) and Nigeria (Chinma et al., 2008).

In the combined-sites analysis, there were no statistically significant differences in proximates, moisture, or calories between whole grain samples of event 709A and control cowpea (Table 19).

**Table 19.** Proximate composition of whole grain harvested from event 709A and control cowpea grown at four locations in West Africa during 2015

| Samples                  | Proximates (%DB) <sup>a</sup> |                    |                     |                                  |                                  |                     |
|--------------------------|-------------------------------|--------------------|---------------------|----------------------------------|----------------------------------|---------------------|
|                          | Crude Protein                 | Crude Fat          | Ash                 | Carbohydrates                    | Calories (kcal/g DB)             | Moisture (%)        |
| Event 709A IT97KT        | 24.56<br>(22.4–26.6)          | 6.59<br>(3.33–8.9) | 3.19<br>(2.75–3.7)  | 65.7<br>(61.3–70.7)              | 4.37<br>(4.23–4.44)              | 8.86<br>(7.43–10.6) |
| Control IT97KN           | 25.06<br>(23.1–28.5)          | 5.80<br>(2.7–8.34) | 3.25<br>(2.76–3.73) | 65.9<br>(59.4–70.1)              | 4.37<br>(4.3–4.41)               | 8.92<br>(7.8–10.6)  |
| <i>p</i> -Value          | 0.211                         | 0.300              | 0.338               | 0.818                            | 0.950                            | 0.679               |
| <b>Literature Values</b> |                               |                    |                     |                                  |                                  |                     |
| Heuzé and Tran (2015)    | 25.2<br>(18.2–30.4)           | 1.6<br>(0.5–3.9)   | 4.1<br>(3.1–5.8)    | 69.1 <sup>b</sup><br>(59.9–78.2) | 4.47 <sup>c</sup><br>(4.13–4.97) | 10.1<br>(5.4–14.2)  |
| JIRCAS (2018)            | 22.1<br>(20.5–24.1)           | 4.5<br>(2.7–5.7)   | 3.7<br>(3.3–4.0)    | 69.8 <sup>b</sup><br>(68.2–71.9) | – <sup>d</sup>                   | –                   |

<sup>a</sup> Values expressed on a dry basis (DB) and represent the LS mean of four replicate samples collected from each of four locations in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015 (n=16 for each entry). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

<sup>b</sup> Carbohydrate by calculation as 100% – (Crude Protein %DB + Crude Fat %DB + Ash %DB).

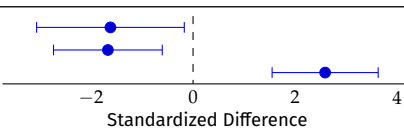
<sup>c</sup> Gross energy was converted from MJ/kg DB to kcal/g DB using the conversion factor of 238.85 kcal per MJ.

<sup>d</sup> Not reported.

Only three statistically significant differences between 709A and control samples were noted from the single-site analyses, including decreased ash (–5.8%) in 709A samples from Bakura, decreased calories (–1.9%) at the Zaria location, and slightly increased calories (0.9%) in 709A samples from Minjibir (Table 20).

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**Table 20.** Statistically significant differences from the single-site analyses of grain proximates

| Analyte              | Loc | 709A Mean <sup>a</sup> | Range       | Control Mean | Range       | p-Value              | Difference Plot with CIs <sup>b</sup>  |
|----------------------|-----|------------------------|-------------|--------------|-------------|----------------------|--|
| ash (% DB)           | BK  | 3.05                   | (2.98-3.25) | 3.24         | (3.13-3.33) | $3.78 \cdot 10^{-2}$ |  |
| calories (kcal/g DB) | ZA  | 4.29                   | (4.23-4.35) | 4.37         | (4.32-4.41) | $1.54 \cdot 10^{-2}$ |  |
| calories (kcal/g DB) | MJ  | 4.41                   | (4.39-4.41) | 4.37         | (4.34-4.38) | $4.33 \cdot 10^{-3}$ |  |

<sup>a</sup> Values represent the LS mean of four replicate samples collected from the indicated location in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> LS means calculated for each respective compositional analyte for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Díaz et al., 2016). When the 95 percent confidence interval (CI) for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).

Additional food and feed uses of cowpea include in-season harvest of cowpea leaves for human food and post-harvest use of above grown plant biomass (haulms) as fodder for livestock feed. In the combined-sites analysis of proximates from leaves and fodder derived from 709A and control cowpea, there were no statistically significant differences (Table 21). Statistically significant differences were noted between 709A and control samples in the single-site analyses for leaf proximates, including lower crude protein (−35% to −7.5%) at the Minjibir, Bakura, and Tamale locations, elevated ash content (13–21%) at the Minjibir and Bakura locations, alternately decreased (−4.1%) and increased (19.6%) carbohydrate content at the Minjibir and Tamale locations, respectively, and decreased calories (−7.6%) at the Minjibir location (Table 22).

**Table 21.** Proximate composition of leaves and fodder harvested from event 709A and control cowpea grown at four locations in West Africa during 2015

| Leaf Samples                     | Proximates (% dry weight) <sup>a</sup> |                     |                      |                                  |                                  |                     |
|----------------------------------|--|---------------------|----------------------|----------------------------------|----------------------------------|---------------------|
|                                  | Crude Protein                          | Crude Fat           | Ash                  | Carbohydrates                    | Calories (kcal/g)                | Moisture (%)        |
| Event 709A IT97KT                | 21.8<br>(11.3–32.1)                    | 6.64<br>(4.19–10.3) | 19.83<br>(7.96–30.5) | 51.74<br>(42.5–76.3)             | 3.73<br>(3.18–4.01)              | 7.38<br>(5.65–8.89) |
| Control IT97KN                   | 24.31<br>(16.3–32.4)                   | 7.30<br>(5.52–9.08) | 19.11<br>(10.8–24.7) | 49.28<br>(43.4–66.5)             | 3.81<br>(3.6–4.08)               | 7.38<br>(6.01–8.39) |
| p-Value                          | 0.181                                  | 0.135               | 0.753                | 0.508                            | 0.340                            | 0.994               |
| <b>Literature Values</b>         |  |                     |                      |                                  |                                  |                     |
| Heuzé et al. (2015) <sup>b</sup> | 17.1<br>(16.1–18.5)                    | 2.8<br>(1.3–4.1)    | 15.8<br>(13.4–18.1)  | 64.3 <sup>c</sup><br>(59.3–69.2) | 3.01 <sup>d</sup>                | 7.5<br>(5.5–11.5)   |
| <b>Fodder Samples</b>            |  |                     |                      |                                  |                                  |                     |
| Event 709A IT97KT                | 16.36<br>(11–26.6)                     | 7.18<br>(5.72–9.63) | 10.82<br>(7.34–18.6) | 65.64<br>(48.7–73.8)             | 4.21<br>(4.03–4.36)              | 7.96<br>(6.39–9.14) |
| Control IT97KN                   | 15.72<br>(9.25–27.3)                   | 6.33<br>(4.34–9.43) | 10.26<br>(6.5–17.1)  | 67.69<br>(48.9–79.7)             | 4.19<br>(3.99–4.4)               | 8.37<br>(7.43–9.83) |
| p-Value                          | 0.388                                  | 0.164               | 0.296                | 0.247                            | 0.65                             | 0.185               |
| <b>Literature Values</b>         |  |                     |                      |                                  |                                  |                     |
| Heuzé et al. (2015)              | 13.7<br>(6.9–18)                       | 2.2<br>(1.3–3.7)    | 11<br>(6.8–15.9)     | 73.1 <sup>c</sup><br>(62.4–85)   | 4.28 <sup>d</sup><br>(4.20–4.68) | 5<br>(4–7)          |

<sup>a</sup> Values represent the LS mean of four replicate samples collected from each of four locations in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015 (n=16 for each entry). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

<sup>b</sup> Values, except fat, were from aerial part, dehydrated.

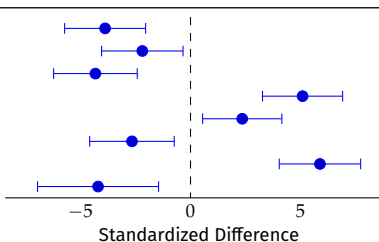
<sup>c</sup> Carbohydrate by calculation as  $100\% - (\text{Crude Protein \%DB} + \text{Crude Fat \%DB} + \text{Ash \%DB})$ .

<sup>d</sup> Gross energy was converted from MJ/kg DB to kcal/g DB using the conversion factor of 238.85 kcal per MJ.

From the single-site analyses for fodder, a small number of significant differences between 709A and control samples were identified, including elevated fat content

## COMPOSITIONAL ANALYSIS

**Table 22.** Statistically significant differences from the single-site analyses of leaf proximates

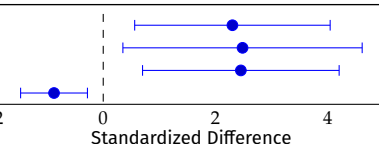
| Analyte              | Loc | 709A Mean <sup>a</sup> | Range       | Control Mean | Range       | p-Value              | Difference Plot with Cis <sup>b</sup>  |
|----------------------|-----|------------------------|-------------|--------------|-------------|----------------------|--|
| crude protein (% DB) | MJ  | 21.22                  | (20.2-21.6) | 23.18        | (22.7-23.4) | $1.41 \cdot 10^{-2}$ |  |
| crude protein (% DB) | BK  | 22.2                   | (21.7-23.1) | 24           | (22.5-24.6) | $3.27 \cdot 10^{-2}$ |  |
| crude protein (% DB) | TA  | 12                     | (11.3-12.9) | 18.58        | (16.3-20.9) | $1.15 \cdot 10^{-2}$ |  |
| ash (% DB)           | MJ  | 28.85                  | (27.3-30.5) | 23.8         | (23.1-24.7) | $6.41 \cdot 10^{-3}$ |  |
| ash (% DB)           | BK  | 25.48                  | (23.1-26.7) | 22.48        | (21.2-23.2) | $4.89 \cdot 10^{-2}$ |  |
| carbohydrates (% DB) | MJ  | 43.68                  | (43.2-44)   | 45.52        | (44-46.2)   | $4.28 \cdot 10^{-2}$ |  |
| carbohydrates (% DB) | TA  | 74.47                  | (73.4-76.3) | 62.28        | (60.1-66.5) | $4.45 \cdot 10^{-3}$ |  |
| calories (kcal/g DB) | MJ  | 3.34                   | (3.18-3.44) | 3.62         | (3.6-3.64)  | $1.67 \cdot 10^{-2}$ |  |

<sup>a</sup> Values represent the LS mean of four replicate samples collected from the indicated location in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> LS means calculated for each respective compositional analyte for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Diaz et al., 2016). When the 95 percent CI for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).

(32–33%) at the Zaria and Minjibir locations, increased ash content (26%) at the Zaria location, and slightly decreased calories (–1.8%) at the Bakura location (Table 23).

**Table 23.** Statistically significant differences from the single-site analyses of fodder proximates

| Analyte              | Loc | 709A Mean <sup>a</sup> | Range       | Control Mean | Range       | p-Value              | Difference Plot with Cis <sup>b</sup>   |
|----------------------|-----|------------------------|-------------|--------------|-------------|----------------------|---|
| crude fat (% DB)     | ZA  | 7.02                   | (6.21-7.62) | 5.31         | (4.58-6.09) | $4.81 \cdot 10^{-2}$ |  |
| crude fat (% DB)     | MJ  | 6.47                   | (5.76-7.24) | 4.87         | (4.34-5.39) | $3.41 \cdot 10^{-2}$ |   |
| ash (% DB)           | ZA  | 8.98                   | (7.81-9.8)  | 7.15         | (6.5-7.94)  | $4.14 \cdot 10^{-2}$ |   |
| calories (kcal/g DB) | BK  | 4.23                   | (4.13-4.36) | 4.3          | (4.21-4.4)  | $1.83 \cdot 10^{-2}$ |   |

<sup>a</sup> Values represent the LS mean of four replicate samples collected from the indicated location in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> LS means calculated for each respective compositional analyte for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Diaz et al., 2016). When the 95 percent CI for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).

### 7.2. Minerals

Cowpea is a rich source of the essential minerals, calcium, magnesium, potassium, iron, zinc, and phosphorus (Muranaka et al., 2016; Pereira et al., 2014). Across trial site locations, there were no significant differences between event 709A and control cowpea for any of the minerals analyzed in grain samples (Table 24).

A small number of significant differences between grain samples from 709A and control cowpea were noted at individual trial sites, including higher copper at Tamale, higher iron at Minjibir and Tamale, higher magnesium at Tamale, higher manganese at Minjibir and Bakura, and lower potassium at Bakura (Table 25). Even in those cases of significant differences between 709A and control cowpea in the single-site analyses, the mean values measured for event 709A were within the range of normal variability reported in the literature.

### 7.3. Phytic Acid

Phytic acid (myoinositol, 1, 2, 3, 4, 5, 6 hexakis-dihydrogen phosphate) is generally regarded as the primary storage form of phosphate and inositol in seeds, and lowers the bioavailability of minerals and limits the digestibility of protein and starch by inhibiting proteases and amylases (Gonçalves et al., 2016). There was no significant difference in

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**Table 24.** Mineral composition of whole grain harvested from event 709A and control cowpea grown at four locations in West Africa during 2015

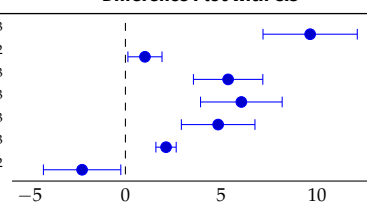
| Samples                  | Minerals (mg/100 g dry weight) <sup>†</sup> |                        |                     |                    |                     |                    |                     |                     |                     |
|--------------------------|---|------------------------|---------------------|--------------------|---------------------|--------------------|---------------------|---------------------|---------------------|
|                          | Ca <sup>a</sup>                             | Cu                     | Fe                  | Mg                 | Mn                  | P                  | K                   | Na                  | Zn                  |
| Event 709A               | 97.7<br>(75.7–114)                          | 0.561<br>(0.45–0.675)  | 5.56<br>(4.65–6.45) | 206.6<br>(177–225) | 2.87<br>(1.53–4.14) | 491.8<br>(443–537) | 1432<br>(1140–1720) | 8.37<br>(3.66–26.5) | 4.55<br>(4.29–5.17) |
| Control IT97KN           | 88.92<br>(72.3–109)                         | 0.532<br>(0.446–0.729) | 5.31<br>(4.49–6.34) | 200.9<br>(170–236) | 2.35<br>(1.46–3.56) | 499.2<br>(432–575) | 1493<br>(1130–1680) | 9.31<br>(2.93–27.9) | 4.55<br>(4.08–5.24) |
| <i>p</i> -Value          | 0.114                                       | 0.632                  | 0.370               | 0.604              | 0.064               | 0.619              | 0.187               | 0.196               | 0.953               |
| <b>Literature Values</b> |   |                        |                     |                    |                     |                    |                     |                     |                     |
| Heuzé and Tran (2015)    | 110<br>(30–270)                             | 0.9<br>(0.6–1.4)       | 4.22<br>(0.96–13.6) | 220<br>(160–280)   | 2.0<br>(1.4–3.2)    | 420<br>(210–540)   | 1500<br>(1280–2150) | 10<br>(10–20)       | 3.8<br>(2.4–4.6)    |
| Belane and Dakora (2012) | 60<br>(37–113)                              | 0.6<br>(0.5–0.8)       | 6.1<br>(4.8–9.7)    | 170<br>(130–240)   | 3.3<br>(2.1–4.3)    | 470<br>(380–470)   | 1330<br>(1140–1640) | – <sup>b</sup>      | 4.3<br>(3.3–6.5)    |
| JIRCAS (2018)            | –   | 0.482<br>(0.34–0.73)   | 5.31<br>(4.14–6.63) | –                  | 2.54<br>(1.47–3.94) | –                  | –                   | –                   | 3.96<br>(3.33–4.73) |

<sup>†</sup> Values represent the least square mean of four replicate samples collected from each of four locations in West Africa where event 709A IT97KT and control IT97KN cowpea were grown in 2015 (n=16 for each entry). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

<sup>a</sup> Ca = calcium; Cu = copper; Fe = iron; Mg = magnesium; Mn = manganese; P = phosphorus; K = potassium; Na = sodium; Zn = zinc.

<sup>b</sup> Not reported.

