



# Bioinformatics analysis to assess potential risks of allergenicity and toxicity of HRAP and PFLP proteins in genetically modified bananas resistant to Xanthomonas wilt disease



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## ABSTRACT

Banana Xanthomonas wilt (BXW) disease threatens banana production and food security throughout East Africa. Natural resistance is lacking among common cultivars. Genetically modified (GM) bananas resistant to BXW disease were developed by inserting the *hypersensitive response-assisting protein* (*Hrap*) or/and the *plant ferredoxin-like protein* (*Pflp*) gene(s) from sweet pepper (*Capsicum annuum*). Several of these GM banana events showed 100% resistance to BXW disease under field conditions in Uganda. The current study evaluated the potential allergenicity and toxicity of the expressed proteins HRAP and PFLP based on evaluation of published information on the history of safe use of the natural source of the proteins as well as established bioinformatics sequence comparison methods to known allergens ([www.AllergenOnline.org](http://www.AllergenOnline.org) and NCBI Protein) and toxins (NCBI Protein). The results did not identify potential risks of allergy and toxicity to either HRAP or PFLP proteins expressed in the GM bananas that might suggest potential health risks to humans. We recognize that additional tests including stability of these proteins in pepsin assay, nutrient analysis and possibly an acute rodent toxicity assay may be required by national regulatory authorities.

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## 1. Introduction

Banana (*Musa* spp.) is an important global food commodity providing about 25% of food energy requirements for more than 100 million people. It is grown in more than 140 countries in the tropics and subtropics with an annual world production of around 145 million tons (FAOSTAT, 2014). Approximately one third of global *Musa* spp. production is from Africa, with more than 40% grown in East Africa (FAOSTAT, 2014). The East African Great Lakes region including Burundi, Rwanda, Democratic Republic of Congo, Uganda, Kenya and Tanzania is the largest banana-producing and -consuming region in Africa. Uganda is the highest producer with annual production of approximately 10.5 million tons. The average daily per capita consumption in Uganda ranges from 0.61 to over

1.6 kg. Food security studies revealed that in Uganda, Rwanda, and Burundi, bananas constitute more than 30% of the daily per capita caloric intake, rising to 60% in some regions (Abele et al., 2007). In East Africa, bananas are produced mostly by smallholder subsistence farmers for local consumption.

The yield of banana has been decreasing in recent years due to parasitic nematodes, bacterial and fungal infections plaguing banana farmers in the East African region. The banana Xanthomonas wilt (BXW) disease caused by *Xanthomonas campestris* pv. *musacearum*, is one of the most important diseases of banana, and is considered the biggest threat to banana production in the Great Lakes region of East Africa (Tripathi et al., 2009). All cultivars of banana grown in this region, including East African Highland banana subgroup (AAA-EA genome) used to make 'matooke' (steamed and mashed banana); brewing cultivar 'Pisang Awak' (ABB genome); the dessert cultivars 'Sukali Ndiizi' (AAB genome) and the Cavendish subgroup (AAA genome); and plantains ('Gonja', AAB genome) are susceptible to BXW disease, which causes premature ripening of the fruit and wilting of the plant (Tripathi et al., 2009). The impact of BXW is both extreme and rapid, unlike those of other diseases, which cause gradually increasing losses over years.

Abbreviations: aa, amino acid; AOL v17, allergenonline database version 2017; BXW, banana Xanthomonas wilt; E values, expectation values; GM, genetically modified; HRAP, hypersensitive response-assisting protein; NCBI, National Center for Biotechnology Information; PFLP, plant ferredoxin-like protein.

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Economic loss due to BXW was estimated to be about \$2–8 billion over the last decade and significant reduction in production has resulted in major price increases (Nkuba et al., 2015; Tripathi et al., 2009).

The disease can be managed by strict adherence to cultural practices including cutting and burying infected plants, restricting the movement of banana planting materials from BXW affecting to disease free areas, removal of male buds and the use of sterilized tools. However, the adoption of such practices has been inconsistent as they are labour intensive. The use of resistant cultivars is a cost-effective method of managing bacterial diseases. As yet there are no banana cultivar known to be completely resistant to BXW. Even if resistant germplasm sources are identified, conventional breeding of banana is a difficult and lengthy process due to sterility of most cultivars and long generation times. As an alternative, researchers at the International Institute of Tropical Agriculture (IITA) and the Uganda-based National Agricultural Research Organization (NARO), worked to develop the genetically modified (GM) banana resistant to BXW by inserting single or stacked genes encoding the hypersensitive response-assisting protein (HRAP) and plant ferredoxin-like protein (PFLP) proteins into different banana cultivars (Namukwaya et al., 2012; Tripathi et al., 2010, 2014). The *Hrap* and *Pflp* genes were isolated from sweet pepper (*Capsicum annuum*) and were previously shown to have anti-microbial activity against certain plant pathogenic bacteria (Chen et al., 2000; Dayakar et al., 2003). Field testing of several of these GM banana events showed 100% resistance to BXW through successive crop cycles (Tripathi et al., 2014). The GM banana plants performed exactly like the disease-free non-modified bananas. Aside from full resistance to BXW, these GM bananas showed yield characteristic comparable to disease-free non-modified varieties, indicating there were no observable unintended effects of the transgenes on crop performance. However, before the GM bananas would be allowed into production for foods, a safety evaluation is needed to ensure that there is no additional risk in consuming the GM bananas. A number of African countries are now implementing safety guidelines and laws to regulate GMOs and so far they are following the Codex Alimentarius Commission guidelines (CODEX, 2003, 2009). Thus the Codex guidelines were followed in this study of pre-market evaluation for food safety.

The primary concerns of food safety of recombinant proteins expressed in GM crops are whether the introduced gene(s) encodes an allergen or potentially cross-reactive protein, or a toxin, and whether the insertion of the gene(s) alters the overall expression level of endogenous allergens of the host organism (CODEX, 2003). Therefore, the key aspects of the safety assessment of the GM crops are evaluation of allergenicity and toxicity of the recombinant protein(s), and altered nutritional properties of food-materials from the GM crops. Yet, there is no single criterion that could predict the potential allergenicity or toxicity of a transgenic protein. The Codex recommended a weight-of-evidence evaluation approach to decide whether the novel protein introduces potential risk or not. Since many allergens and toxins have been identified nowadays, a combination of a literature review and bioinformatics sequence comparison has become a primary tool for evaluating the potential risk of allergenicity or toxicity (Ladics et al., 2011). The review of published information should carefully seek information regarding the history of human exposure to the protein encoded by the gene as well as the source of the gene regarding the evidence of allergy or toxicity. Then the amino acid (aa) sequence of the introduced protein is compared to sequences of known or putative allergens using the best available curated database (Goodman, 2006; Goodman et al., 2016) and if appropriate, the general National Center for Biotechnology Information (NCBI) Protein database using keyword search limit. If a significant identity match of

the protein to a known allergen is found, additional tests such as IgE binding tests may be necessary (Goodman, 2008). If the GM plant species is a common source of food allergy (e.g., milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, soybean), the guideline also recommends evaluation for potential changes in the overall food allergenicity, which is measured by specific serum IgE tests of the food materials from the modified plant (Goodman et al., 2008).

Potential cross-reactivity between a novel protein and a known allergen is hard to predict. Therefore, there are multiple criteria of sequence comparison to known allergens. The CODEX (2003) is specific in setting a conservative limit for allergenicity with matches having > 35% identity over any segment of 80 or more aa requiring specific allergic human serum IgE testing if appropriately allergic subjects can be identified. The 80 aa search was included by CODEX to identify short motifs that might contain sequential or conformational IgE epitopes that might be naturally transferred by genetic recombination by nature or human technology. Meanwhile, Aalberse (2000) suggested that proteins having < 50% identity in aa sequence over full-length with an allergen are unlikely to be cross-reactive. Similarly, Goodman et al. (2008) suggest that full FASTA sequence alignment with at least 50% identity to a known allergen is the most predictive comparison for risk evaluation of potential cross-reactivity. The rationale is that genetic changes might transfer a structural motif, which could include conformational IgE-binding epitopes. Conformational epitopes are capable of cross-linking IgE on receptors of sensitized mast cells or basophils which could induce allergic mediator release (Gieras et al., 2011). Besides, some regulatory authorities require a search comparison for 8 aa segment identity matches to allergens to identify possible cross-reactive targets (Ladics, 2008). The rationale is based on an assumption that individual epitopes may be represented by peptide segments as short as 8 aa.

There are no specific cutoff scores for toxicity comparisons, therefore, it requires a relative identity comparison to evaluate potential risk and safety. There are also no broadly applicable public toxin databases. The toxicity evaluation is normally performed using the NCBI protein database with keyword limit of toxin. Significantly matched “toxin”, if there is any, will be searched against NCBI database without keyword limit for evaluating whether there are other proteins from commonly consumed sources with higher identity matches than the introduced protein. Searches were also performed for each protein sequence without any keyword limit to investigate whether homologous or similar proteins exist in other organisms that might provide information of risk in allergenicity or toxicity. Additional review of publications reporting studies of matched allergens and toxins are often required to interpret potential risks.

This study evaluated the potential allergenicity and toxicity of HRAP and PFLP proteins based on published literature search on the source of the genes and bioinformatics analysis (sequence comparisons) of the two proteins with known allergens and toxins. This study will guide decisions regarding whether additional safety tests are needed for evaluating these proteins as potential sources of allergy or toxicity in GM bananas for human consumption.

## 2. Methods

### 2.1. Scientific literature search

The PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed>) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy and toxicity. The key questions are whether banana is a commonly allergenic food, and whether the source of the genes sweet pepper (*Capsicum*

*annuum*) are linked to allergy or toxicity as well as the proteins. The data (authors, publication, date, PubMed identity number, and abstracts) from searches were saved to an archive file for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles.