**Table 25.** Statistically significant differences from the single site analysis of grain minerals

| Analyte <sup>a</sup> | Loc | 709A Mean <sup>b</sup> | Range         | Control Mean | Range         | <i>p</i> -Value      | Difference Plot with CIs <sup>c</sup>  |
|----------------------|-----|------------------------|---------------|--------------|---------------|----------------------|--|
| Cu (mg/100 g DW)     | TA  | 0.67                   | (0.659–0.675) | 0.48         | (0.457–0.524) | $1.10 \cdot 10^{-3}$ |  |
| Fe (mg/100 g DW)     | MJ  | 4.93                   | (4.65–5.13)   | 4.69         | (4.49–5.01)   | $3.51 \cdot 10^{-2}$ |  |
| Fe (mg/100 g DW)     | TA  | 6.13                   | (5.86–6.39)   | 5.24         | (5.15–5.4)    | $5.41 \cdot 10^{-3}$ |  |
| Mg (mg/100 g DW)     | TA  | 207                    | (203–212)     | 177.9        | (172–185)     | $2.86 \cdot 10^{-3}$ |  |
| Mn (mg/100 g DW)     | MJ  | 3.5                    | (3.21–3.87)   | 2.59         | (2.49–2.72)   | $8.56 \cdot 10^{-3}$ |  |
| Mn (mg/100 g DW)     | BK  | 3.79                   | (3.43–4.14)   | 3.14         | (2.8–3.56)    | $1.05 \cdot 10^{-3}$ |  |
| K (mg/100 g DW)      | BK  | 1,380                  | (1290–1440)   | 1,531        | (1470–1620)   | $3.78 \cdot 10^{-2}$ |  |

<sup>a</sup> Cu=copper; Fe=iron; Mg=magnesium; Mn=manganese; K=potassium.

<sup>b</sup> Values represent the LS mean of four replicate samples collected from each of four locations in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>c</sup> LS means calculated for each respective compositional analyte for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Diaz et al., 2016). When the 95 percent CI for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).

the phytic acid content of grain harvested from event 709A and control cowpea in the combined-sites analysis (Table 26) nor were there any significant differences noted in the analyses of individual site data.

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**Table 26.** Phytic acid content of whole grain harvested from event 709A and control cowpea grown at four locations in West Africa during 2015

| Samples                  | Phytic Acid (% dry weight) |
|--------------------------|----------------------------|
| Event 709A IT97KT        | 1.17<br>(0.887–1.37)       |
| Control IT97KN           | 1.19<br>(0.716–1.66)       |
| <i>p</i> -Value          | 0.701                      |
| <b>Literature Values</b> |                            |
| Gonçalves et al. (2016)  | 0.264–1.52                 |
| Muranaka et al. (2016)   | 2.83<br>(2.18–3.7)         |

<sup>†</sup> Values represent the least square mean of four replicate samples collected from each of four locations in West Africa where event 709A IT97KT and control IT97KN cowpea were grown during 2015 (n=16 for each entry). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

### 7.4. Conclusions from Composition Analyses

From the combined-sites analysis across the four locations, there were no statistically significant differences in concentrations of proximates, moisture, calories, minerals, or phytic acid between grain samples collected from event 709A and control cowpea. Similarly, there were no significant differences in concentrations of proximates, moisture, and calories between 709A and control cowpea leaf and fodder samples when analyzed using the combined-sites model.

While some significant differences were observed at individual trial site locations, no consistent patterns emerged to suggest that biologically meaningful changes in composition or nutritive value of the grain, leaves, or fodder had occurred as a consequence of the genetic modification or expression of the Cry1Ab and NPTII proteins in 709A cowpea.

The conclusion based on these data was that whole grain, leaves, and fodder from 709A cowpea were compositionally equivalent to these same products from non-transgenic control cowpea. Processing is unlikely to alter the compositional components of cowpea grain, thus, products derived from 709A grain will also be compositionally equivalent to their conventional counterparts.

## 8. Agronomic and Phenotypic Characterization

The genetic modification resulting in cowpea event 709A was not intended to affect a specific agronomic or phenotypic characteristic, except to confer resistance to lepidopteran pests, such as cowpea pod borer. To confirm that 709A cowpea was otherwise agronomically equivalent to its conventional counterpart, yield and other agronomic (phenotypic) measurements were collected from multi-location confined field trials (CFTs) conducted during 2014 and 2015 at three different locations in Nigeria, and in three locations in Ghana in 2016. Each of these trial series is discussed separately in the following sections.

The conventional (non-transgenic) cowpea comparator used in these trials was IT97K-499-35, which is an improved high-yielding variety released by IITA that is known

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to be resistant to *Striga* (Kamara et al., 2008) and well-adapted to cultivation in West and Central Africa (Singh, 2006).

The purpose of the CFTs was not to demonstrate the yield and performance advantage of 709A cowpea, but rather to assess whether the genetic modification process had resulted in any unexpected, unintended, effects on the plant phenotype as part of the biosafety assessment. Therefore, the conduct of the trials included pesticide applications to control *Maruca vitrata* in order to minimize the confounding effects that pest damage on control plants would have on phenotypic measurements. Under these conditions, yield differences between 709A IT97KT and control IT97KN were generally not significant, as expected.

### 8.1. Multi-Location Confined Field Trials in Nigeria during 2014

Trials were conducted at three locations in Nigeria during 2014 as described by Utono et al. (2018). Cowpea seed containing event 709A, from the BC<sub>5</sub>F<sub>3</sub> generation following introgression and repeated back-crossing with IT97K-499-35, and non-transgenic BC<sub>5</sub>F<sub>3</sub> null segregant seed, obtained during back-cross selection using IT97K-499-35, were planted at three sites located in typical cowpea production areas in Nigeria (Figure 17). Four blocks (replicates) of each entry (event 709A IT97KT and control IT97KN) were established at each test site in a randomized complete block design. Trials were maintained weed-free and otherwise conducted according to local agronomic practice, including the routine insecticide applications to control insect pests.



**Figure 17.** Map showing field trial locations in Nigeria used during 2014 and 2015.

There were no significant differences observed between 709A IT97KT and control IT97KN in the combined sites analysis (Table 27). When analyzed under the single-site model, there were significant reductions in the number of *Maruca* damaged pods on 709A (IT97KT) plants compared to control cowpea at each trial site (Table 28), significantly reduced *Maruca* damaged seed (grain) per plant measured at the Zaria

## PHENOTYPIC CHARACTERIZATION




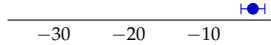
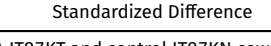
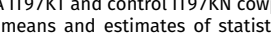
location, and a reduced number of pods per plant exhibiting sucking insect damage recorded at the Bakura and Zaria locations.

**Table 27.** Combined sites analysis of agronomic characteristics of 709A IT97KT and control IT97KN cowpea grown at three locations in Nigeria in 2014

| Entries             | Germination (%)                       | Days to 50% Flowering    | Plant Height (cm)                 | Pods per Plant                            | Maruca Damaged Pods per Plant |
|---------------------|---------------------------------------|--------------------------|-----------------------------------|---|-------------------------------|
| Event 709A IT97KT   | 82.2<br>(60–100)                      | 41.9<br>(35.1–45.3)      | 35.9<br>(17.5–72.8)               | 22.7<br>(8.1–39.3)                        | 0.008<br>(0–0.1)              |
| Control IT97KN      | 76.1<br>(46.7–93.3)                   | 41.7<br>(32.3–47.1)      | 31.8<br>(16.7–54)                 | 17.0<br>(5.7–26)                          | 1.88<br>(0.4–4.5)             |
| Sites with Data (N) | 3                                     | 3                        | 3                                 | 3   | 3                             |
| <i>p</i> -Value     | 0.388                                 | 0.897                    | 0.473                             | 0.076                                     | 0.223                         |
| Entries             | Sucking Insect Damaged Pods per Plant | Total Seed per Plant (g) | Maruca Damaged Seed per Plant (g) | Sucking Insect Damaged Seed per Plant (g) | Healthy Seed per Plant (g)    |
| Event 709A IT97KT   | 0.49<br>(0–1.7)                       | 30.5<br>(11.5–54.1)      | 0.03<br>(0–0.12)                  | 0.17<br>(0.03–0.38)                       | 30.04<br>(10.1–53.6)          |
| Control IT97KN      | 2.03<br>(0.1–3.6)                     | 27.7<br>(7.3–44.8)       | 0.34<br>(0.03–0.57)               | 0.18<br>(0.03–0.52)                       | 26.9<br>(6.7–43.8)            |
| Sites with Data (N) | 3                                     | 3                        | 3                                 | 3   | 3                             |
| <i>p</i> -Value     | 0.084                                 | 0.396                    | 0.091                             | 0.699                                     | 0.348                         |

<sup>a</sup> Values represent LS means of four replicate measurements from event 709 (IT97KT) and control (IT97KN) plants grown at three locations in Nigeria in 2014. Minimum and maximum values recorded at any location are shown in parentheses. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

**Table 28.** Statistically significant differences from the single site analysis of agronomic parameters

| Parameter                   | Loc | 709A Mean <sup>a</sup> | Range       | Control Mean | Range       | <i>p</i> -Value      | Difference Plot with CIs <sup>b</sup>   |
|-----------------------------|-----|------------------------|-------------|--------------|-------------|----------------------|---|
| Maruca Damaged Grain Weight | ZA  | $1.5 \cdot 10^{-2}$    | (0.01–0.02) | 0.54         | (0.51–0.57) | $4.33 \cdot 10^{-5}$ |  |
| Maruca Damaged Pods         | BK  | $3.14 \cdot 10^{-16}$  | (0–0)       | 1.13         | (0.6–1.4)   | $8.23 \cdot 10^{-3}$ |  |
| Maruca Damaged Pods         | MJ  | $2.5 \cdot 10^{-2}$    | (0–0.1)     | 0.53         | (0.4–0.6)   | $2.67 \cdot 10^{-3}$ |  |
| Maruca Damaged Pods         | ZA  | 0                      | (0–0)       | 4            | (3.2–4.5)   | $8.87 \cdot 10^{-4}$ |  |
| Sucking Insect Damaged Pods | BK  | 1.18                   | (0.8–1.7)   | 2.85         | (1.2–3.5)   | $4.46 \cdot 10^{-2}$ |  |
| Sucking Insect Damaged Pods | ZA  | 0.18                   | (0–0.5)     | 2.45         | (1–3.6)     | $2.45 \cdot 10^{-2}$ |  |

<sup>a</sup> Values represent the LS mean of measurements taken from four replicate plots of event 709A IT97KT and control IT97KN cowpea grown during 2014. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> LS means calculated for each respective parameter for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Díaz et al., 2016). When the 95 percent CI for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).

There were no differences observed in percent germination (emergence), days to 50 percent flowering, plant height, pods per plant, total seed weight, sucking insect damaged seed weight, or healthy seed weight between 709A (IT97KT) and control IT97KN cowpea at any location.

There were no differences noted between 709A (IT97KT) and control cowpea with respect to plant vigour at the Bakura location (rating of 5 for each entry), and no pod shattering or lodging observed for either entry at the Bakura and Zaria locations. Overall, the transgenic and control entries exhibited normal growth and development at each trial location.

### 8.2. Multi-Location Confined Field Trials in Nigeria during 2015

In 2015, trials were again conducted at the same locations as used in 2014 using the same seed for planting in the same plot layouts (Abdourhamane et al., 2018a).

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In the combined-sites analysis, the only statistically significant difference between 709A IT97KT and control IT97KN in measured agronomic parameters was in the percentage of *Maruca* damaged pods per plant, which were near-zero for 709A plants and ca. 13 percent (range: 5.9–20.9) for control plants ( $p = 0.0244$ ) (Table 29). This observation was entirely predictable and consistent with the intended function of the Cry1Ab protein expressed in 709A cowpea, which was to reduce damage from cowpea pod borers.

**Table 29.** Combined sites analysis of agronomic characteristics of 709A and control cowpea grown at three locations in Nigeria in 2015

| Entries             | Days to First Flowering | Days to 50% Flowering | Plant Height at Harvest (cm) | Pods per Plant      | <i>Maruca</i> Damage (%) |
|---------------------|-------------------------|-----------------------|------------------------------|---------------------|--------------------------|
| Event 709A IT97KT   | 38.1<br>(33–44)         | 46.8<br>(39–54)       | 24.2<br>(12.5–45.9)          | 18.8<br>(12.2–22.9) | 0.01<br>(0–0.12)         |
| Control IT97KN      | 40<br>(35–50)           | 48.9<br>(39–54)       | 29.4<br>(13.4–67.8)          | 16.3<br>(12.9–19)   | 13.0<br>(5.9–20.9)       |
| Sites with Data (N) | 3                       | 3                     | 3                            | 3                   | 3                        |
| <i>p</i> -Value     | 0.1232                  | 0.1529                | 0.2331                       | 0.2083              | <b>0.0244</b>            |





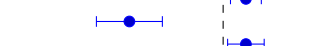
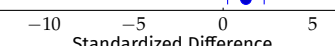
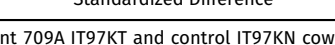
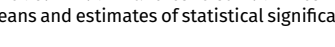
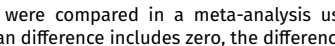

  

| Entries             | Pod Length (cm)     | Total Seed Weight per Plant (g) | Healthy Seed Weight per Plant (g) | Hundred Seed Weight (g) | Fodder Yield per Plant (g) |
|---------------------|---------------------|---------------------------------|-----------------------------------|-------------------------|----------------------------|
| Event 709A IT97KT   | 14.8<br>(12.4–17.2) | 21.8<br>(14.2–26)               | 21.5<br>(13.7–25.5)               | 13.8<br>(11.2–18.6)     | 20.5<br>(11.5–35.8)        |
| Control IT97KN      | 14.6<br>(13.3–15.5) | 19.2<br>(17.3–23.8)             | 18.4<br>(15.6–22.6)               | 14.2<br>(12.8–15.5)     | 18.1<br>(11.7–27.4)        |
| Sites with Data (N) | 3                   | 3                               | 3                                 | 3                       | 3                          |
| <i>p</i> -Value     | 0.733               | 0.1416                          | 0.0968                            | 0.7348                  | 0.5714                     |

<sup>a</sup> Values represent LS means of four replicate measurements from event 709 (IT97KT) and control (IT97KN) plants grown at three locations in Nigeria in 2015. Minimum and maximum values recorded at any location are shown in parentheses. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

There were a number of statistically significant differences observed at individual trial site locations. These included reduced days to first flowering at Bakura, increased pods per plant at Zaria, significantly reduced percentage of *Maruca* damaged pods at the Bakura and Minjibir locations, increased total and healthy seed weight at the Zaria and Minjibir locations, and reduced 100-seed weight and increased fodder yield per plant at Minjibir (Table 30).