Searches using individual term “HRAP” and “PFLP” were performed on May 10, 2017 to seek publications related to the history of safe use of these two proteins in foods. Searches using combinations of the source term either “*Capsicum*” or “*Capsicum annuum*” and term either “allergen” or “allergy” were performed on May 10, 2017 to identify publications related to risks of allergy caused by sweet pepper. Similar, searches using the combinations of the source term either “*Capsicum*” or “*Capsicum annuum*” and term either “toxin” or “toxicity” were performed on May 12, 2017 to evaluate possible risks of toxicity. Searches using the combinations of the term “banana” and term of either “allergen” or “allergy” were performed on May 15, 2017 to seek banana endogenous allergens and the prevalence of allergy to banana in order to decide if banana is a common source of food allergy. In addition, World Health Organization and International Union of Immunological Societies Allergen Nomenclature Subcommittee database ([www.allergen.org](http://www.allergen.org)) and AllergenOnline (AOL) database ([www.AllergenOnline.org](http://www.AllergenOnline.org)) were used to search for the endogenous allergens and allergens known from sweet peppers.

## 2.2. Bioinformatics search

### 2.2.1. AA sequences of query proteins

The HRAP (269 aa) and PFLP (144 aa) sequences encoded by the genes introduced in banana were described in [Chen et al. \(2000\)](#) and [Dayakar et al. \(2003\)](#). These sequences have been confirmed as expressed in the GM bananas. The aa sequences are presented in [Table 1](#).

### 2.2.2. AllergenOnline version 2017 (AOL v17)

The bioinformatics comparisons of HRAP and PFLP to allergens were performed using a well-documented allergen database (AOL, <http://www.allergenonline.org/>) on May 16, 2017. The AOL database is an annually updated, peer-reviewed allergen list and sequence database maintained by the Food Allergy Research and Resource Program of the University of Nebraska ([Goodman et al., 2016](#)). The current version was updated in January 18, 2017 and contains sequences of 2035 known (binding IgE and positive measurement of biological activity) or putative (binding IgE, but no measure of biological activity) allergens from food, airway, contact and venom sources. The annual update includes an evaluation of publications describing allergic human subjects, characterization of proteins, test methods and results with a review by a panel of allergy and allergen experts. Three comparison methods were used: an overall FASTA3 full-length search using expectation values (*E*

values) of 10 and 1.0, a sliding 80 aa window FASTA search with *E* value of 10 (identity > 35%), and a search of 8 aa exact matches. These criteria and methods have been tested and found to identify proteins with low sequence identity matches, but no evidence of cross-reactivity ([Goodman, 2006](#); [Silvanovich et al., 2009](#); [Cressman and Ladics, 2009](#)).

### 2.2.3. Bioinformatics search algorithms in AOL v17

The full-length FASTA3 was the primary search which gives optimum alignments using the default criteria defined by [Pearson \(1999\)](#). The default scoring matrix is BLOSUM 50 ([Henikoff and Henikoff, 1992, 1996](#)). The penalty for each gap inserted into query or searched sequences to obtain optimal alignments is calculated as  $(q + r \cdot k)$ , where  $q$  (10) is an initial penalty for each independent gap,  $r$  (2) is a penalty for each aa position within the gap and  $k$  is the number of aa positions within the gap ([Reese and Pearson, 2002](#)). The default word size (k<sub>cut</sub>) is 2 ([Pearson, 1999](#)). The FASTA3 used in these searches was version 35.04 updated in Jan. 15, 2009 (<ftp://ftp.virginia.edu/pub/fasta/>). Statistical values were calculated for each search and compared to expected values, as illustrated in the histogram of the computer output. Alignment of regions containing low sequence complexity may lead to irrelevant alignments and are expected to show skewed distributions and should be reanalyzed after removing the low complexity regions ([Pearson, 1999](#)). Very small *E* values indicate probable evolutionary homology, and structural similarity. While the *E* value default for FASTA3 was set to 1, a value that does not indicate significant similarity, distantly related sequences generally have *E* values less than 0.01, and highly similar sequences that probably represent close homology are more likely to have *E* values less than  $1e-07$  ([Siruguri et al., 2015](#)).

Based on the recommendation of Indian Council of Medical Research ([ICMR, 2008](#)) and [CODEX \(2003\)](#) all possible contiguous 80 aa segments of each of the two proteins, HRAP and PFLP were compared against all sequences in AOL v17 ([Siruguri et al., 2015](#)). Searches began with aa 1–80, then 2–81, 3–82 and so on until the last 80 aa segment of each protein was compared, using the same FASTA3 algorithm used for the overall-comparison. In this case, only the *E* values and percent identities [(# identical residues/80 or more aa) \* (100%)] were evaluated to consider potential cross-reactivity. Alignments of less than 80 aa in length were recalculated to normalize the identity to an 80 aa score, by increasing the denominator to 80, without altering the numerator. Therefore, an alignment with 38 identical aa over a length of 40 (= 95%), would be recalculated to 47.5%. The adjustment was based on the rationale that alignments less than 80 aa long may have very high identities, and would therefore be more likely to act as a cross-reactive allergen if the matched region represented an IgE epitope, than longer alignments of markedly lower identity scores.

Eight aa identity matches was also performed by searching for any 8 contiguous aa exact matches in AOL v17.

**Table 1**  
Amino acid sequences of the recombinant proteins HRAP and PFLP.

Protein	Accession #	# aa	Description	aa sequence
HRAP	NP_001312018.1	269	hypersensitive response-assisting protein from sweet pepper ( <i>Capsicum annuum</i> )	<u>MKMKNL</u> SLLLLLLTLTLLFISVCSQVQQTQFVYRWPETYCEKATPTVACTKIPLQFTLVGFWGTDSSGGIQGCGQDTGKYDWA KVFTTETANKLTAFWPSLSTQDPTMWWKAAWTTYGTCLISKFKTPTQYFNRAIRLSAIGGGLFQGGQLIGKDGIVPCDSAT YTNAEILKSLTAVTTVNNQKEKKVSFTCSNYSIETHAYLNQVTCYTNNAQYYTDCPTTVISKRCNVPNIIVPRPPTPTARASSL QDLLTGEKIGPNVLWETLGLQLF
PFLP	Q9ZTS2.1	144	plant ferredoxin-like protein from sweet pepper ( <i>Capsicum annuum</i> )	MASVSATMISTSFMPKRPKPAVTSKPIPNVGEALFGLKSANGGKVTMCASYKVKLITPDGPIEFDCPDNVYILDQAEAEAGHDL PYSRAGSCSSCAGKIAGAVDQTDGNFLDDDDQLEEGVWLTCVAYPQSDVVTIETHKEAELVG

Underlined sequence is the predicted signal peptide based on [www.cbs.dtu.dk/cgi-bin](http://www.cbs.dtu.dk/cgi-bin) (SignalP 4.1).

### 2.2.4. BLASTP in NCBI Entrez protein database

The non-redundant general protein database (<http://www.ncbi.nlm.nih.gov/BLAST/>), maintained by the National Library of Medicine, National Institute of Health was also used for sequence comparisons of HRAP and PFLP using keyword “allergen”. The NCBI database was used as supplemental verification for matches to potential allergens that may have been recently published after the last AOL update v17. It was also used with keyword “toxin” to find high scoring identity matches to proteins that indicate a potential risk of toxicity. In addition, BLAST searches were conducted with HRAP and PFLP sequences, but without and keyword limit to show the closest related proteins in order to evaluate the relevance of any matches. The purpose was to find the highest identity matches to other proteins and sources that might have a history of safe use, or a history of allergy or toxicity. The criteria used for BLASTP were default parameters: BLOSUM62 scoring matrix, Word size 6, Expect value 10, hitlist 100, Gapcosts 11,1, window size 40, threshold 21. The NCBI database is updated every few days and therefore the sequence searches were recorded based the performing date May 22, 2017. However, matches would need to be verified by locating published evidence that the NCBI entry has valid data demonstrating allergenicity.

## 3. Results and discussion

### 3.1. Scientific literature search results

#### 3.1.1. Literature search results for Musa/banana

A total of 140 and 215 references were identified in the PubMed searches using keywords of “banana AND allergen” and “banana AND allergy” respectively (Table 2). Each abstract was carefully reviewed to exclude the irrelevant references, and then the ones actually related to banana allergen or allergy was further investigated using the full texts. The most recent publication was the study performed by Inam et al. (2016) investigating the prevalence of sensitization to food allergens in 689 allergic patients from two major cities in Pakistan. Their data shows that only 7.1% of these patients were skin prick test positive for banana while the numbers for wheat, egg, milk and soybean were 22.6%, 21.48%, 20.03% and 12% respectively. Another relevant paper investigated food tolerance on 33 children with food allergy in Turkey for 5 years (Doğruel et al., 2016). Their study showed that only 1 child was allergic to banana and developed complete tolerance, while 20 and 17 were allergic to cow's milk and eggs. Popescu (2015) cited ragweed-melon-banana syndromes as one of the examples of cross-reactivity between aeroallergen and food allergen from plant sources. More specifically, Mus xp 1, a profilin in banana and Mus a

3, a lipid transfer protein in *Musa acuminata* may be the causes for the cross-reactivity between banana and other food sources, pollen or latex. Palacin et al. (2011) showed that among children with allergic symptoms after banana ingestion, although over 70% showed IgE positive to Mus a 5, a glycosylated protein, and Mus a 4, a thaumatin-like protein and not a glycosylated protein, only 1 out of 12 allergic children was skin prick test positive to Mus a 5 while 6 were positive to Mus a 4. Another study showed that children with IgE to Phl p 12, grass profiling, may be provoked with oral allergic syndrome by many fruits including banana (Asero et al., 2015). Overall, the search results indicate that banana is a very rare cause of food allergy or cross-reactivity, thus should not be considered as commonly allergenic food.

#### 3.1.2. Literature search results for *Capsicum annuum* source of the *Hrap* and *Pflp* genes

A total of 24 and 62 references were identified using keywords of “*Capsicum*” combined with “allergen” and “allergy” respectively (Table 2). Adding the species name “*annuum*” after genus “*Capsicum*” did not make a difference.