**Table 30.** Statistically significant differences from the single site analysis of agronomic parameters

| Parameter                | Loc | 709A Mean <sup>a</sup> | Range       | Control Mean | Range       | <i>p</i> -Value       | Difference Plot with CIs <sup>b</sup>   |
|--------------------------|-----|------------------------|-------------|--------------|-------------|-----------------------|---|
| Days to 1st Flowering    | BK  | 33                     | (33-33)     | 35           | (35-35)     | $1.77 \cdot 10^{-53}$ |  |
| Pods per Plant           | ZA  | 21.15                  | (19.8-22.9) | 16.7         | (14.8-18.4) | $1.59 \cdot 10^{-2}$  |  |
| <i>Maruca</i> Damage (%) | MJ  | $3.03 \cdot 10^{-2}$   | (0-0.121)   | 17.15        | (11.2-20.9) | $4.78 \cdot 10^{-3}$  |  |
| <i>Maruca</i> Damage (%) | BK  | 0                      | (0-0)       | 10.88        | (7.37-13)   | $3.08 \cdot 10^{-3}$  |  |
| Total Seed Wt. (g)       | ZA  | 25.26                  | (24.8-26)   | 20.7         | (18.4-23.8) | $2.91 \cdot 10^{-2}$  |  |
| Total Seed Wt. (g)       | MJ  | 21.29                  | (19.1-25.4) | 18.87        | (17.6-21.5) | $3.56 \cdot 10^{-2}$  |  |
| Healthy Seed Wt.         | ZA  | 24.87                  | (24.5-25.5) | 19.87        | (17.8-22.6) | $1.82 \cdot 10^{-2}$  |  |
| Healthy Seed Wt.         | MJ  | 21.06                  | (18.9-25)   | 18.26        | (17.1-20.8) | $1.82 \cdot 10^{-2}$  |  |
| 100-Seed Wt (g)          | MJ  | 12.22                  | (11.2-13.1) | 15.07        | (14.6-15.5) | $6.12 \cdot 10^{-3}$  |  |
| Fodder (g/plant)         | MJ  | 18.72                  | (15.7-21.8) | 14.84        | (11.7-18.1) | $2.90 \cdot 10^{-2}$  |  |

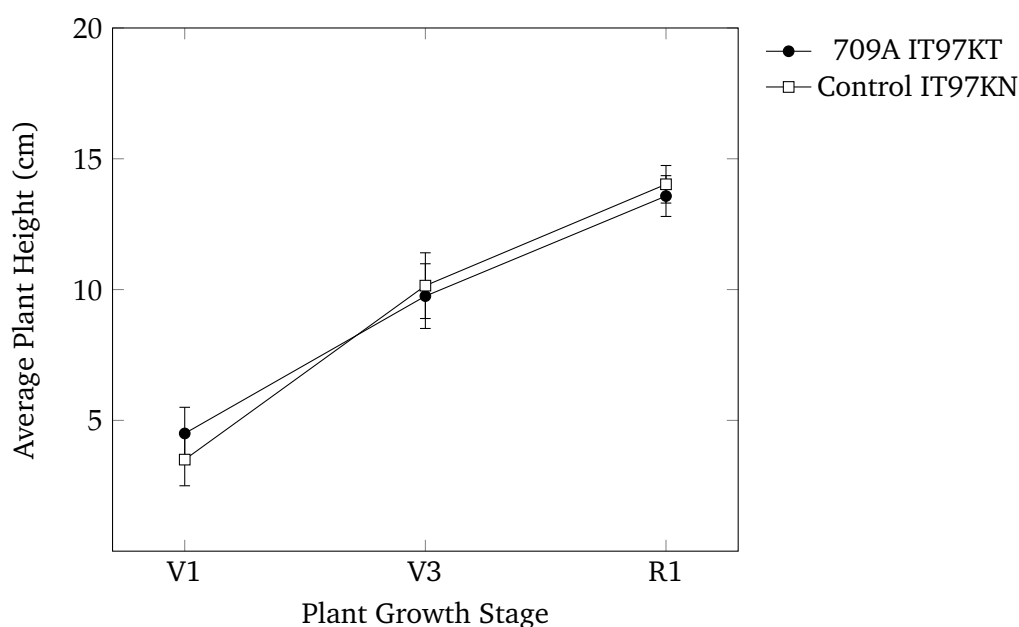
<sup>a</sup> Values represent the LS mean of measurements taken from four replicate plots of event 709A IT97KT and control IT97KN cowpea grown during 2015. Data were subjected to linear mixed model analysis to generate LS means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> LS means calculated for each respective parameter for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Díaz et al., 2016). When the 95 percent CI for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).

There were no differences observed in days to 50 percent flowering, plant height, or pod length between 709A and control cowpea at any location. Measurements of plant

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height were collected from the Zaria location at different growth stages and there were no significant differences between 709A and control cowpea in average plant heights determined at the V1, V3, and R1 growth stages (Figure 18).



**Figure 18.** Average plant heights were determined at three different growth stages at the Zaria location. Within each plot, heights were measured for 10 consecutive plants in a representative row of each entry (709A IT97KT and control IT97KN). Average plant heights  $\pm$  standard deviation are shown.

Plant vigour ratings taken at different growth stages were similar between 709A IT97KT and control IT97KN plots at each trial location, and across growth stages differences were only significant at the Bakura location (Table 31). Overall, plant growth rate and vegetative vigour were not significantly different between event 709A and control cowpea.

**Table 31.** Plant vigour ratings at different growth stages

| Entry  | Plant Growth Stage <sup>a</sup> |     |     |     |     |     |     |
|--|---------------------------------|-----|-----|-----|-----|-----|-----|
|  | VE                              | V1  | V3  | R1  | R2  | R3  | R8  |
| <b>Zaria</b>   |                                 |     |     |     |     |     |     |
| 709A IT97KT  | 4.5 <sup>b</sup>                | 4.5 | 4.5 | 3.5 | 3.5 | 3   | 3   |
| Control IT97KN   | 4.5                             | 3.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| Median of 709A: 3.5. Median of Control: 4.5. Significance of differences across growth stages: P-Value = 0.1678 <sup>c</sup> |                                 |     |     |     |     |     |     |
| <b>Minjibir</b>  |                                 |     |     |     |     |     |     |
| 709A IT97KT  | 5                               | 5   | 5   | 5   | 5   | 5   | 5   |
| Control IT97KN   | 5                               | 5   | 5   | 5   | 5   | 5   | 5   |
| Median of 709A: 5. Median of Control: 5. Significance of differences across growth stages: P-Value > 0.9999                  |                                 |     |     |     |     |     |     |
| <b>Bakura</b>  |                                 |     |     |     |     |     |     |
| 709A IT97KT  | 5                               | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| Control IT97KN   | 5                               | 5   | 5   | 5   | 5   | 5   | 5   |
| Median of 709A: 4.5. Median of Control: 5. Significance of differences across growth stages: P-Value = 0.0047                |                                 |     |     |     |     |     |     |

<sup>b</sup> Composite score; 1-5 scale, 1 = relatively small and weak, pale yellow in appearance; 3 = acceptable growth and development; 5 = normal size, strong and erect. Rating is representative of the entire plot.

<sup>c</sup> Data were analyzed using the non-parametric Mann Whitney test (Mann and Whitney, 1947).

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### 8.3. Multi-Location Confined Field Trials in Ghana during 2016

In 2016, trials were conducted at three sites (Figure 19) located in typical cowpea production areas in northern Ghana (Abudulai et al., 2019) using the same event 709A IT97KT and control IT97KN entries that were planted in previous trials in Nigeria.



**Figure 19.** Map showing approximate field trial sites at Damongo, Manga, and Nyankpala in Ghana during 2016.

In the combined-sites analysis, significant differences between event 709A (IT97KT) and control IT97KN entries were observed in pods per plant, *Maruca* damaged pods per plant, and *Maruca* damaged seed per plant (Table 32). The significant difference in *Maruca* damaged pods per plant was consistently observed at each of the three trial locations (Table 33), while significant differences between 709A and control cowpea in pods per plant, *Maruca* damaged seed per plant, and total and healthy seed per plant were observed only at the Nyankpala location.

Across locations, there were no differences observed between 709A (IT97KT) and control IT97KN cowpea in percent germination (emergence), days to first flowering, plant height at harvest, total seed weight, sucking insect damaged seed weight, or healthy seed weight. Measurements of plant height were collected from the Damongo and Nyankpala locations at the V1, V3, R1, and R8 (harvest) growth stages (Figure 20). At Damongo, there were no significant differences in average plant height at each growth stage between event 709A and control cowpea (Figure 20, panel A), while at Nyankpala, average plant height of control IT97KN plants was significantly greater than event 709A (IT97KT) plants at the R1 and R8 growth stages, but not at V1 and V3 growth stages (Figure 20, panel B).

There were no differences noted between event 709A (IT97KT) and control cowpea with respect to plant vigour at any trial site location (rating of 5 for each entry), and no pod shattering or lodging observed for either entry at the three locations. Across the three locations, the transgenic and control entries exhibited normal growth and development.

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**Table 32.** Combined sites analysis of agronomic characteristics of 709A IT97KT and control IT97KN cowpea grown at three locations in Ghana in 2016

| Entries             | Germination (%)    | Days to First Flowering | Plant Height (cm)   | Pods per Plant      | Maruca Damaged Pods per Plant |
|---------------------|--------------------|-------------------------|---------------------|---------------------|-------------------------------|
| Event 709A IT97KT   | 94.3<br>(81.7–100) | 43.8<br>(27–61)         | 38.6<br>(30.0–60.8) | 10.7<br>(5.97–20.9) | 0.15<br>(0–0.97)              |
| Control IT97KN      | 98.1<br>(87.5–100) | 44.3<br>(28–60)         | 39.7<br>(29.3–72.7) | 7.42<br>(5.25–12.5) | 1.22<br>(0.63–2.53)           |
| Sites with Data (N) | 3                  | 2 <sup>b</sup>          | 3                   | 3                   | 3                             |
| <i>p</i> -Value     | 0.078              | 0.357                   | 0.688               | <b>0.005</b>        | <b>&lt;0.001</b>              |

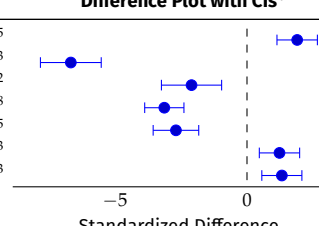
| Entries             | Sucking Insect Damaged Pods per Plant | Total Seed per Plant (g) | Maruca Damaged Seed per Plant (g) | Sucking Insect Damaged Seed per Plant (g) | Healthy Seed per Plant (g) |
|---------------------|---------------------------------------|--------------------------|-----------------------------------|---|----------------------------|
| Event 709A IT97KT   | 2.99<br>(1.6–8.23)                    | 11.6<br>(6.1–25.5)       | 0.13<br>(0–0.48)                  | 1.26<br>(0.62–2.35)                       | 9.89<br>(4.16–24.4)        |
| Control IT97KN      | 2.13<br>(0.87–3.47)                   | 9.02<br>(4.98–15.5)      | 1.14<br>(0.5–1.97)                | 1.31<br>(0.5–2.55)                        | 7.34<br>(1.8–13.4)         |
| Sites with Data (N) | 1 <sup>c</sup>                        | 3                        | 1                                 | 1   | 3                          |
| <i>p</i> -Value     | 0.223                                 | 0.068                    | <b>&lt;0.001</b>                  | 0.831                                     | 0.069                      |

<sup>a</sup> Values represent estimated marginal (EM) means of four replicate measurements from event 709A (IT97KT) and control (IT97KN) plants grown at three locations in Ghana in 2016. Minimum and maximum values recorded at any location are shown in parentheses. Data were subjected to linear mixed model analysis to generate EM means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> Data from Damongo and Nyankpala locations.

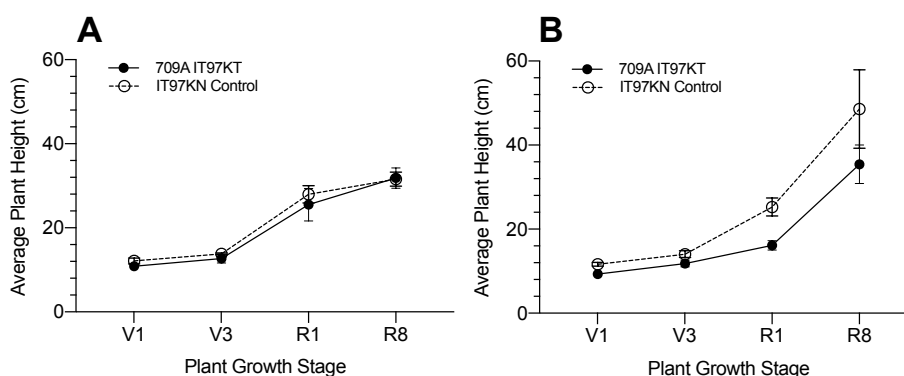
<sup>c</sup> Data were collected only at the Nyankpala site from an insecticide reduction trial conducted in parallel with the agronomic performance trial.

**Table 33.** Statistically significant differences from the single site analyses of agronomic parameters

| Parameter                               | Loc 709A <sup>a</sup> | Range             | Control | Range        | <i>p</i> -Value      | Difference Plot with CIs <sup>b</sup>   |
|---|-----------------------|-------------------|---------|--------------|----------------------|---|
| Pods per Plant (count/plant)            | NY                    | 14.92 (8.45–20.9) | 8.71    | (5.37–12.5)  | $6.10 \cdot 10^{-5}$ |  |
| Maruca Damaged Pods (count/plant)       | DA                    | 0.03 (0–0.0833)   | 0.93    | (0.667–1.08) | $1.46 \cdot 10^{-3}$ |   |
| Maruca Damaged Pods (count/plant)       | MA                    | 0.10 (0–0.25)     | 1.06    | (0.633–2.02) | $3.65 \cdot 10^{-2}$ |   |
| Maruca Damaged Pods (count/plant)       | NY                    | 0.31 (0–0.967)    | 1.67    | (0.767–2.53) | $1.87 \cdot 10^{-8}$ |   |
| Maruca Damaged Seed (g/plant)           | NY                    | 0.13 (0–0.483)    | 1.14    | (0.5–1.97)   | $1.46 \cdot 10^{-5}$ |   |
| Total Seed Weight per Plant (g/plant)   | NY                    | 15.72 (9.62–25.5) | 10.69   | (5.5–15.5)   | $4.17 \cdot 10^{-3}$ |   |
| Healthy Seed Weight per Plant (g/plant) | NY                    | 14.13 (8–24.4)    | 8.62    | (1.8–13.4)   | $2.36 \cdot 10^{-3}$ |   |

<sup>a</sup> Values represent EM means of four replicate measurements from event 709A (IT97KT) and control (IT97KN) plants grown at Damongo (DA), Manga (MA), or Nyankpala (NY) during 2016. Data were subjected to linear mixed model analysis to generate EM means and estimates of statistical significance for any differences ( $p < 0.05$ ).

<sup>b</sup> EM means calculated for each respective agronomic parameter for 709A and control cowpea were compared in a meta-analysis using standardized differences (Heredia Diaz et al., 2016). When the 95 percent CI for the mean difference includes zero, the difference is not statistically significant at the five percent level ( $p < 0.05$ ).



**Figure 20.** Average plant heights were determined at four different growth stages at the Damongo (panel A) and Nyankpala (panel B) locations. Within each plot, heights were measured for 10 consecutive plants in a representative row of each entry (709A IT97KT and control IT97KN). Average plant heights  $\pm$  standard deviation are shown.

#### 8.4. Conclusions from Agronomic and Phenotypic Characterization

An evaluation of the agronomic and phenotypic data generated for 709A cowpea grown at multiple locations under a range of environmental conditions in Ghana and in Nigeria did not identify trends of significant differences with the recurrent parental variety that would have any negative environmental consequences. Collectively, the nine location-years (three in Ghana and six in Nigeria) of comparative agronomic and phenotypic data support the conclusion that the genetic modification resulting in event 709A did not have an unintended, unexpected, effect on plant growth habit and general morphology, vegetative vigour, or grain yield. From the data and observations, there were no indications that 709A cowpea would be more invasive or persistent in the environment, or have altered susceptibility to pests and diseases, compared to conventional cowpea. Except for the intended resistance to *Maruca* pod borer, 709A cowpea is agronomically and phenotypically equivalent to conventional cowpea.

## 9. Environmental Risk Assessment

As noted by Raybould (2010a), the effectiveness of an environmental risk assessment (ERA) should not be measured by the amount of data collected on a transgenic crop (i.e., the size of the bucket of observations), but by the power of the risk hypothesis searchlights to clarify the risks that may arise from cultivation of that crop. Unfortunately, research into the risks posed by the cultivation of transgenic crops often appears to apply the bucket theory; many data are produced, but knowledge of risk is not advanced.

Well formulated ERAs begin with an explicit problem formulation, which involves among other steps: (1) formally devising plausible pathways to harm that describe how the deployment could be harmful; (2) formulating risk hypotheses about the likelihood and severity of such events; (3) identifying the information that will be useful to test the risk hypotheses; and if necessary, (4) developing a plan to acquire new data when the existing scientific literature is insufficient for decision-making (Devos et al., 2019).

In the case of 709A cowpea, the ERA was structured along four dimensions, which included:

- the potential of 709A cowpea to become a weed of agriculture or be invasive of natural habitats;
- the potential for gene flow to sexually compatible plants whose hybrid offspring may become more weedy or move invasive;
- the potential of 709A cowpea to become a plant pest; and
- the potential impact on non-target species.

Each of these areas is discussed in more detail in the sections that follow.

#### 9.1. Potential Weediness and/or Invasiveness of 709A Cowpea

The weediness of a plant is its ability to persist and spread in managed ecosystems, while invasiveness is its ability to do the same in unmanaged ecosystems (OECD, 2018b).