The most recent case related to “*Capsicum* allergen” was the identification of two new occupational respiratory allergens, defensin J1 from paprika and vicilin from cayenne by IgE binding (Airaksinen et al., 2015). But this patient only developed work-related rhinoconjunctivitis, but not food allergy. Some publications reported only aa sequence comparisons of a suspected allergenic protein to protein sequences in the general public database (NCBI), and implied potential cross-reactivity without any further investigation (Lee et al., 2006). True IgE mediated allergic reactions to peppers are likely due to some common cross-reactive proteins including profilins that are common in pollen (Willeroider et al., 2003), and possibly two pathogenesis related proteins, Bet v 1 and the osmotin-like protein P23 (Jensen-Jarolim et al., 1998). Those studies demonstrated *in vitro* IgE binding to purified proteins, but the biological significance of binding is rarely tested. Another paper reported a case study of a woman with three severe anaphylactic reactions to raw green bell pepper (García-Menaya et al., 2014), but no protein was definitively identified. There were a few reports of severe allergic reactions following the consumption of raw bell pepper (Rüger et al., 2010). There were a few other reports of different potential allergenic proteins from pepper plants including to a beta-1,3-glucanase as well as profilin (Wagner et al., 2004); osmotin-like proteins (Sharma et al., 2011) and other IgE binding proteins that have not been identified (van der Walt et al., 2010; van der Walt et al., 2013).

Switching the second term to “allergy” increased the number of references (Table 2). Also studies on anti-inflammatory or allergic properties of some non-protein compounds present in peppers (Jang et al., 2011). Some reports are of individuals who have allergic reactions to pepper due to occupational exposure, but that is also confused by irritation with capsaicin molecule, the organic compound responsible for spicy sensation and burning due to vasoactive properties (Dutta and Deshpande, 2010). The basic structure of the capsaicin molecules is an 8-methyl-*N*-vanillyl-6-nonenamide, and it is a volatile, hydrophobic compound and sometimes considered toxic compound (Huynh and Teel, 2005). Although these active compounds might act as haptens, binding to endogenous human proteins as fixed targets of IgE binding, these are not related to the introduced proteins. Determining the reactive molecules in spices is complex as sensitivities seem to span across broad taxonomic groups that would not normally be expected to be involved in cross-reactivity (Sastre et al., 1996). The search of “*Capsicum annuum* allergy” also led to the study by Tripathi et al. (2010) regarding the use of the *Hrap* gene in GM crops. However, Tripathi et al. (2010) clearly did not describe any tests for

**Table 2**  
PubMed literature search results.

Search term	Search result
Banana AND allergen	140
Banana AND allergy	215
<i>Capsicum</i> AND allergen	24
<i>Capsicum</i> AND allergy	62
<i>Capsicum annuum</i> AND allergen	24
<i>Capsicum annuum</i> AND allergy	62
<i>Capsicum</i> AND toxin	117
<i>Capsicum</i> AND toxicity	116
<i>Capsicum annuum</i> AND toxin	117
<i>Capsicum annuum</i> AND toxicity	116
Plant ferredoxin-like protein	36
PFLP	50
Hypersensitive response-assisting protein	5
HRAP	69

allergenicity or allergy. The reason this paper was identified in the search was due to the automated extrapolation of MeSH (Medical Subject Heading) terms that translate “hypersensitive” response element, or protein as causing allergy (hypersensitivity).

The conclusion of the literature search in terms of allergenicity is that peppers produce a few small molecule irritants as well as a few common and cross-reactive allergenic proteins (e.g., profilin, Bet v 1 homologues, possibly osmotin like-proteins and glucanase), but food allergic reactions to peppers are fairly rare (Callero et al., 2012; Jensen-Jarolim et al., 1998; Sharma et al., 2011) and no studies have implicated HRAP or PFLP.

In total 117 and 116 references were found with “*Capsicum* (annuum) AND toxin” and “*Capsicum* (annuum) AND toxicity” respectively (Table 2). However, none identified a protein produced by *Capsicum annuum* that is toxic to mammals. Most of the publications relevant to toxin reported studies of mycotoxins or the impact of pepper on plant pathogens. Most of the reports of toxicity appear to be due to contamination of *Capsicum* sp. by toxins from fungal infection and not because of inherent toxins of *Capsicum* sp. For example, Yang et al. (2011) evaluated mycotoxins in greenhouse grown peppers. Early reports of toxicity associated with processed peppers identified aflatoxins associated with the use of processed peppers in food products (Guegue and Ramirez, 1977). Searching with “toxicity” for the second term again expanded the number of publications. The final report of Generally Recognized as Safe for extracts of *Capsicum annuum* used for cosmetic purposes and in foods was published (GRAS, 2007) and a 13-week subchronic rat toxicity study feeding paprika oleoresin extracted from pepper fruits to F344 rats at up to 5% of the diet did not show any toxic effects (Kanki et al., 2003). Other studies of extracts did not reveal specific toxicity of proteins from *Capsicum annuum*. An interesting publication by Petersen (2011) reviewed toxicity reports for the U.S. and the state of Ohio and showed that almost all reports are due to oxalates or other non-protein-molecules that cause malabsorption or other toxic responses. *Capsicum* sp. ranked number six on the list of common plants causing some kind of toxic response. A review of all identified articles failed to uncover any new studies demonstrating possible toxicity of pepper proteins. One of the publications of interest, Chen et al. (2003) reported results from animal studies conducted using rats and mice fed with GM sweet pepper and conventional sweet pepper. They reported no adverse effects from ingestion of sweet pepper.

### 3.1.3. Literature search results for HRAP and PFLP

Searching the terms “plant ferredoxin-like protein” and “PFLP” yielded 36 publications and 50 publications respectively, with none related to toxin or toxicity. Similarly, searching using “hypersensitive response-assisting protein” and “HRAP” yield 5 and 69 reports without any documentation on toxin or toxicity.