Although the establishment of feral populations of domesticated cowpea is theoretically possible, as reported in Japan (Berville et al., 2005), this has rarely been observed

in Africa. A few small transient feral populations have been reported in coastal Kenya, which were not seen in consecutive years (OECD, 2015). Cowpea cultivars are generally not capable of creating long-lived seed banks in the soil because their seeds are permeable to water and lack dormancy (Lush et al., 1980). The domestication of cowpea has resulted in loss of many primitive traits typically associated with weediness, such as perenniality, hairiness, small size of seeds and pods, hard seeds, pod shattering, and significant out-crossing (OECD, 2015).

Field comparisons between 709A cowpea and its near-isogenic non-transgenic counterpart over multiple locations and years did not identify any significant differences in plant growth and morphology, reproductive biology characteristics, seed set or shattering, that would indicate any unintended change in the weediness or invasiveness of conventional cowpea cultivars (see sections 8.1, 8.2, and 8.3) (Abdourhamane et al., 2018a; Utono et al., 2018; Abudulai et al., 2019).

### 9.2. Potential Impact of Pollen-Mediated Gene Flow from 709A Cowpea

Pollen-mediated gene flow is a natural part of the reproductive biology of all plants and is important in the maintenance of genetic variation in populations, as well as in the spread of new traits among populations and across species boundaries (Tzotzos et al., 2009). Pollen movement between plants is dependent upon wind or animal vectors (pollinators), and while it occurs with all crop species, the amount of gene flow (i.e., out-crossing) is a function of crop biology. Gene flow between cultivated plants and their wild relatives occurs naturally for all crops with relatives that are sexually compatible and grow in proximity to the crop (Ellstrand et al., 1999; Raybould, 2010b; Raybould and Wilkinson, 2005).

Cowpea is cleistogamous, producing viable pollens and receptive stigma before anthesis, meaning that cowpea reproduction is entirely via self-pollination. However, out-crossing mediated by insects can occur naturally in the field, and while different insect species visit cowpea flowers, not all are responsible for pollen movement associated with out-crossing. As reported by Asiwe (2009), only honey and bumblebees are responsible for insect-vectored pollen movement because only such heavy insects could depress the wings of cowpea flowers and expose their stamens and stigmas for pollination. Out-crossing rates between cultivated cowpea varieties are low, ranging from 0.5–0.85 percent when cowpea was planted in alternate rows 1 meter apart, and between 0.01–0.13 percent when planted in concentric circles around a pollen source (Asiwe, 2009). There are no reports of hybridization between *V. unguiculata* and other *Vigna* species (Huesing et al., 2011).

The wild relatives of cultivated crop species typically represent greater genetic variability than their domesticated descendants and the maintenance of this variability is important for conservation and genetic improvement programs. For example, Souleymane et al. (2013) were able to identify sources of resistance to damage from cowpea aphid (*Aphis craccivora* Koch) from screening wild cowpea accessions.

Wild relatives can also exist in crop-weed complexes with domesticated plants, which is the case for cultivated cowpea (*Vigna unguiculata* ssp. *unguiculata* var. *unguiculata*) and its wild progenitor, *Vigna unguiculata* ssp. *unguiculata* var. *spontanea*. In West Africa, wild cowpea is primarily found in disturbed habitats, such as fields, field

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margins, roadsides, and fallows but have not been reported as a significant weed problem in sub-Saharan Africa (Huesing et al., 2011). Notwithstanding the high degree of self-pollination within both domestic and wild cowpea populations (Kouam et al., 2012), the relatively close proximity of domesticated and wild cowpeas, well within the distance over which cowpea pollen can be transported, means that some degree of gene flow is certain.

The secondary gene pool of wild relatives of *V. unguiculata* includes five perennial outcrossing (allogamous) subspecies, *ssp. baoulensis*, *ssp. burundensis*, *ssp. letouzeyi*, *ssp. aduensis* and *ssp. pawekiae*, which are adapted to humid environments, with only *V. unguiculata ssp. baoulensis* likely to occur in rain forest areas of southern Nigeria. An assessment of intra-species hybridization among wild and cultivated cowpea subspecies using an arbitrarily primed polymerase chain reaction (AP-PCR) technique found that the cultivated subspecies (var. *unguiculata*) and most of its wild progenitors (var. *spontanea*) could be placed in a single cluster, along with *ssp. pubescens* and *ssp. stenophylla*, with a second cluster containing var. *spontanea* grouped with *ssp. alba* and *ssp. tenuis*, and finally a more distantly related cluster containing only two subspecies, *ssp. alba* and *ssp. baoulensis* (Vijaykumar et al., 2012). These data suggest significant genetic divergence between cultivated cowpea and perennial subspecies. In crossing studies, the wild allogamous forms gave the lowest rates of success with the cultivated forms (3.1–40 percent) showing that they are genetically more distant compared to the other wild groups (Kouadio et al., 2007). In crosses between *V. unguiculata ssp. unguiculata* cv. Biflora and *ssp. baoulensis*, the rate of crossing success was ca. 20 percent when *ssp. baoulensis* was the female parent and only ca. 3.1 percent when *ssp. baoulensis* was the pollen donor (Kouadio et al., 2007).

For those farmers in the West African Sahel who are primarily growing cowpea for fodder, the fodder from wild cowpea (including progeny from domestic × wild crosses) is equivalent to that from cultivated cowpea (Feleke et al., 2006), implying that wild cowpea may be tolerated in such agro-ecosystems. Hybrid progenies may even end up being used by farmers for sowing and may be considered as fodder landraces (OECD, 2015).

Based on current understanding of relationships between cultivated and wild cowpeas, the likelihood of gene introgression from cultivated to nearby (i.e., less than three meters and assuming synchronous flowering) wild relatives can be considered high for *V. unguiculata ssp. unguiculata* var. *spontanea* and moderate for the perennial wild *V. unguiculata ssp. baoulensis* (Andersson and de Vicente, 2010).

Thus, some level of gene flow between domesticated and wild cowpea does occur, and will occur at the same frequency between transgenic 709A cowpea and other domestic and wild cowpea. What is important to consider are the likely consequences of gene flow and whether these represent hazards to the environment, human or animal health, or biodiversity. While much of the focus is on assessing the consequences of transgene flow (i.e., *cry1Ab* and *nptII* in the case of 709A cowpea), it is recognized that gene flow is an ongoing phenomenon that did not begin with the introduction of genetically engineered plants and is not limited to the movement of only transgenes. Thus the consequences of transgene movement and introgression cannot be considered in isolation of the movement of other genes.

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As noted by Huesing et al. (2011), there are several potential consequences of pollen-mediated gene flow and these are further elaborated below in the context of gene flow from 709A cowpea to wild cowpea.

### 9.2.1. Genetic Swamping

“Genetic swamping” is a phenomenon that could occur if gene flow from a cultivated crop to a wild population is high enough that the wild populations are genetically uniform with the crop population, thus resulting in loss of genetic diversity.

The mere coexistence of wild and domestic cowpea today is ample evidence that genetic swamping is unlikely. Genetic swamping by domesticated genes would lead to the disappearance of wild types, which is obviously not the case for cowpea. The addition of two new genes (*cry1Ab* and *nptII*) to the 709A cowpea genome does not change this calculus.

### 9.2.2. Loss of Genetic Variation

The introgression of the *cry1Ab* gene from 709A cowpea into wild cowpea populations could be advantageous. Strong selection for wild cowpeas carrying the *cry1Ab* gene, in combination with linkage disequilibrium (co-inheritance of alleles linked to the *cry1Ab* gene) due to the predominately selfing mating system in cultivated cowpea could result in replacing large portions of the wild cowpea genome with the cultivated crop alleles linked to the *cry1Ab* gene.

This potential outcome depends on two conditions: positive selection for the *cry1Ab* gene in wild cowpea populations; and significant linkage disequilibrium between the *cry1Ab* locus and other crop alleles in 709A cowpea. There is no genetic information that can answer the question of linkage disequilibrium, but some information is available regarding the potential value of resistance to *Maruca vitrata* in wild populations.

Wild cowpea in West Africa are infrequent in non-agricultural settings, and in those cases are closely associated with cowpea fields where they are likely to be subject to the same level of pest pressure as cultivated plants (Pasquet, 2012). If *Maruca* pod borer was the pest mainly responsible for pre- and/or post-dispersal seed predation in West Africa, then the acquisition of an insect-resistance trait could confer some selective advantage.

While *Maruca* inflicts its damage to cowpea plants by being one of the most damaging pests during flowering and pod development, it does not appear to be a major predator of seeds in East Africa, where a beetle in the family Curculionidae (Coleoptera), and a bean fly in the family Agromyzidae (Diptera) are primarily responsible for seed destruction (Pasquet, 2012). Seed predators of cowpea in the beetle families Curculionidae and Bruchidae are also present in Nigeria (Prevett, 1961). With respect to post-dispersal seed predation, Pasquet (2012) observed that in Kenya, rodents destroyed almost all the seeds produced by cowpea plants. In Nigeria as well, rodents and other non-insect pests may even be more important in the field than insects (Kitch et al., 1997). The main agents of seed predation and thus the ability of wild cowpeas to establish and survive in wild habitats does not appear to be *Maruca*, or other species that are susceptible to *Cry1Ab*. Therefore, it is unlikely that populations of wild cowpea

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that acquire the *cry1Ab* gene would increase in abundance. Consequently, a loss of genetic variation in wild cowpea populations is unlikely.

Furthermore, hybrids derived from crosses between domesticated cowpea, including 709A cowpea, and wild cowpea are likely to acquire one or more domestication traits that significantly reduce their persistence in wild ecosystems. The white seed colour makes seeds more visible to seed predators, the thinner seed coat makes seed less dormant, and reduced seed shattering could reduce dispersal distances.

### *9.2.3. Loss or Reduced Abundance of a Valued Species*

If the *cry1Ab* gene confers a selective advantage that increases the abundance of wild cowpeas in populations in wild habitats such that the wild cowpea out-competes other plant species, there could be a reduction in the abundance of those plant species, especially those that are considered valued in Nigeria. However, wild cowpea currently has low invasive tendencies that are unrelated to *Maruca* pod borer pressure, so this consequence is unlikely. As noted in section 9.2.2, gene flow from 709A cowpea is unlikely to increase the abundance of wild cowpea.

### *9.2.4. Loss of Ecosystem Services*

If there is an increase in wild cowpeas to the point where the species became invasive, there could be a reduction in other resources that service the ecosystem, such as soil nutrients, water and light. The evidence presented in section 9.2.2 is also relevant to this potential consequence. Since an increase in abundance of wild cowpea is unlikely, the loss of ecosystem services due to that increase is unlikely as well.

### *9.2.5. Loss of Crop Yield and Quality*

If there is an increase in wild cowpeas in agricultural fields, there could be a reduction in both crop yield and quality, particularly if wild cowpea seeds are mixed with cowpea seeds at harvest. Seeds derived from wild cowpea, including F1 hybrids between domestic and wild cowpea, will be smaller with a thick and dark testa (seed coat) and are unlikely to be selected by farmers for the next sowing. Once again, the information presented in section 9.2.2 supports the conclusion that an increase in the abundance of wild cowpea in agricultural fields as a consequence of gene flow from 709A cowpea is unlikely.

## *9.3. Altered Plant Pest Potential of 709A Cowpea*

The intended effects of the introduced trait in 709A cowpea is unrelated to plant pest potential, and cowpea itself is not a plant pest. In addition, agronomic and phenotypic characteristics of 709A cowpea were shown to be within the range of values displayed by conventional cowpea, and indicate that the growing habit of cowpea was not inadvertently altered. Field observations did not indicate modifications to disease and pest susceptibilities, other than to *Maruca* pod borer, which is not known to be a limiting factor in the establishment and spread of cowpea (see section 9.2.2).

Some of the genetic elements introduced into 709A cowpea were derived from known plant pathogens, but in all cases the genes responsible for the pathogenic qualities of the pathogen were not introduced. Therefore, the introduction of this genetic

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material would not be expected to result in 709A cowpea expressing novel pathogenic characteristics.

Based on these points, it can be concluded that 709A cowpea does not display any altered plant pest potential.

### 9.4. Potential Impact of 709A Cowpea on Non-Target Organisms

An important consideration in the environmental risk assessment of a transgenic plant is the potential for interactions with non-target organisms (NTOs), operationally defined as those organisms that are not intended to be affected by the expressed active ingredient (i.e., Cry1Ab protein in the case of 709A cowpea). The initial evaluation is at the level of individual species of concern, and only if abundance, reproductive biology, or behaviour are adversely affected, are broader ecological impacts expected (OECD, 2018b).

As briefly reviewed in section 2.2.1 (page 23), insecticidal formulations containing *Bacillus thuringiensis* bacteria have an extensive history of safe use spanning several decades. Similarly, as described in section 6.1.1 (page 44), the mode of action and specificity of Cry1Ab protein are well-known and there is extensive information relevant to assessing the environmental, food, and feed safety of Cry1Ab in the scientific and regulatory literature, as reviewed by CERA (2011) and ILSI-RF (2016).

As noted by Huesing et al. (2011), the current safety data and history of safe use of Cry1Ab and closely related Cry proteins provide all the necessary NTO data for 709A cowpea. Also, there do not appear to be any unique species (taxa) in the African receiving environment for which additional testing of their sensitivity to Cry1Ab would be warranted because they belong to higher taxa that are adequately covered already (Huesing et al., 2011).

Since the publication by Huesing et al. (2011), there have been additional publications reviewing the food and environmental safety of Bt crops (Koch et al., 2015; Pellegrino et al., 2018), including a re-evaluation of the potential risk of Bt cowpea to non-target organisms in West Africa (Ba et al., 2018). In Pellegrino et al. (2018), a meta-analysis of 76 publications over 21 years found no adverse effects of Bt crops on non-target organism families, including Anthocoridae, Aphididae, Araneae, Carabidae, Chrysopidae, Coccinellidae, Nabidae, Nitidulidae and Staphylinidae. The exception was for Braconidae, represented by a parasitoid of European corn borer, the target organism of Bt maize, where parasitoid abundance was not unexpectedly reduced.

As noted by Ba et al. (2018), their conclusion remains that “*there are no unique arthropod species in the likely deployment area for Bt cowpea in Africa that have not been assessed elsewhere for other GE crops*” and that no additional testing should be required to come to a determination of negligible risk to non-target organisms.

The existing data on the environmental safety of Cry1Ab to non-target organisms are entirely sufficient to complete the environmental risk assessment of 709A cowpea. Thus, for 709A cowpea, the assessment of potential NTO effects focuses on examining potential exposure pathways to Cry1Ab for selected organisms that have been commonly used as sentinel species to represent pollinators and pollen feeders, soil-dwelling organisms, aquatic organisms, and wild mammals and birds. Where there is a likelihood

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**Table 34.** Summary of ecotoxicological tests of Cry1Ab on non-lepidopteran non-target organisms

| Species <sup>a</sup>                                  | Method of Exposure   | Exposure Duration | Results   |
|---|--|-------------------|---|
| <i>Apis mellifera</i> (honeybee) larvae               | Single dose exposure to protein at 20 ppm (MRID <sup>b</sup> 434392-02)                        | single dose       | NOEL <sup>c</sup> > 20 ppm  |
| <i>Apis mellifera</i> (honeybee) adult                | Single dose exposure at 20 ppm (MRID 434392-03)  | single dose       | No statistically significant difference observed between test and control populations |
| <i>Chrysoperla carnea</i> (green lacewing) larvae     | Exposure at 16.7 ppm (MRID 434680-03)  | 7 days            | NOEL > 16.7 ppm   |
| <i>Hippodamia convergens</i> (ladybird beetles)       | Exposure at 20 ppm (MRID 434680-05)  | single dose       | NOEL > 20 ppm   |
| <i>Brachymeria intermedia</i> (parasitic hymenoptera) | Exposure at 20 ppm (MRID 434680-04)  | single dose       | NOEL > 20 ppm   |
| <i>Folsomia candida</i> (Collembola)                  | Lyophilized leaf tissue (estimated 50.6 µg Cry1Ab/g) (MRID 442715-01)                          | 28 days           | NOEL > 50% of diet  |
| <i>Daphnia magna</i> (water flea)                     | Exposure to Cry1Ab in Bt176 maize pollen at multiple concentrations (MRID 433236-10)           | 48 hours          | NOEC <sup>d</sup> > 150 µg/ml   |
| <i>Eisenia fetida</i> (earthworm)                     | Exposure to purified Cry1Ab in artificial soil substrate (MRID 438879-02)                      | 14 days           | NOEL > 200 ppm  |
| <i>Colinus virginianus</i> (bobwhite quail)           | 50,000–100,000 ppm Cry1Ab in transgenic cornmeal derived from event MON 80187 (MRID 435332-05) |                   | No observed adverse effects   |
| <i>Mus musculus</i> (mouse)                           | Acute oral gavage at 3280 mg/kg body weight (MRID 433236-08)                                   | single dose       | No observed adverse effects   |

<sup>a</sup> Extracted from CERA (2011); Huesing et al. (2011); EPA (2010).

<sup>b</sup> master record identification number (MRID).

<sup>c</sup> NOEL = No observed effect level.

<sup>d</sup> NOEC = No observed effect concentration.

of significant exposure to Cry1Ab, estimated environmental concentrations will be placed in the context of existing NTO studies, such as those listed in Table 34, and the implied safe margins of exposure.

### 9.4.1. Potential Impacts on Pollinators and Pollen Feeders

Cultivated cowpea is highly self-pollinated, and therefore reproduction does not depend on pollinators (OECD, 2015). Nevertheless, there is some opportunity for out-crossing when pollinators are present, and while pollinators of cowpea have not been definitively identified, some have been implicated as possible pollinators of cultivated cowpea. In Nigeria, *Apis mellifera* (specifically *Apis mellifera adansonii*, the African honeybee) has been mentioned as a possible pollinator (Ige et al., 2011). In Burkina Faso, there is evidence that bees from the genus *Xylocopa*, and the family *Megachilidae* are pollinators (Pasquet, 2012), and in particular the megachilid *Chalcidoma cincta cincta* may play a role in pollinating *V. unguiculata* flowers in Cameroon (Pando et al., 2014). The only organisms that could potentially ingest Cry1Ab contained in pollen are bees (Huesing et al., 2011). Therefore, since *Apis mellifera* is an important pollinator of agricultural crops in Nigeria, and may be exposed to pollen from cultivated 709A cowpea, it serves as an important species for consideration itself and as a representative of other pollinator species that could be similarly exposed.

Adult honeybees feed on pollen from a variety of different plant species as a major source of protein and also consume nectar for sugar and carbohydrates (Brodsgaard et al., 2003; Malone and Pham-Delègue, 2001). The likely route of exposure to the insecticidal proteins expressed in 709A cowpea is through pollen as it generally contains 8–40 percent protein, while nectar does not have significant protein content

(Malone and Pham-Delègue, 2001). The existing data show that exposure will be low as, notwithstanding the implication of Ige et al. (2011), *Vigna unguiculata* is rarely if ever visited by *A. mellifera adansonii* when compared to other species. Omoloye and Akinsola (2006) observed that 41 plant species were visited by the honeybee in southwest Nigeria, but *V. unguiculata* was not among them. Similarly, Mbah and Amao (2009) observed visitation of 28 plant species by the honeybee from September 2006 to January 2008 in northern Nigeria, none of which was *V. unguiculata*. Finally, the most extensive survey of plants visited by *Apis mellifera* was conducted by Dukku (2013) in the Sudan Savanna zone of northeastern Nigeria. Of the 61 plant species visited by *A. mellifera* in that study, *V. unguiculata* was not included. Therefore, the contribution of pollen from 709A cowpea to the total pollen exposure of adult *Apis mellifera* in Nigeria is likely insignificant.

Honeybee larvae are regarded as the most sensitive life stage for assessing the risk from exposure to Cry1Ab protein expressed in transgenic plants, as they require a complex diet and have limited food choice in the brood cells (Brodsgaard et al., 2003; Hendriksma et al., 2011). Honeybee larvae feed on small amounts of pollen from a variety of plant sources and a nutrient rich jelly (Brodsgaard et al., 2003; Malone and Pham-Delègue, 2001). Appearance of Cry1Ab protein in jelly is unlikely because these proteins would have to pass the insects guts and become incorporated into the hypopharyngeal glands. Studies with insecticides that are much smaller molecules showed only traces in the hypopharyngeal glands of worker bees when fed the test compound (Babendreier et al., 2004). Therefore, ingestion of pollen would present the greatest exposure. The amount of total protein ingested by larvae in a study where transgenic maize plants were the only pollen source was less than five percent in terms of the total protein required to complete larval development (Babendreier et al., 2004).

Furthermore, Bt proteins generally do not persist and are not stable in the environment, e.g., Clark et al. (2005); Icoz and Stotzky (2008). Environmental conditions, such as heat, relative humidity, and ultra-violet radiation, will likely compromise the presence and integrity of the pollen capsule and may also impact protein stability. Rainfall or heavy dew will likely wash pollen from the host plant leaves (Pleasants et al., 2001) and diverse microbial communities colonizing the phyllosphere of plant leaf surfaces (Lindow and Brandl, 2003; Yang et al., 2001) may degrade pollen proteins. In addition, pollen grains are known to desiccate rapidly in the environment (Fonseca and Westgate, 2005; Luna V et al., 2001). These factors likely further limit exposure of pollinators and pollen feeders, including non-target Lepidoptera, to Cry1Ab via ingestion of 709A cowpea pollen.

In a recent meta-analysis of 64 studies conducted with purified Bt toxins or transgenic pollen, under laboratory conditions or in fields or semi-field trials, Ricroch et al. (2018) concluded that current Bt crops have no adverse effects on larvae or adult honey bees. Toxins produced by current Bt plants have no lethal effects on survival and no demonstrated sub-lethal effects (studied as midgut bacteria population, foraging activity, or olfactory learning).

In conclusion, it is highly unlikely that there will be an impact of 709A cowpea on pollinators and pollen feeders, especially *A. mellifera*. Cry1Ab itself is non-toxic to honeybee adults and larvae, and the level to which adults and larvae would be exposed is negligible.

**Table 35.** Bt protein persistence in the soil

| Persistence <sup>a</sup>   | Protein           | Source                       | Endpoint                               |
|--|-------------------|------------------------------|--|
| DT <sub>50</sub> <sup>b</sup> < 1 day  | Cry1F             | microbial                    | <i>H. virescens</i> bioassay           |
| DT <sub>50</sub> < 30 days   | Cry1Ab and Cry1Ac | microbial and cotton tissue  | ELISA                                  |
| Half-lives of 2.2, 22, 40, and 46 days   | Cry1Ab and Cry1Ac | microbial and cotton tissue  | ELISA                                  |
| DT <sub>50</sub> = 1.6 days (in soil) and 15 days (no soil)  | Cry1Ab            | maize tissue                 | <i>H. virescens</i> bioassay           |
| DT <sub>50</sub> = 15.5 days (lab) and 31.7 days (field), 20–25% of bioactivity remained after 120 days  | Cry2A             | cotton tissue                | <i>H. virescens</i> bioassay           |
| Insecticidal activity of extracts retained for 6 months  | Btk(Cry1)         | DiPel (sporular formulation) | <i>M. sexta</i> bioassay               |
| Detection and insecticidal activity at termination of tests 28 days (Cry1Ab) and 56 days (Cry1Ac)  | Cry1Ab and Cry1Ac | cotton tissue                | ELISA and <i>H. virescens</i> bioassay |
| Decreased to 20% of initial in 60 days (buried) and to 38% of initial in 40 days (soil surface); protein remained detectable post-harvest through following spring | Cry1Ab            | maize tissue                 | ELISA                                  |

<sup>a</sup> Extracted from Table 2 in Clark et al. (2005).

<sup>b</sup> DT<sub>50</sub> = Number of days to result in 50% degradation or 50% loss of activity.

#### 9.4.2. Potential Impacts on Soil-Dwelling Organisms

Soil-dwelling organisms (e.g., earthworms) may be directly exposed to Cry1Ab protein that enters the soil matrix via root exudates, sloughing, and decomposition of 709A cowpea plants. The magnitude and duration of soil-dweller exposure to Cry1Ab will be highly dependent on protein accumulation and dissipation. Most proteins do not persist or accumulate in the soil because they are inherently degradable in soils that have healthy microbial activity (Burns, 1982; Marx et al., 2005). As noted previously, Bt proteins typically do not persist or accumulate in soil over time (Table 35) Clark et al. (2005); Icoz and Stotzky (2008) and thus the potential of environmental exposure to soil dwelling organisms is minimized.

In a study conducted by Dubelman et al. (2005), soil samples were collected from agricultural fields in five regions of the United States where Bt corn hybrids (MON 810 or Bt11) had been planted for at least three consecutive years and assayed for the presence of active Cry1Ab protein using a European corn borer (*Ostrinia nubilalis*) bioassay. The limit of detection for Cry1Ab in the soil was 0.03 µg/g, and only one soil sample collected after pollen shed from a field site that had also been treated with carbofuran insecticide showed insect toxicity. Based on these data, there was no evidence of persistence or accumulation of Cry1Ab protein in soils following successive planting of Bt maize.

A multi-year study of the environmental fate and agronomic effects of Cry1Ab and synthetic insecticides [tefluthrin (pyrethroid) and clothianidin (neonicotinoid)] concluded that while Cry1Ab was present soil and runoff water, it dissipated quickly (Whiting et al., 2014). Cry1Ab was not found in any groundwater or soil pore water samples, indicating little movement of the protein below the surface of the soil; however, it could be detected in runoff water from both Bt and non-Bt maize fields, with the highest concentration measured of 0.129 µg/l in one sample. Cry1Ab was also detectable in soil samples and the highest average concentration in a monthly sample was 0.009 µg/g, which was during pollination.

As described, several lines of evidence indicate that Cry1Ab rapidly dissipates in the environment and that the risk to non-target species from this exposure is very low.

#### Calculation of estimated environmental concentration (EEC):

Worst-case assumptions regarding the amount of protein that could enter the soil matrix were used to estimate an EEC for soil dwelling organisms, and included the following assumptions:

- A whole-plant Cry1Ab concentration of 12.7 mg/kg fresh weight tissue (FWT), the same concentration as used for estimating livestock animal exposure to Cry1Ab from 709A forage and hay (section 5.3, page 42).
- A cowpea planting density for the Nigeria savannah region of 133,333 plants/hectare, which results in a total dry matter yield ranging from 3,136 to 4,453 kg/ha depending on variety and location (Kamara et al., 2018). The higher value of 4,453 kg/ha was assumed for EEC calculations.
- Soil incorporation of 709A cowpea biomass into the top 15 cm of soil, which for a 1-hectare plot corresponds to  $10,000 \text{ m}^2 \times 0.15 \text{ m} = 1,500 \text{ m}^3$  of soil. Soil densities reported in Nigeria range from 1,200–1,520 kg/m<sup>3</sup> (Aina, 1979; Olaoye, 2002), and the highest value was used. Therefore, Cry1Ab will be incorporated into 2,280,000 kg of soil.

The EEC was calculated according to equation 4, below:

$$\text{EEC (mg/kg soil)} = \frac{\text{Total cowpea tissue (kg/ha)} \times \text{Cry1Ab amount (mg/kg tissue)}}{\text{Soil weight (kg/ha)}} \quad (4)$$

$$\text{EEC (mg/kg soil)} = \frac{4,453 \text{ (kg soil/ha)} \times 12.7 \text{ (mg/kg tissue)}}{2,280,000 \text{ (kg/ha)}}$$

$$\text{EEC} = 0.025 \text{ (mg/kg soil)} = 0.025 \text{ (}\mu\text{g/g soil)}$$

#### *Eisenia fetida*:

Earthworms (*Eisenia fetida*) are arguably the most important soil biota in maintaining soil structure, function and fertility (Edwards, 2004). The contribution of earthworms to soil fertility can be considerable (Killham, 1994) and earthworms are intimately involved in organic matter decomposition (for example of surface leaf litter and the remineralization and humification of organic matter). They also contribute to soil aeration and have large direct effects on nitrogen cycling (Killham, 1994; Forbes and Kure, 1997). Earthworms are also a preferred species for ecological risk assessment because they are more highly exposed to soil contaminants through ingestion and dermal contact than other soil and leaf litter invertebrates (Ma, 1994). The earthworm was chosen as a representative non-target soil-dwelling invertebrate within the agroecosystem that could be exposed to the Cry1Ab protein via ingestion of soil.

From Table 34 (page 75), the no observed effect level (NOEL) of Cry1Ab for *E. fetida* was greater than 200  $\mu\text{g/g}$ , which translates to a margin of exposure (MOE) of >8000-fold

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(200  $\mu\text{g/g}$  / 0.025  $\mu\text{g/g}$ ). Thus, the existing Cry1Ab NTO data for earthworms as a representative soil-dwelling invertebrate are applicable to 709A cowpea, and support the very low likelihood of risk.

### 9.4.3. Potential Impacts on Predators and Parasitoids

Because a predator or parasitoid does not feed directly on the plant, the magnitude of exposure to Bt proteins via ingestion of a prey item is reduced relative to the prey item's direct ingestion of plant tissue, as long as there is no bioaccumulation. Secondary exposures are influenced not only by the rates of ingestion, digestion, and excretion of plant material by the prey item, but also by the stability of proteins within the prey item.

Proteins, in general, lack stability in environmental matrices and do not have the chemical characteristics needed to bioaccumulate (Spacie et al., 1995). This has been confirmed in several studies that were conducted to characterize the potential for Bt proteins to bioaccumulate in prey items (Meissle and Romeis, 2012). Bt proteins have been detected in some prey species reared on Bt crops. For example, thrips (*Frankliniella tenuicornis*) transiently contain up to  $0.35 \times$  the concentration of Cry1Ab in Bt maize; adults contain about half this amount and pupae less than  $1/40^{\text{th}}$  the concentration in larvae (Obrist et al., 2005). Cry3Bb1 proteins were detected in spider mites (*Tetranychus urticae*; Li and Romeis (2010)), and Bt proteins have been detected in aphids reared on Bt crops (Romeis and Meissle, 2011). Generally, the concentrations reported in these studies were lower than those detected in the crop tissues, thereby indicating a lack of bioaccumulation. Other studies demonstrate that Bt proteins do not accumulate in prey species (Lundgren and Wiedenmann, 2004; Meissle and Romeis, 2009). The exposure of predatory species to Bt proteins through trophic transfer is low due to the short half-life of the protein in prey (Obrist et al., 2005) and the low concentration of protein in prey (Li and Romeis, 2010; Obrist et al., 2005). To date, there have been no studies using validated and robust ELISA or western immunoblot methods that have indicated bioaccumulation of Bt proteins in prey foods. If Bt proteins do not bioaccumulate or persist in prey foods, then predators and parasitoids will receive minimal exposure to the proteins of interest through trophic transfer.

As most predators are generalist feeders that do not depend on a single prey species as a food source, it is difficult to compute a precise EEC. However, setting the EEC at  $0.2 \times$  the maximum Bt protein concentration in any above-ground 709A cowpea tissue at the highest expressing developmental stage is reasonably conservative, and is an approach that has been used previously in the context of Bt maize (Raybould et al., 2007).

For Cry1Ab protein expressed in 709A cowpea, the highest concentrations were measured in flower tissue (22.8  $\mu\text{g/g}$  FWT; Table 7, page 39), resulting in an EEC for above-ground non-target arthropods of 4.56  $\mu\text{g/g}$  prey diet.

#### ***Chrysoperla rufilabris*:**

Green lacewings (Neuroptera: Chrysopidae) are important beneficial predators in many cropping systems (McEwen et al., 2001). Protecting the biological control function provided by lacewings and other natural enemies is a goal that should not be compromised by the introduction of new pest control products (Sanvido et al., 2012). Characteristics that make this test organism suitable for toxicity testing include

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availability, ease of culturing, and possibility of exposure to the protein via prey or plant pollen ingestion.

From early tier NTO testing with green lacewing larvae, the NOEL for Cry1Ab was greater than 16.7  $\mu\text{g/g}$  (Table 34; EPA (2010)), providing a 3.7-fold MOE relative to the worst-case EEC from 709A cowpea.