## 3.2. Sequence comparison results of HRAP and PFLP to allergens in AOL v17 database

### 3.2.1. Full FASTA search of HRAP and PFLP against allergens in AOL v17

Results of the full-length FASTA3 searches of the HRAP against AOL v17 did not identify any significant alignment with an allergen for either protein (Table 3). Scoring results for the HRAP showed only one alignment with an *E* value less than 1. The “full-length” alignment of 24.2% identity is with Japanese cedar class IV chitinase. The identity score is markedly below the 50% identity that is likely to indicate cross-reactivity (Aalberse, 2000). This alignment required insertion of 6 gaps of 1–5 aa over 162 aa, which means the overall structures are unlikely to be similar. The longest contiguous identity match was only two aa long. Scoring results for the PFLP

showed only one alignment with an *E* value less than 1 (Table 3). The first alignment was with 30% identity over a 60 aa segment of *Phaseolus vulgaris* (kidney bean) non-specific lipid transfer protein. The identity score is also below the level that indicates likely cross-reactivity. This alignment required insertion of one gap of 3 aa over the 60 aa alignment, meaning the overall structures are unlikely to be highly similar. The longest contiguous identity match was only two aa long, which would not trigger serum IgE binding tests. In total 4 and 21 alignments were found for searches using HRAP and PFLP sequences with *E* value 1–10 (Table 3), and no identity was as significant as above 50%.

### 3.2.2. Sliding 80 mer window search of HRAP and PFLP against allergens in AOL v17

The comparison of either protein sequence did not identify any possible match of >35% identity with any known or putative allergen in the database (Fig. 1). Thus the risk of cross-reactions for allergic individuals is very low, and the data indicate there is no reason to perform serum IgE testing as there is not a target allergen to suspect cross-reactivity.

### 3.2.3. Exact 8 mer search of HRAP and PFLP proteins against allergens in AOL v17

The results of both searches were negative, not a single 8 aa identity was found (results not shown).

In sum, the full-FASTA comparisons, sliding window of 80 mer comparisons and the searches for identity matches of 8 contiguous aa of HRAP and PFLP were all negative.

## 3.3. BLASTP results of HRAP and PFLP in NCBI Entrez database

### 3.3.1. BLASTP with keyword limit “allergen”

The search result with keyword limit “allergen” yielded only one aligned protein match to HRAP (Table 4) with *E* value values below 10, suggesting very minimal evolutionary homology. It was Barwin-like endoglucanase with length of 256 aa from plant *Cynara cardunculus* var. *scolymus* with 33% identity over 55 aligned aa. The low identity does not raise concerns of potential cross-reactivity (Aalberse, 2000; Goodman, 2006; Goodman et al., 2008). The BLASTP search with the aa sequence of PFLP showed that no significant similarity was found using “allergen” as the query limit.

### 3.3.2. BLASTP with keyword limit “toxin”

No significant similarity was found with HRAP sequence using keyword “toxin” (results not shown). A few matches were found with PFLP sequence with keyword “toxin” (Table 5) but no sequence was demonstrated to have a significant match by further investigating high identity matches when no keyword was used. The best three scoring alignments of PFLP were to bacterial proteins from *Bordetella bronchiseptica* and *Bordetella pertussis* that produce bacterial toxins, CDP-6-deoxy-delta-3,4-glucose reductase and phenylacetic acid degradation NADH oxidoreductase. The *E* values were 3e-11 and 5e-10, which was significant, but the percent identity were only 42% over 77 aa segment of a 348 aa long protein, and only 46% over 65 aa segment of a 362 aa long protein, respectively. Ferredoxin-like proteins from many other bacteria that express toxins were found with slightly less significant matches. However, the comparisons to alignments with ferredoxins from commonly consumed plant sources were much higher, indicating that the alignments to the bacterial proteins are not indicative of toxicity. The conclusion from this section is that there is no need for specific additional toxicity testing for the HRAP or PFLP proteins.