### ***Coleomegilla maculata*:**

The ladybird beetle *Coleomegilla maculata* (DeGeer) is a common and abundant predator found in many cropping systems worldwide. Both larvae and adults of *C. maculata* are predaceous, feeding on aphids, thrips, and lepidopteran eggs and young larvae (Duan et al., 2002; Lundgren and Wiedenmann, 2002). Additionally, *C. maculata* also feeds on plant tissues, such as pollen (Lundgren and Wiedenmann, 2004), and thus can be directly or indirectly exposed to Cry proteins in different ways when feeding in Bt crops. Since *C. maculata* represents an important group of predatory arthropods that is exposed to transgenic plant-expressed insecticidal proteins in the field and since the species is suitable for laboratory studies, it is commonly used in Tier-I testing of potential non-target impacts of insecticidal proteins.

NTO testing with ladybird beetles resulted in a NOEL for Cry1Ab of greater than 20  $\mu\text{g/g}$  (Table 34; EPA (2010)), providing a 4.4-fold MOE relative to the worst-case EEC from 709A cowpea.

The early tier NTO studies with sentinel predators provide an adequate MOE relative to any realistic exposure to Cry1Ab protein from 709A cowpea, thus providing assurance of the safety of 709A cowpea to non-target predator and parasitoid organisms.

### 9.4.4. Potential Impacts on Aquatic Organisms

There are different routes through which 709A cowpea plant material or insecticidal protein may enter an aquatic system, and each entry route is influenced by a number of factors including, crop management practices, the spatial relationship between cultivation fields and aquatic ecosystems, wind, rain, and soil runoff events. Potential entry routes include erosion of soil and adsorbed protein; surface runoff of freely soluble protein; and movement of plant material. Aerial deposition of pollen is not a significant exposure pathway as *V. unguiculata* is predominantly self-pollinated.

Both soil-bound and freely soluble proteins may potentially enter an aquatic ecosystem following a rain event via erosion of the soil and/or surface runoff, thus understanding the terrestrial fate of Bt proteins is important for assessing potential exposure to aquatic organisms. As described by Carstens et al. (2012), numerous studies have demonstrated that while Bt proteins may enter the soil through root exudates, root sloughing, or tissue decomposition, they have been shown to degrade rapidly in soils and bind tightly to soil particles. As shown by field studies, such as those of Whiting et al. (2014), concentrations of Cry1Ab in surface runoff water from Bt maize fields is less than 0.13  $\mu\text{g/l}$ .

The transfer of 709A4 cowpea plant material and/or senescent crop residue into aquatic systems surrounding fields may occur. While pieces of cowpea leaf tissue may enter the aquatic systems throughout the growing season, input is more likely from post-harvest crop residues in a manner similar to that reported for maize (Jensen et al., 2010).

However, the concentrations of Cry1Ab protein are expected to be lower in senescent tissue, the developmental stage from which post-harvest plant debris will originate, and are unlikely to result in ecologically relevant concentrations in water bodies because of leaching from intact plant tissues. This contribution will be even further reduced if vegetative material is harvested for animal feed.

### Calculation of EEC:

Worst-case assumptions of potential exposure of aquatic organisms to Cry1Ab protein from 709A cowpea as a result of soil erosion, surface runoff, or movement of plant material into aquatic systems, were calculated using the US EPA standard agricultural field-farm pond model (also called the US EPA standard pond). The standard pond model assumes runoff from a 10-hectare field is deposited in a 1-hectare pond (2 m deep; equivalent to 20,000,000 l of water). Because it is highly unrealistic to assume that all of the above-ground 709A cowpea biomass from a 10-hectare field would simultaneously enter a 1-hectare pond, an assumption from the EPA generic estimated environmental concentration (GENEEC) pond model (i.e., 10 percent runoff from 10 ha field into a 1 ha pond) was used in the calculations. Worst-case assumptions for calculating the EEC were:

- A pond (1 ha, 2 m deep, containing 20,000,000 l of water) receives 10 percent of the above ground cowpea biomass from a 10 ha field, equivalent to 4,453 kg dry weight (DWT) plant tissue (4,453 kg DWT/ha × 10 ha × 0.1)
- Cry1Ab protein 709A cowpea tissue is freely soluble and instantaneously bioavailable to aquatic non-target organisms. No protein degradation (biotic or abiotic) occurs. No binding of the protein to soil occurs.
- A whole-plant Cry1Ab concentration of 12.7 mg/kg FWT, the same concentration as used for estimating livestock animal exposure to Cry1Ab from 709A forage and hay (section 5.3, page 42).

The EEC was calculated according to equation 5, below:

$$\text{EEC (mg/l water)} = \frac{4,453 \text{ (kg tissue)} \times 12.7 \text{ (mg/kg tissue)}}{20,000,000 \text{ (l water)}} \quad (5)$$

$$\text{EEC} = 0.0028 \text{ (mg/l water)} = 2.8 \text{ (}\mu\text{g/l water)}$$

This worst-case EEC is approximately 20-fold higher than actual concentrations of Cry1Ab measured in surface runoff water from Bt maize fields (Whiting et al., 2014). Under more realistic conditions, it is unlikely that: (a) even 10 percent of the plant biomass from a 709A cowpea field would end up in an adjacent water body; (b) that Cry1Ab protein within plant tissue would not rapidly degrade; and (c) that Cry1Ab protein within the tissue would become freely soluble and not subject to biotic and abiotic degradation, and binding to organic matter.

### *Daphnia magna*:

*Daphnia magna* (water flea) is a crustacean (phylum: Arthropoda) invertebrate that inhabits ponds and lakes in most regions of the world. It is a common inhabitant of ponds in agricultural landscapes and will, like many other zooplankton and benthic

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arthropods, receive pollen and detritus from drainage water from agricultural fields. *Daphnia magna* feed non-selectively on a broad range of particles in the size range 1–50  $\mu\text{m}$ , and where 709A cowpea plants are grown, they might receive this in their diet in the form of detrital particles.

Early tier studies with *D. magna* exposed to Cry1Ab in maize pollen have found no observed effects at concentrations of  $>150 \mu\text{g}$  Bt176 maize pollen/ml (Table 34). Considering the concentration of  $12.36 \mu\text{g}$  Cry1Ab/g Bt176 pollen (EPA, 2010), the corresponding no observed effect concentration (NOEC) is  $>1.9 \mu\text{g}$  Cry1Ab/l water, or approximately 68 percent of the extreme worst-case EEC for Cry1Ab from 709A cowpea using the EPA farm pond model.

In comparison with more realistic environmental concentrations of Cry1Ab in aquatic systems based on measurements from field studies (e.g.,  $<0.13 \mu\text{g/l}$  water; Whiting et al. (2014)), the minimum NOEC of  $1.9 \mu\text{g}$  Cry1Ab/l water for *D. magna* represents at least a 14.6-fold MOE.

Under realistic exposure scenarios, early tier NTO studies with the sensitive sentinel species, *D. magna*, provide a sufficient MOE to demonstrate the safety of 709A cowpea to likely exposed aquatic organisms.

### 9.4.5. Potential Impacts on Wild Mammals

The most significant route of exposure of wild mammals to the Cry1Ab protein produced in 709A cowpea would be through consumption of cowpea grain. In Kenya, Pasquet (2012) observed that rodents were responsible for predating seed that might be left after harvest or buried, and the situation is likely to be similar in Nigeria. Therefore, the impact on wild mammals will primarily be on rodents, who are likely to be the highest consumers of cowpea grain.

Ratios of food intake rate to body weight have been reported for a number of rodent species (Crocker et al., 2002), and the highest ratio of 0.33 for the harvest mouse (*Micromys minutus*) was used to estimate conservative DDE for the Cry1Ab protein. The highest measured concentration of Cry1Ab in 709A cowpea grain was  $2.9 \mu\text{g/g}$  (Table 7, page 39), yielding a worst-case DDE of  $0.33 \text{ kg grain/kg body weight} \times 2.9 \text{ mg/kg grain} = 0.957 \text{ mg Cry1Ab/kg body weight}$ .

Acute oral toxicity testing with mice (*Mus musculus*) using Cry1Ab protein found no observed adverse effects following a single dose of  $3,280 \text{ mg/kg body weight}$  (Table 34), which is 3,427-fold greater than the worst-case DDE. The lack of acute oral toxicity of the Cry1Ab protein to mice supports the conclusion that consumption of 709A grain would present minimal risk to rodents and small mammals.

### 9.4.6. Potential Impacts on Wild Birds

Wild birds will be exposed to the Cry1Ab protein expressed in 709A cowpea primarily via consumption of seed (grain), either as germinating or spilled seed from shattered pods. However, wild birds are highly unlikely to consume a diet comprised solely of cowpea seed and it is more realistic to assume a diet containing less than 50 percent 709A cowpea seed. Similar assumptions have been made in estimating potential exposure of wild birds to kernels (seed) derived from Bt maize (Raybould et al., 2007). Therefore,

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a diet comprised of *ca.* 50 percent 709A cowpea seed can be considered as equivalent to a  $1 \times$  EEC for wild birds.

Assuming that wild birds can consume up to their weight in seed per day, and assuming that half the seed is comprised of 709A cowpea seed containing up to 2.9 mg/kg Cry1Ab (Table 7, page 39), the worst-case DDE to Cry1Ab for wild birds is 1.45 mg/kg body weight.

An avian oral toxicity study conducted in Northern Bobwhite quail resulted in no treatment related mortality or differences in food consumption, body weight, or behaviour following feeding of up to 100,000 mg/kg body weight Bt maize (MON 80187) meal containing Cry1Ab protein (Table 34; EPA (2010)). Assuming a Cry1Ab concentration of 5  $\mu$ g/g in the Bt maize meal, bobwhite quail were exposed to *ca.* 500 mg Cry1Ab/kg body weight.

The dietary intake of Cry1Ab resulting in no adverse effects to bobwhite quail corresponded to  $345 \times$  the worst-case DDE to Cry1Ab from 709A cowpea seed for wild birds. The lack of oral toxicity of the Cry1Ab protein to bobwhite quail at 345-fold MOE supports the conclusion that consumption of 709A cowpea seed would present minimal risk to wild birds.

### 9.4.7. Conclusions on Potential Non-Target Effects

This risk assessment has considered the wealth of existing data on the potential for adverse impacts of Cry1Ab on a range of non-target organisms, including, but not limited to: honeybee (*Apis mellifera*) larvae and adults; ladybird beetle (*Hippodamia convergens*); Daphnia (*Daphnia magna*); earthworms (*Eisenia foetida*); green lacewing (*Chrysoperla carnea*) larvae; wild mammals, and birds. In every case, the conclusion is that unconfined environmental release would not result in altered impacts on interacting organisms, with the exception of target lepidopteran insect species (e.g., *Maruca vitrata*). Reviews of the environmental safety of Cry1Ab (CERA, 2011; Huesing et al., 2011) have concluded that, based on toxicity testing with a range of representative non-target organisms, cultivation of genetically engineered plants expressing Cry1Ab would not significantly affect the abundance of non-target arthropods in comparison with alternative insect management practices.

## 10. Product Stewardship

### 10.1. Insect Resistance Management Plan

#### 10.1.1. Introduction to IRM

Insect resistance management (IRM) is a set of practices intended to slow or reduce the evolution of resistant insect populations. IRM plans do not generally specify a target number of years until resistance develops but do outline a set of practices that if implemented should extend the useful life of a particular insect protection trait or Bt crop under conditions of field use. The intention is to balance the science of resistance evolution with practical and economic realities, as impractical plans are unlikely to be implemented. It is also recognized that IRM plans developed to accommodate large-scale industrialized agriculture in a highly monocropping production system are

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unlikely to be well suited to smallholder agriculture typified by diverse cropping systems and landscapes.

The factors affecting the rate at which resistant insect populations develop are generally well understood and include: the biology and ecology of the insect species; the genetics of resistance; the insecticidal properties of the crop; and the characteristics of the agricultural production system.

Each of these factors, described in more detail in the following sections, was considered in developing the proposed IRM plan for 709A cowpea.

### *10.1.2. The Biology and Ecology of Maruca vitrata*

Included in the consideration of pest biology and ecology are: the insect's use of alternative host plants, including crops and natural vegetation; seasonal migration and survival biology; adult insect dispersal within and among fields; larval feed behaviour, including dispersal within and among neighbouring plants; and density-dependent and density-independent factors affecting mortality, fecundity, and dispersal.

#### Native Range in West Africa:

Although previously thought to be endemic only in the coastal areas of southern Benin, Ghana, and Nigeria, *M. vitrata* also appears to survive year-round in southern Burkina Faso (the Sudanian zone) (Ba et al., 2009; Traore et al., 2014). The insect prefers high humidity and low to moderate temperatures (Jakai and Adalla, 1997), e.g., 20–25°C and >80 percent relative humidity (RH) for mating (Jackai et al., 1990).

In drier northern parts of West Africa, *M. vitrata* is found on cowpea during the cultivation period but appears to migrate in from wetter regions each year rather than being present year-round (Bottenberg et al., 1997; Ba et al., 2009; Margam et al., 2010). Bottenberg et al. (1997) did not observe *M. vitrata* associated with either cowpea or alternative hosts during the dry season in northern Nigeria.

#### Alternative Hosts:

There are a number of reports of alternative hosts of *M. vitrata* in West Africa (Bottenberg et al., 1997; Arodokoun et al., 2003; Margam et al., 2010; Traore et al., 2014). In areas where *M. vitrata* persists year-round, some alternative hosts enable the pest to survive when cowpea is not cultivated, whereas other alternative hosts may be present at the same time as the crop. In Burkina Faso, the wild host plants *Mucuna poggei* Taub. and *Daniellia oliveri* (Rolfe) Hutch. & Dalziel maintained a permanent *M. vitrata* population during the off-season, whereas other alternative hosts, such as *Sesbania pachycarpa* DC. and several species of *Tephrosia*, were infested at the same time as cowpea (Traore et al., 2014). In southern and central Benin, populations of *M. vitrata* are maintained year-round through survival on a series of wild host plants (Arodokoun et al., 2003), some of which flower at the same time as the cowpea crop.

In an extensive survey of areas near cowpea fields in northern Ghana, northern Nigeria, and Burkina Faso, alternative hosts were found in association with all of the 43 surveyed sites in Burkina Faso, in 24 of 25 sites in northern Ghana, in 22 of 25 sites in Bunkure (Kano State in northern Nigeria), and in 11 of 19 sites in Bomo (near Zaria in Kaduna State, northern Nigeria). The alternative host plants included species of *Crotalaria*, *Sesbania*, *Tephrosia*, *Vigna*, *Senna*, and *Centrosema*. In most cases, flowering and

## PRODUCT STEWARDSHIP

podding of the alternative hosts and that of nearby (within 200 m) cultivated cowpea overlapped. In Ghana, one of the alternative hosts was pigeon pea (*Cajanus cajan* [L.] Millsp.), which could be planted as both a refuge and a food source. An example similar to the latter was studied in Australia at a time when single-gene Bt cotton was still being grown (Baker et al., 2008). In that study, pigeon pea was found to be an effective refuge species for *Helicoverpa* spp., as measured by production of pupae.

### Number of Generations:

In areas where *M. vitrata* is endemic, it can survive up to seven generations per year (Huesing et al., 2011), with fewer generations occurring in areas where the insect does not persist year-round. In the Kano area in northern Nigeria, *M. vitrata* invades cowpea during the rainy season (July to October), where three generations occur annually (Bottenberg et al., 1997). In more northern, drier areas (e.g., Niamey, Niger, and Kamboinsé, Burkina Faso), only one generation of *M. vitrata* has been observed per year (Bottenberg et al., 1997; Ba et al., 2009).

### Life Cycle:

Eggs of *M. vitrata* are most often laid on the leaf, particularly on the lower leaf surface (Okeyo-Owuor and Ochieng, 1981), and hatch within three to five days. The insect has five instars, which can be distinguished on the basis of head capsule width (Taylor, 1967; Odebiyi, 1981; Adati et al., 2004). Larval development has been reported to take around eight to fourteen days, with variation across different studies e.g., (Taylor, 1967; Okeyo-Owuor and Ochieng, 1981; Adati et al., 2004).

During the pre-pupation stage, the larvae bury themselves in loose soil near the host plant and spin a silken cocoon (Okeyo-Owuor and Ochieng, 1981). Pupation can also occur within the pod (Okeyo-Owuor and Ochieng, 1981). The pre-pupal stage is one to two days, and pupation is about three to fourteen days depending on temperature.

The adult life span is typically three to ten days, with females living longer than males. Adult emergence and mating both occur primarily at night, with mating peaks reported three to five days after emergence, with some variation among studies (Lu et al., 2007). Oviposition in moths paired soon after emergence occurred 24–48 h after mating (Okeyo-Owuor and Ochieng, 1981). Egg, larval, and pupal survival are reduced to below 10 percent by temperatures of 34.3°C or above (Adati et al., 2004).

### Larval Movement:

Larvae move from flower to flower, and older larvae (3<sup>rd</sup>–5<sup>th</sup> instars) can bore into pods and stems (Taylor, 1967). Larvae can move between different peduncles by producing silken threads (Taylor, 1967). There is very little published information on how often *Maruca* larvae move between plants. It has been suggested that older larvae can move between adjacent plants when resources are exhausted (Okeyo-Owuor et al., 1983), perhaps by means of a silken thread (personal communication, Prof. Rabiou and Dr. Utono, Institute for Agricultural Research, Ahmadu Bello University). Young larvae (1<sup>st</sup>–2<sup>nd</sup> instar) tend to remain sheltered in flowers and floral buds but can be blown from one plant to another via silken threads they spin (personal communication, Dr. L. Jackai, North Carolina A&T State University). Larvae that fall off a plant can travel to another one within about one meter, although most larvae that fall to the ground are likely to die or become prey.

### Adult Movement and Migration:

In humid areas such as southern and central Benin, *M. vitrata* adults move readily from cowpea to alternative hosts (Bottenberg et al., 1997; Arodokoun et al., 2003). When cowpea is present, it is the preferred host (personal communication, Prof. Rabiou and Dr. Utono, Institute for Agricultural Research, Ahmadu Bello University).

In drier areas such as southern Niger and northern Nigeria, there are few alternative hosts for *M. vitrata* (Margam et al., 2010). Outside of the cultivation season, *M. vitrata* does not occur because of lack of alternative hosts and dry (unfavourable) conditions. Consequently, *M. vitrata* migrates into these regions each year and is not continuously present (Margam et al., 2010).

Diapause, a period of suspended development, has not been observed in *M. vitrata* in Africa e.g., Bottenberg et al. (1997); Okeyo-Owuor and Ochieng (1981); Arodokoun et al. (2003), so it cannot survive in the absence of a host.

### 10.1.3. Effective Dose

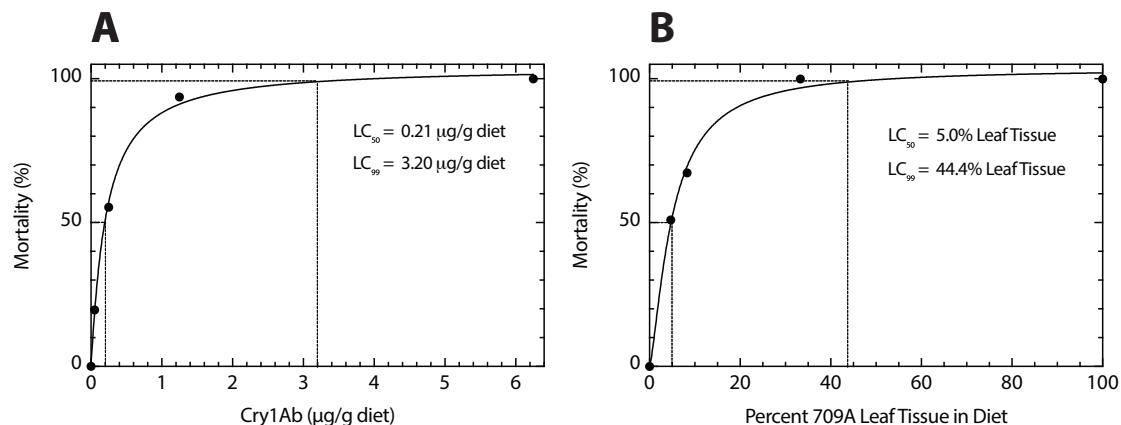
An important consideration in the design of effective IRM is the potency (i.e., dose) of the active ingredient(s) in the pesticidal plant.

To truly determine the dose of a Bt plant relative to the target insect requires resistant insect populations, as it is essential to experimentally compare the fitness of resistant (*RS*) heterozygotes with susceptible (*SS*) homozygotes by challenging them with the Bt crop (or Bt active ingredient). Plant tissue must be sufficiently toxic to ensure that any resistance allele is functionally recessive. Ideally, this would be done by dose-response bioassay on *SS*, *RS*, and *RR* genotypes. The Bt plant would be considered high-dose if the survival rate of *RS* heterozygotes was not significantly different than the survival rate of *SS* homozygotes.

However, resistant insect populations are rarely present at the time of introduction of a new Bt crop, making it impossible to empirically determine whether the new Bt plant is high-dose or not. Instead, a provisional definition of high-dose is used, such as that proposed by Gould and Tabashnik (1998), which is that the plant must express toxin at a concentration that is  $25\times$  the lethal concentration ( $LC_{99}$ ) for the target pest. This operational definition of high-dose has been adopted by US EPA as an integral component of the high-dose/refuge strategy even though the supporting scientific evidence is weak.

In order to determine whether 709A cowpea met the high-dose criterion, insect bioassays with *Maruca vitrata* were conducted using either purified Cry1Ab protein incorporated into insect diet (Figure 21, panel A) or using different proportions of 709A leaf tissue in the insect diet (Figure 21, panel B) (Abdourhamane et al., 2018c). The  $LC_{50}$  and  $LC_{99}$  for purified Cry1Ab against *M. vitrata* were estimated at ca.  $0.21\ \mu\text{g/g}$  diet and  $3.2\ \mu\text{g/g}$  diet, respectively. The estimated  $LC_{50}$  from this study agreed closely with the value of 0.207 ppm reported by Srinivasan (2008).

Using an  $LC_{99}$  of  $3.2\ \mu\text{g/g}$  diet for purified Cry1Ab, the high-dose threshold for 709A cowpea would be  $25 \times 3.2 = 80\ \mu\text{g/g}$  FWT, which is ca. 4.9-fold greater than the mean concentration of  $16.4\ \mu\text{g}$  Cry1Ab/g FWT measured in samples of flower tissue from field-grown 709A (IT97K-499-35 genetic background) (see Table 7, page 39).



**Figure 21.** The susceptibility of *Maruca vitrata* larvae to Cry1Ab was investigated by two methods, diet incorporation of different concentrations of purified Cry1Ab protein (Panel A) and incorporation of different proportions of 709A leaf tissue into the insect diet (Panel B). Insect mortality data were analyzed by non-linear regression to a four-parameter logistic curve to derive estimates of LC<sub>50</sub> and LC<sub>99</sub>, expressed in terms of µg Cry1Ab protein/g diet (Panel A) or percent 709A leaf tissue (Panel B). Average Cry1Ab concentration in 709A leaf tissue was 5.1 µg/g FWT.

Another way to determine if a Bt plant expresses at a high enough concentration is to dilute the tissue 25× and determine if SS survival is >1 percent or <1 percent at this dilution. Using data from the *M. vitrata* bioassay with leaf tissue, incorporation of 4 percent (i.e., 1/25<sup>th</sup> dilution) 709A leaf tissue resulted in ca. 44 percent mortality and to achieve LC<sub>99</sub> (i.e., <1 percent SS survival) required incorporation of ca. 44.4 percent 709A leaf tissue. Even accounting for a 3–4-fold higher Cry1Ab concentration in flower vs. leaf tissue, the amount of incorporated 709A tissue required to achieve <1 percent SS survival would be greater than 10 percent. Using the average Cry1Ab concentration in leaf tissue of 5.1 µg/g FWT, the estimated LC<sub>50</sub> for *in planta* expressed Cry1Ab was ca. 0.26 µg/g FWT, which was similar to the LC<sub>50</sub> of 0.21 µg/g diet estimated for purified Cry1Ab incorporated in artificial insect diet.

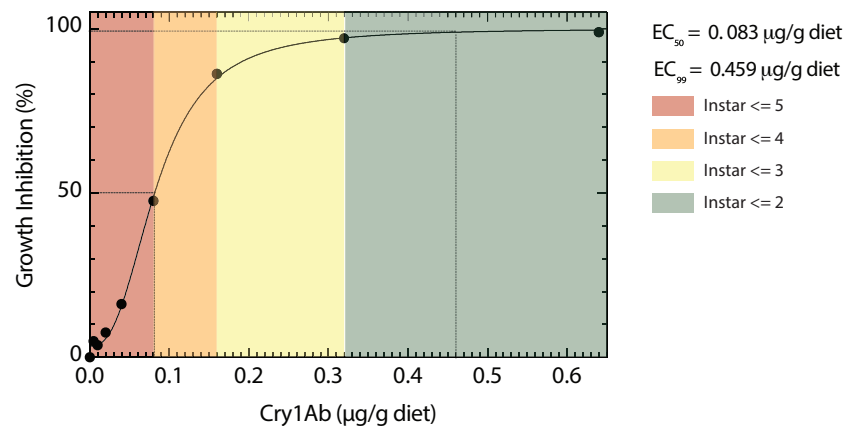
The results of *M. vitrata* bioassays using either diet-incorporated purified Cry1Ab protein or 709A leaf tissue were consistent and supported the conclusion that 709A cowpea did not meet the EPA operational criterion for high-dose.

In addition to measuring levels of insect mortality, it is also useful to consider the effect of insect-control proteins on insect growth and development as insects whose development is arrested before reproductive maturity are effectively dead. An analysis of Cry1Ab effect on the development of *Maruca vitrata* found that the effective concentration (EC) of purified Cry1Ab resulting in 50 percent inhibition of insect growth was 0.083 µg/g diet, with an EC<sub>99</sub> of ca. 0.46 µg/g diet (Figure 22). At EC<sub>99</sub>, *Maruca vitrata* larvae fail to develop beyond 2<sup>nd</sup>–3<sup>rd</sup> instar and are reproductive dead-ends. From these results, the mean concentration of Cry1Ab in 709A (IT97K-499-35 background) flowers and pods of 16.4 µg/g FWT and 9.4 µg/g FWT, respectively, represent respective margins of effective control of ca. 36-fold and 20-fold.

#### 10.1.4. Summary of IRM Considerations

Considerations for the proposed IRM plan for 709A cowpea have included:

- a) While 709A cowpea does not meet the operational definition of high-dose, it does provide a significant margin of effective control based on inhibition of larval growth and development.



**Figure 22.** The effect of Cry1Ab on growth and development of *Maruca vitrata* was studied with an insect bioassay using different concentrations of diet-incorporated purified Cry1Ab protein. Percent growth inhibition was calculated based on mean survivor weights, and each data point represents the mean of up to 44 larvae, depending on mortality. Growth inhibition data were analyzed by non-linear regression to a four-parameter logistic curve to derive estimates of EC<sub>50</sub> and EC<sub>99</sub>, expressed in terms of µg Cry1Ab protein/g diet.

- b) The presence of alternative hosts, both cultivated non-Bt cowpea and native plants, helps mitigate the need for extensive structured refuge. Genetic examination of *M. vitrata* samples collected from cowpea and several wild hosts in Benin did not show a clear population structure related to either host type or collection site (Agunbiade et al., 2014). This apparent lack of differentiation favours the possibility of gene flow between resistant survivors from 709A cowpea and susceptible insects from wild host plants, which would be necessary if wild plants are to function as a natural refuge.
- c) Although there are fewer alternative hosts in regions where *M. vitrata* is not endemic, the lack of year-round persistence of the pest in these areas means resistance evolution is less likely. However, there remains some uncertainty on the boundaries between endemic and non-endemic regions, which may be changing over time, and there will likely be isolated *M. vitrata* populations persisting year-round in river valleys where alternative hosts are present.
- d) The lack of information on the frequency of *M. vitrata* resistance alleles and the susceptibility of *RS* heterozygotes adds to the uncertainty around appropriate refuge size.
- e) The presence of small diversified cropping systems and expected low rate of release of new cowpea varieties containing event 709A will help delay the onset of resistant populations. At least initially, adoption rates are expected to be low because of limited seed supply and simply the time it takes to build farmer awareness. For example, Mbavai et al. (2015) studied adoption of improved (conventionally bred) cowpea varieties in northern Nigeria. When such varieties were actively promoted by the Sudan Savannah task-force, the adoption rate was about 36 percent after three years of promotion.
- f) The development of new cowpea varieties with pyramided modes of action (e.g., Cry1Ab, Cry2Ab2, Vip3Bb2) is actively underway and the first products may be available within five years. The effectiveness of such products containing Cry1Ab is reliant on preserving the durability of 709A cowpea varieties during the interim period.
- g) The experience with other non-high dose Bt traits has indicated a high risk of resistance evolution in the absence of sufficient refuge (Tabashnik and Gould, 2012).

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The 2002 report of the EPA Scientific Advisory Panel reviewing corn rootworm resistant maize concluded that products expressing Cry3Bb1 were not high-dose and a majority of the panel members recommended a minimum 50 percent refuge, stating that “modeling suggested that a 50% refuge would net at least twice the time to resistance as the proposed 20% refuge” (EPA, 2002). With no cases of field-evolved resistance to any Bt product at that time, and mainly relying on the experience of high-dose products with European corn borer, the EPA ultimately required adoption of a 20 percent refuge. Well-documented resistance to Cry3Bb1 maize occurred in 2009 (Gassmann et al., 2011), the seventh year of registration of this product, and only the third year during which this type of maize was planted on more than 5 million ha (ca. 16 percent of total maize area) in the United States.

### 10.1.5. The IRM Plan for 709A Cowpea

The following IRM requirements are applicable to cowpea varieties containing event 709A:

- 1) At a village or community level, a minimum of 50 percent of total cowpea area will be planted to non-Bt cowpea to serve as a refuge for Bt cowpea plantings. This requirement does not necessarily impose additional requirements on Bt cowpea farmers as it is expected that significant areas of non-Bt cowpea, particularly cowpea grown for fodder production, will continue to be present. In addition to non-Bt cowpea, an alternative host food crop, such as pigeonpea, may be planted to serve as a refuge.
- 2) The non-Bt cowpea serving as a refuge must be located within 400 m of the field planted with Bt cowpea and may be a shared community refuge (e.g., a neighbouring farm planting non-Bt cowpea) or may be an area of unmanaged native plants that could serve as alternative hosts for *Maruca vitrata* provided the abundance of such plants is comparable to cowpea planting densities.
- 3) Seed mixtures of Bt and non-Bt cowpea are not permitted.
- 4) The refuge and Bt cowpea must be of similar maturity.

Additionally, AATF and CSIR will ensure that there are documented procedures established and implemented to:

- 1) Undertake annual village-level assessments to survey the relative proportions of Bt and non-Bt cowpea planted and the abundance of other alternative host species.
- 2) Monitor for the emergence of *Maruca vitrata* resistant pest populations during each growing season.
- 3) Respond with mitigation and/or remediation procedures in the case of confirmed development of insect resistance.
- 4) Immediately report to the NBA any discoveries of confirmed insect resistance.
- 5) Educate growers on the importance of implementing the appropriate IRM program for products containing 709A cowpea.
- 6) Ensure that all education and training materials developed for products containing 709A cowpea are complete and consistent with all IRM requirements.
- 7) Review and modify the procedures put in place to address items 1–6 (above) in response to any issues identified during compliance monitoring.



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### 10.2. Farmer Communication and Response Plan

Comprehensive farmer education and communication programs are essential for the successful implementation of sound IRM strategies. Farmers should have a clear understanding of the importance of these strategies to preserve the long-term efficacy of the Bt technology and realize that their participation in IRM stewardship is vital to prolonging the success and benefits of Bt cowpea varieties.

This grower communication and response plan will be implemented for all cowpea varieties containing event 709A. AATF believes this plan provides for efficient communication from growers and an effective response to possible problems of unexpected damage from *Maruca vitrata*.

A product use guide will be developed and where possible translated into major local languages and provided to seed companies and agro-dealers, who in turn will give it to each purchaser of Bt cowpea seed with information on the recommendations for the IRM strategy. This information will provide guidance on how to distinguish pod-borer damage from other types of injury and indicate the level of damage considered “unexpected” and necessary to report.

#### 10.2.1. Definition of Unexpected Damage

Unexpected *Maruca vitrata* damage is defined as more than five (5) percent of the Bt-protected plants showing any of the following:

- Damaged flowers and newly formed pods with *Maruca* holes;
- Presence of fecal remains of adults and larvae, frass of larvae and malformed pods;
- Presence of young caterpillars among flowers and foliage;

#### 10.2.2. Response to Unexpected *Maruca vitrata* Damage

As part of a farmer education program, AATF and CSIR will ensure that farmers are encouraged to monitor their Bt cowpea plantings for the presence of *Maruca* pod borer populations and any feeding damage.