**Table 3**  
Summary of sequence alignments for HRAP and PFLP identified in AOL v17.

Quary Protein	Sequence GI # [Acc#]	Organism	Description	aa Length	E value	Identity (%)	aa Alignment length
HRAP	56550550 [BAD77932.1]	<i>Cryptomeria japonica</i> (Japanese cedar)	Class IV chitinase	281	0.91	24.2	161
	60418848 [AAX19851.1]	<i>Malus domestica</i> (apple)	Mal d 2, thaumatin-like protein precursor	246	1.8	29.2	96
	60418842 [AAX19848.1]	<i>Malus domestica</i> (apple)	Mal d 2, thaumatin-like protein precursor	246	2.4	29.2	96
	30316292 [Q9FSG7.1]	<i>Malus domestica</i> (apple)	Mal d 2, thaumatin-like protein precursor	246	2.7	29.2	96
	1313966 [CAA96534.1]	<i>Malus domestica</i> (apple)	Mal d 1, major allergen	159	5.7	27.0	68
	PFLP	289064179 [ADC80503.1]	<i>Phaseolus vulgaris</i> (kidney beans)	Pha v 3, non-specific lipid transfer protein 1b precursor	118	0.43	30.0
1398915 [BAA07711.1]		<i>Oryza sativa, Japonica</i> group (Japanese rice)	Oryza Trypsin alpha-amylase inhibitor	160	2.1	42.9	42
114152865 [Q01882.2]		<i>Oryza sativa, Japonica</i> group (Japanese rice)	Oryza Trypsin alpha-amylase inhibitor	166	2.2	42.9	42
9929163 [CAC05258.1]		<i>Cupressus arizonica</i> (Arizona cypress)	Cup s 3, a major allergen from <i>Cupressus arizonica</i> pollen	199	2.4	35.5	31
38456226 [AAR21073.1]		<i>Cupressus sempervirens</i> (Mediterranean cypress)	PR5 allergen Cup s 3.1 precursor	225	2.5	35.5	31
218193 [BAA01998.1]		<i>Oryza sativa, Japonica</i> group (Japanese rice)	Oryza Trypsin alpha-amylase inhibitor	165	3.5	39.0	41
571256597 [CCK33472.1]		<i>Cannabis sativa</i> (hemp)	Can s 3, lipid transfer protein precursor	91	4.3	26.6	64
38456224 [AAR21072.1]		<i>Juniperus rigida</i> (cedar)	PR5 allergen Jun r 3.2 precursor	225	5.6	32.3	31
38456228 [AAR21074.1]		<i>Cupressus sempervirens</i> (Mediterranean cypress)	PR5 allergen Cup s 3.2 precursor	225	5.6	32.3	31
38456222 [AAR21071.1]		<i>Juniperus rigida</i> (cedar)	PR5 allergen Jun r 3.1 precursor	225	5.6	32.3	31
50659889 [AAT80664.1]		<i>Malus domestica</i> (apple)	allergen Mal d 3 lipid transfer protein precursor	115	5.7	21.7	60
50199132 [CAH03799.1]		<i>Citrus sinensis</i> (sweet orange)	Allergen Cit l 3 lipid transfer protein	91	6	26.5	68
14531020 [AAK63089.1]		<i>Theragra chalcogrammu</i> (Alaska pollock)	parvalbumin	109	6.5	33.3	33
51316532 [Q9LD79.2]		<i>Juniperus virginiana</i> (Red cedar)	Putative major pollen allergen Jun v 3	110	6.5	32.3	31
209979542 [ACJ04729.1]		<i>Gallus gallus</i> (chicken)	Gal d 1, ovomucoid	210	7.5	34.0	47
124757 [P01005.1]		<i>Gallus gallus</i> (chicken)	Gal d 1, ovomucoid	210	7.5	34.0	47
9087177 [P81295.1]		<i>Juniperus ashei</i> (Ozark white cedar)	Pollen allergen Jun a 3	225	7.7	32.3	31
559797763 [AHB19225.1]		<i>Punica granatum</i> (pomegranate)	Pun g 1, non-specific lipid transfer protein	120	8	30.2	43
37778438 [AAO73305.1]		<i>Eriocheir sinensis</i> (Chinese mitten crab)	Eri s 2 ovary development-related protein	252	8.2	28.9	97
112745 [P23110.1]		<i>Helianthus annuus</i> (sunflower)	Methionine-rich 2S protein	141	8.6	20.8	53
23616947 [BAC20650.1]		<i>Oryza sativa, Japonica</i> group (Japanese rice)	Oryza Trypsin alpha-amylase inhibitor	160	9.1	39.0	41
338930686 [CBM42667.1]		<i>Paspalum notatum</i> (Bahia grass)	group 13 grass pollen allergen	169	9.4	26.0	104

### 3.3.3. BLASTP without keyword limit

The HRAP aa sequence from *Capsicum annuum* aligned with a number of proteins (results not shown), but the highest two scoring matches (except matching to itself) were 75% and 68% identity to a 263 aa protein (ribonuclease MC-like) and a 260 aa protein (ribonuclease S-4-like), both from *Capsicum annuum*. The first match was over a 273 aa alignment and the second was over a 261 aa alignment, both including gaps. The third highest match was also a ribonuclease MC-like protein from tomato *Lycopersicon pennellii*. A PubMed search of “*Solanum lycopersicum*” and “allergy” on May 22, 2017 yielded 112 publications. Demonstrated IgE allergy to tomato is not a common occurrence (Asero, 2013; Asero et al., 2008, 2010). Other matches dropped in significance of alignments quickly. There is no proof of allergy or toxicity to the ribonucleases identified in any of the publications. There is also a reasonable likelihood of safe exposure as ribonucleases are common enzymes in eukaryotic cells.

The PFLP aa sequence from *Capsicum annuum* aligned with many other plant ferredoxin proteins (results not shown). Out of 100 alignments, 36 alignments were >70% identity and 64 alignments ranging from 60% to 69% identity. No evidence was found that these proteins are allergens or toxins. The common alignments with greater than 70% identity included proteins from tobacco, pepper, potato, tomato, soybean and grape. Proteins with alignments between 60% and 70% identity were matched to those in citrus, poplar (tree), jack bean, prunes, apple, pears, sunflowers and many other plant sources. Although it would take considerable effort to sort out actual exposures, it is probably safe to conclude that similar proteins from safe sources are present in the foods of potato, soybean and other common agricultural plants. It is likely that most consumers are exposed to a wide variety of these proteins and there is no obvious history of harmful consumption.