The following describes the steps regarding unexpected damage from *M. vitrata* in this process:

- 1) An issue of unexpected insect damage, or any agronomic issue, is reported to the local seed distributor, retailer, or extension officer by a farmer. The issue will be flagged to CSIR and AATF staff for their information and necessary action.
- 2) Field staff from the seed dealer will perform an on-site follow up, normally within two days of the reported issue. The purpose of this field site visit is to verify that *M. vitrata* caused the damage, confirm the extent of the damage and whether the damage is at an unexpected level.
- 3) If the unexpected damage is found on greater than 5 percent of the plants, contact will be made with the CSIR Savanna Agricultural Research Institute (SARI), who will advise and provide assistance in determining if the plants in question are positive for the Cry1Ab trait using the appropriate lateral-flow dipstick assay, according to the manufacturer’s instructions. Plants that are sampled will be marked for subsequent

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- identification in the field. If the damaged plants are positive for the Cry1Ab trait, additional samples will be collected and forwarded to CSIR-SARI.
- 4) Additional information pertinent to the issue (e.g., variety name, growth stage, pesticide applications if any, presence of insect populations, etc) is collected from the farmer and forwarded to AATF and CSIR-SARI.
  - 5) Based on a review of all relevant information, AATF will decide on the necessary course of action, which on a case-by-case basis could include communication with product licensees, or other third-parties as provided for in contractual and/or licensing agreements. In general, the following steps will be followed:
    - a. A site visit will be scheduled as soon as possible to collect surviving *M. vitrata* larvae from damaged plants and the surrounding area. As many larvae as possible (preferably >50 larvae) will be collected from the damaged plants, by removing entire sections of plants and transporting these promptly to CSIR-SARI.
    - b. *M.vitrata* larvae will be reared and pupated at CSIR-SARI according to standard procedures. The *M. vitrata* adults which subsequently emerge will lay eggs and it is the neonate larvae (<24 hours old) that will be used in lab bioassays for resistance assessment.
    - c. The offspring of the field-collected *M. vitrata* population will be tested in laboratory bioassays at CSIR-SARI. A discriminating concentration assay (LC<sub>95</sub>), derived from baseline susceptibility data for Cry1Ab, will be used to confirm resistance. Realistically, acquiring adequate numbers of *M. vitrata* offspring in the lab may take from three weeks to three months.
  - 6) When confirmed resistance has been demonstrated to occur, an effective local mitigation plan approved by the NBA will be immediately implemented.

### 10.3. Event-Specific Detection Method

Unambiguous identification of 709A cowpea can be accomplished using the event-specific PCR method described in section 4.3, page 33, and detailed in Moore and Higgins (2018c). Using the event-specific primer pair (see Table 4, page 34), genomic DNA prepared from the non-transgenic parental line (IT86D-1010) and 18 transgenic events created using plasmid pMB4 was subjected to PCR amplification and analysis (Figure 23, panel B). Only DNA from 709A cowpea yielded the expected 936-bp amplicon, while the non-transgenic control and 18 other sister events were negative. All of the transgenic events tested positive for the presence of the *cry1Ab* gene (Figure 23, panel A, lanes 2–19).

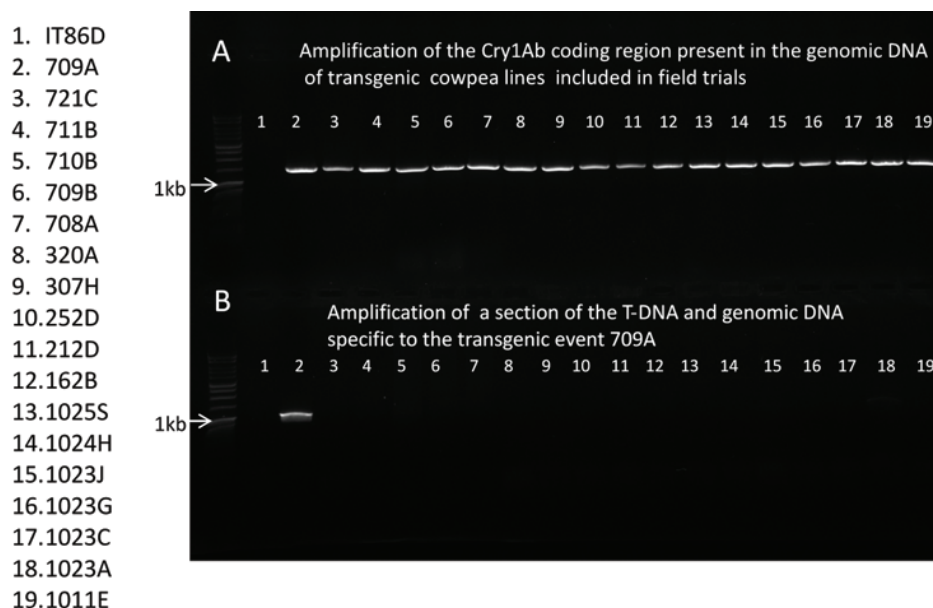
Upon request by the NBA, AATF will provide verified positive and negative control tissue samples, PCR primers, and training as required to facilitate diagnostic identification of 709A cowpea.

## 11. Other Information

### 11.1. Potential Socio-Economic Impacts from the Adoption of 709A Cowpea

An *ex ante* assessment of the potential impacts of releasing and adopting event 709A cowpea varieties in Ghana using an economic surplus partial equilibrium model was

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**Figure 23.** Samples of genomic DNA prepared from the non-transgenic parental control IT86D-1010 (lane 1) and 18 transgenic events created using plasmid pMB4 (lanes 2–19) were subjected to PCR analysis using primer pairs designed to amplify the *cry1Ab* gene present in every transgenic event (panel A) or the LB spanning 936-bp amplicon specific for event 709A (panel B).

recently published by the International Food Policy Research Institute (IFPRI) (Dzanku et al., 2018).

In developing the economic model, consultations with local experts and product performance data were used to derive estimates for anticipated yield change, rates of adoption, and changes in input costs. Considerations around each of these parameters are briefly elaborated below:

- **Yield change:** Based on the results of CFTs, the yield advantage of 709A cowpea was estimated at 13.3 percent under low *Maruca* pressure, and up to 67.6 percent under high pest pressure. Taking into account the probabilities of high and low pest pressure (80 percent and 20 percent, respectively) and the likelihood of lower yield advantages on farmer-managed farms, estimates of 5 percent and 30 percent were used for scenario modelling.
- **Adoption rates:** Based on (limited) data documenting adoption rates for improved cowpea varieties, the expert group estimated that if event 709A were introgressed into only one variety, Songotra (which is cultivated mainly in the Northern Region), adoption would range between 10–20 percent. However, if the event was introgressed into multiple varieties, it was estimated that adoption could be up to 70 percent. Values of 15 percent and 70 percent were chosen to represent the low and high adoption rate scenarios.
- **Cost change:** Partial budget analysis was employed to estimate the incremental cost effects of adopting 709A cowpea, comparing the cost implications to the farmer of adopting the new variety compared with the existing situation. The main factors affecting farmer costs are the cost of seed and expenses related to pesticide applications. Considering a saving of 2–3 pesticide applications, but a seed premium between 0–50 percent, the expert group estimated that the reduction in production

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costs for varieties containing event 709A could range between -4.7 percent (baseline estimate) and -10 percent.

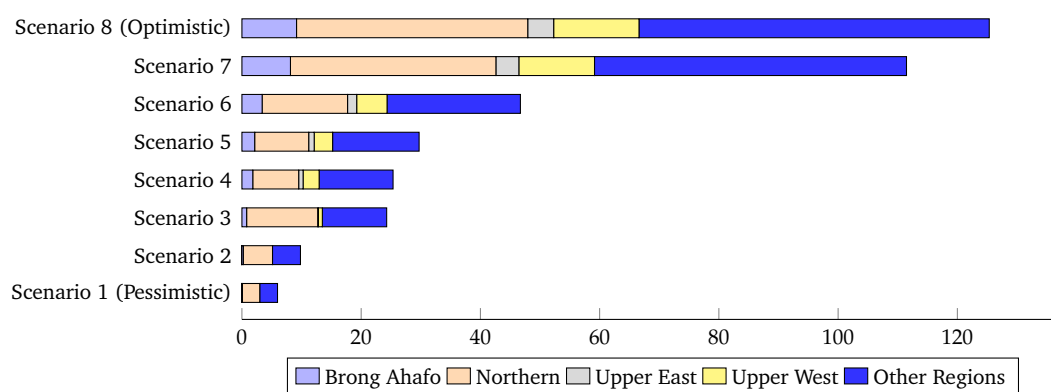
The underlying assumptions and scenario descriptions used by Dzanku et al. (2018) for the *ex ante* Dynamic Research Evaluation for Management (DREAM) simulations are shown in Table 36.

**Table 36.** DREAM scenario descriptions and rankings

| Change in cost of production | Increased Yields <sup>a</sup> |                |                               |                         |
|------------------------------|-------------------------------|----------------|-------------------------------|-------------------------|
|                              | Low: 5%                       |                | High: 30%                     |                         |
|                              | Maximum Adoption <sup>b</sup> |                | Maximum Adoption <sup>b</sup> |                         |
|                              | 15%                           | 70%            | 15%                           | 70%                     |
| -10% Lower insecticide use   | Scenario 2                    | Scenario 6     | Scenario 3                    | Scenario 8 "Optimistic" |
| No increase in seed costs    | -10%, 5%, 15%                 | -10%, 5%, 70%  | -10%, 30% 15%                 | -10%, 30%, 70%          |
| -4.7% Lower insecticide use  | Scenario 1 "Pessimistic"      | Scenario 4     | Scenario 5                    | Scenario 7              |
| Higher seed costs            | -4.7%, 5%, 15%                | -4.7%, 5%, 70% | -4.7%, 30% 15%                | -4.7%, 30%, 70%         |

<sup>a</sup> Source: Dzanku et al. (2018). Scenarios are ranked from 1 (pessimistic) to 8 (optimistic) according to the estimated DREAM net present value (NPV) benefits.

<sup>b</sup> The maximum adoptions rates vary by region: Northern, Upper East, Upper West, Brong Ahafo, and Other.

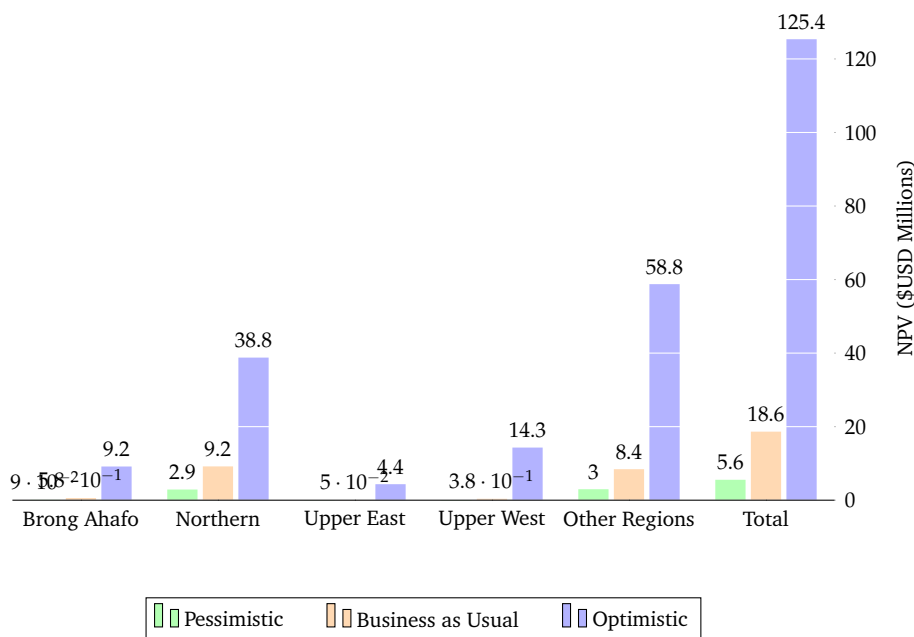


**Figure 24.** Regional net present value benefits for each scenario were estimated using DREAM simulations. All values are in millions of \$USD.

The regionally disaggregated total NPVs for all eight scenarios are illustrated in Figure 24. Over the 30-year simulation period, the total estimated NPV of adopting 709A cowpea varieties in Ghana ranged from \$USD 5.6 million under the “pessimistic” scenario to \$USD 125.4 million under the “optimistic” scenario (Figure 25). When regionally disaggregated, the highest net benefits under six of the eight scenarios accrue to “Other Regions”, which represent regions that are net consumers of cowpea, accounting for *ca.* 71 percent of total consumption. Thus, consumers receive a higher share (*ca.* 57 percent) of the benefits than producers, who are mainly concentrated in the Northern and Upper West regions, which together account for *ca.* 84 percent of cowpea production.

The magnitude of the benefits from 709A cowpea adoption are very sensitive to assumptions around yield advantage, cost change, and rate of adoption. In reality, the

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**Figure 25.** Regional net present value benefits for both optimistic and pessimistic scenarios were estimated using DREAM simulations. In the pessimistic scenario, the NPV estimates for the Upper East and Upper West regions were \$USD -0.12 million and \$USD -0.30 million, respectively. All values are in millions of \$USD.

most likely scenario lies between the two extremes of “pessimistic” and “optimistic”, and Dzanku et al. (2018) also developed a “business as usual” scenario that considered the current situation and the state of product deployment planning. Under this scenario, the most likely adoption rate was estimated as 15 percent, as the *Maruca*-resistant trait from 709A cowpea had only been introgressed into Songotra germplasm without plans for further breeding into other varieties (also supported by the observation that the Songotra variety is preferred in the main cowpea productions areas of the north). The “business as usual” scenario also assumes that the decrease in production costs will be intermediate between 4.7 percent and 10 percent, averaging *ca.* 7.3 percent, and a *ca.* 24 percent increase in yield. Under the “business as usual” scenario, the total NPV was estimated at \$USD 18.6 million (Figure 25).

Dzanku et al. (2018) also presented an analysis of potential gender differentiated welfare effects. Depending on scenario, female shares of potential producer benefits ranged between *ca.* 18–24 percent, while female shares of consumer benefits were *ca.* 43 percent under all scenarios. The lower proportion of female producer benefits reflects the lower female share of production (*ca.* 33.5 percent) combined with gender differences in anticipated rates of adoption (e.g., 12.6 percent vs. 16.8 percent for females and males, respectively, if event 709A was introgressed into only a single cowpea variety). The female share of consumer benefits reflects their lower energy requirement on average (i.e., although females comprise about 51.2 percent of the population, FAO estimate that 1 male equivalent adult is equal to 0.79 female equivalent adults).

The analysis by Dzanku et al. (2018) shows that the estimated potential benefits of *Maruca*-resistant 709A cowpea varieties to producers and consumers in Ghana can be substantial.

## 12. Conclusions

The purpose of this evaluation of Lepidopteran insect-protected 709A cowpea was to determine whether the environmental release or use of 709A cowpea in food or feed could raise any new safety concerns relative to conventional cowpea.

The assessment of 709A cowpea has included a complete description of the genetic modification (e.g., gene sources, characterization of inserted DNA and site of integration within the host genome, stability, and inheritance), the safety of the newly expressed proteins (e.g., history of use in food, function, potential toxicity, potential allergenicity, and patterns and levels of expression), a nutrient compositional assessment, and phenotypic characterization to identify whether there were any unintended, unexpected, effects of the genetic modification.

Collectively, the data presented in this submission have not identified potential environmental hazards or health and safety concerns. Thus, cowpea varieties containing event 709A will not pose an altered risk to the environment relative to conventional cowpea, and food and feed derived from 709A cowpea are as safe as food and feed derived from conventional cowpea varieties.

The deployment of new *Maruca* pod borer resistant cowpea varieties containing event 709A will follow responsible product stewardship principles. Significant components of the stewardship plan include farmer education and outreach, implementing a quality management system to ensure seed quality and trait purity, following IRM best-practices, and having procedures in place to respond quickly to farmer complaints or reports of unexpected damage, including the implementation of appropriate risk mitigation measures if necessary.

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