## 80mer Sliding Window Search Results

Database	AllergenOnline Database v17 (January 18, 2017)
Input Query	>query MKMKNLSLLLLLTLFLFISVCSQVQQTQFVYRWPETYCEKATPTVACTKIPLQFTLVGFW GTDSSGGIQQGCQDTGKYDWAKVFTTETANKLTAFWPLSLTQDPTMKAAWTTYGTCLI SKFKTPTQYFNRAIRLSDAIGGGNLFQGLIGKDGIVPCDSATYTNAEILKSLTAVTTVN NQKEKVSFTCNISYSTHAYLNQVTFCYTNNAQYYTDCPTTVISKRCNVPNIIVPRPPTP TARASSLQDLLTGEKIGPNVLWETLGQLF
Length	269
Number of 80 mers	190
Number of Sequences with hits	0

A

## No Matches of Greater than 35% Identity Found

AllergenOnline Database v17 (January 18, 2017)

## 80mer Sliding Window Search Results

Database	AllergenOnline Database v17 (January 18, 2017)
Input Query	>query MASVSATMISTSFMPKPAVTSKPIPNVGEALFGLKANGGKVTCMASYKVKLITPDGP IEFDPCDNVYILDQAEAGHDLPSYCRAGSCSSCAGKIAGGAVDQTDGNFLDDQLLEEGW VLTCVAYPQSDVTIETHKEAELVG
Length	144
Number of 80 mers	65
Number of Sequences with hits	0

B

## No Matches of Greater than 35% Identity Found

AllergenOnline Database v17 (January 18, 2017)

Fig. 1. Sliding 80 mer window search results for HRAP (A) and PFLP (B) proteins in AOL v17.

Table 4

BLASTP of NCBI Entrez with HRAP (269 aa) using the keyword "allergen".

Accession #	Organism	Description	aa Length	E value	Identity (%)	aa Alignment length
KVI11513.1	<i>Cynara cardunculus</i> var. <i>scolymus</i>	Barwin-like endoglucanase	256	8.8	33	55

Table 5

BLASTP of NCBI Entrez with PFLP (144 aa) using the keyword "toxin".

Accession #	Organism	Description	aa Length	E value	Identity (%)	aa Alignment length
BAO69767.1	<i>Bordetella bronchiseptica</i> (bacterium)	CDP-6-deoxy-delta-3,4-glucoseen reductase	348	3e-11	42	77
ALX21340.1	<i>Bordetella pertussis</i> (bacterium)	CDP-6-deoxy-delta-3,4-glucoseen reductase	348	3e-11	42	77
BAO69988.1	<i>Bordetella bronchiseptica</i> (bacterium)	phenylacetic acid degradation NADH oxidoreductase	362	5e-10	46	65
OTC31281.1	<i>Escherichia coli</i>	phenylacetic acid degradation protein	119	1e-09	41	70
EIL15879.1	<i>Escherichia coli</i>	putative multi-component oxygenase/reductase subunit for phenylacetic acid degradation	98	2e-09	41	70
KNG12114.1	<i>Escherichia coli</i>	phenylacetic acid degradation protein	356	4e-09	34	116
OTB70872.1	<i>Escherichia coli</i>	phenylacetic acid degradation protein	356	6e-09	41	70
EZA16213.1	<i>Escherichia coli</i>	phenylacetic acid degradation protein	356	7e-09	41	70
OTC52187.1	<i>Escherichia coli</i>	phenylacetic acid degradation protein	356	7e-09	41	70
KNF42592.1	<i>Escherichia coli</i>	phenylacetic acid degradation protein	356	7e-09	41	70

## 4. Conclusions

The results of literature searches for the sources of the genes transferred into GM banana were challenging due to the some extensive annotations that suggest allergy or toxicity associated with the source organisms. Careful evaluation of the abstracts and publications as well as refined searches did not identify publications with sufficient evidence to suspect the HRAP or PFLP proteins

represent risks of allergy or toxicity.

None of the results from the bioinformatics searches of the HRAP or PFLP proteins in AOL v17 database or in NCBI database by BLASTP identified evidence that would lead to specific tests for evaluating allergy or toxicity of these proteins.

Results from the search for similarities to known toxic proteins did not indicate significant matches, especially since the identity of many proteins from safe sources had higher identity matches to

HRAP or PFLP than did the potentially toxic proteins identified by BLASTP.

No convincing evidence was found to suggest that the HRAP or PFLP proteins expressed in the transgenic banana represent risks of allergy or toxicity to humans. Based on the guidelines of the Codex Alimentarius Commission (CODEX, 2003, 2009), and on common practices for evaluation of potential risks of allergy or toxicity from GMO (plants, animals or microbes), there is no reason to perform serum IgE binding tests or additional toxicity tests to evaluate potential risks from consuming these proteins. Although the results obtained in this study showed that PFLP and HRAP are not potential allergens or toxins, some additional tests such as evaluating the stability of the proteins in pepsin assay may be required in order to meet the national regulatory requirements to approve the commercial release of GM banana.

### Conflict of interests

The authors declare no conflict of interest.

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### Transparency document

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