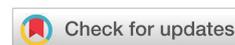


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Short Communication

A Preliminary analysis of potential allergens in a GMO Rice: A Bioinformatics approach

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Abstract

This study uses an *in silico* approach in screening nascent allergens in GMO and conventionally-bred rice. The protein sequences analyzed were taken from published microarray data from GMO and conventionally-bred rice. To determine the proteins' potential allergenicity, we used allergen databases and algorithms such as Allermatch and Allgpred. Our analysis revealed the following putative allergenic proteins in GMO rice, namely: a cysteine proteinase precursor, a putative germin A, a glycosyl hydrolase, a subtilisin-like serine proteinase and an unknown protein. These genes are related to stress and defense response, metabolism, and moving, modifying, storing, and degradation of proteins.

Introduction

Large protein molecules, called allergens, frequently trigger an allergy. It involves a series of intrinsic and extrinsic reactions that contribute to triggering the symptoms (Somvanshi, et al., 2008). A typical allergy (Type I hypersensitive reaction) is induced by allergens that trigger specific IgE antibodies between common homologous allergens from different sources. Allergy symptoms include asthma, atopic dermatitis, and rhinitis, but severe reactions may also occur like an anaphylactic shock, which could lead to death.

Over half of the world's population consumes rice as a staple food because it contains carbohydrates and proteins as energy sources. With the advent of novel foods, the number

of patients with allergy to some foods has been increasing in recent years, and rice is one of these foods. Symptoms of rice allergy are asthma (Arai et al., 1998; Hoffman, 1975; Shibasaki, et al., 1979), diarrhea and vomiting (Cavataio, et al., 1996), and atopic dermatitis with ocular complications (Uchio, et al., 1998).

Developing new cultivars by agricultural biotechnology has paved the way for crops that can adapt to poor environmental conditions and increased nutritional properties. However, these can change the properties of proteins found in food, thus increasing its allergenic potential. The Codex Alimentarius Commission states that safety assessments of genetically-modified (GM) foods need to include an investigation of tendencies to provoke allergy that might result from gene

insertion (Haslberger, 2003). Comprehensive analysis, such as bioinformatics, may be useful tools in assessing allergenicity of newly expressed proteins in GMOs versus conventionally-bred crops. It covers various bioinformatics methods available, including allergen databases and algorithms for the search of sequence identity of newly expressed proteins with known allergens and assessing the relevance of alignments observed (Kaiserlian, et al., 2010).

Chemical comparison of a new protein with known allergens is a useful method for assessing a protein's allergenic potential. Proteins are made up of long chains of amino acids, but their allergenicity is determined by only a few residues which serve as a binding site for antibodies. The ability of a protein to induce both humoral and cellular Th2 immune responses, leading to the release of allergen-specific IgE and Th2 cytokines, is a measure of its allergenicity [1]. Such property is usually assessed by *in vitro* and *in vivo* tests. No protein can be classified as allergenic without at least the evidence of the ability to bind *in vitro* IgEs from sera of sensitized individuals. If antibodies can attach themselves to a new protein and elicit a hypersensitivity cascade, there is a high chance that an allergic reaction can occur. The amino acid sequences of a protein are compared with the known allergens to determine homologies. A minimal requirement for sequence homology with a known allergen is a 35% sequence identity over a window of at least 80 amino acids [2].

GM crops undergo rigorous assessment for food, feed, and environmental safety before commercialization. However, detecting potential allergens, either *in vivo* or *in vitro* using molecular biology techniques, is challenging, time-consuming, and costly [3]. Additionally, eliciting an immune response is very complicated as the body responds to allergens by inducing many processes [4]. Possible development of potentially life-threatening allergic reactions or even the lack of sensitivity due to genetic factors could be some of these methods' challenges. These limitations make the *in silico* (or bioinformatics) an acceptable, but a preliminary approach for identifying cross-reactive epitopes and allergenicity [5].

The allergenicity assessment for GM plants includes two approaches: (1) the assessment of the entire GM plant, and (2) the evaluation of the newly expressed proteins [6]. Based on the latter, in this study, an *in silico* based allergenicity screening is demonstrated as a quick and straightforward approach to identify potential allergens from newly expressed proteins based on differentially-expressed genes that arise from a transgenic variety of rice.

Materials and methods

Microarray data information

The protein sequences used in this study were obtained from the differentially expressed genes from the rice microarray data by Batista et al. [7]. Briefly, the gene expression profiles of GSE12069 were downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/>). The dataset was based on the GPL2025 platform (Affymetrix Rice Genome Array, Thermo

Fisher Scientific, Inc., Waltham, MA, USA). GSE12069 included data from a well-characterized transgenic rice line (cv. Bengal) and its non-transgenic mother plant as control (submission date, 10 July 2008). The stable transgenic line is on its third generation of self-pollination after transformation. The plant expresses a ScFV antibody (ScFvT84.66) against the carcinoembryonic antigen [7].

We used the GEO2R tool (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>) to identify differentially expressed mRNAs from the GEO series [8]. Each sample within a GEO series was first classified into either normal or mutant variety. Then the defined groups were inputted into GEO2R. GEO2R provided a list of DEGs ranked according to differential expression levels. DEGs up-regulated significantly (>2.0-fold) and with significance ($p < 0.01$) were collected. Finally, the sequences were extracted from the David Functional Annotation Bioinformatics Microarray Analysis (<http://david.abcc.ncifcrf.gov>). The sequences analyzed in this study can be found in the DAVID Bioinformatics database with the following Accession Numbers: Q7F3A8, P0C5A4, Q6YZA4, Q0JDD4, Q0E256, Q7XVQ2, Q0IYV1, Q65XV2, Q0D652, Q6Z127, Q6Z127, Q6Z102, Q6K6Q0, Q75WV3, Q0INZ2, Q0JEB7, Q7XEZ6, Q0J525, Q6Z7P9, B7E541, Q6F2N5, Q6K1S6, Q53JL2, Q8H7P2, Q5N818, Q6YUS6, Q0J294, Q2R0M8, Q2R1H2, Q0IRH6, Q7XES5, Q2QXJ4, Q6H5C7, Q6PU50, Q0DVR9, Q10QUO, Q0DM56, Q7XUK3, Q0JBU0, Q0JA08, Q7XIE4, Q6Z4MO, Q8LIR5, Q6H421, Q0IYV1, Q33AW3, Q53PZ7, Q0ITQ0, Q0IQ30, Q2QNX3. The FASTA protein sequences of the differentially expressed genes were obtained from UniProt database (<https://www.uniprot.org/>) using *Oryza sativa* subsp. *japonica* [39947] as background.

In silico prediction of allergenicity

The protein sequences were subjected to allergenicity screening using AlgPred and Allermatch. AlgPred is an online tool that allows the prediction of allergens based on the similarity of known epitope with any region of a query protein (<http://www.imtech.res.in/raghava/algpred/>). Three modules were used: the SVM module based on amino acid composition prediction, the MEME/MAST motif prediction approach, and mapping of IgE epitopes. AlgPred allows the mapping of IgE epitope(s) on epitope in a given protein. It has a search function for MEME/MAST allergen motifs using MAST, which assigns if a protein is a possible allergen. MEME (Multiple Em for Motif Elicitation) is a tool for discovering motifs in related protein sequences. MAST (Motif Alignment and Search Tool) is a tool for searching biological sequence databases for sequences that contain one or more of a group of known motifs. AlgPred also allows the prediction of allergenicity based on SVM modules using amino acid or dipeptide composition. SVM is implemented using SVM_light [9] with input vectors on amino acid composition (20 vectors) and dipeptide composition (400 vectors) of each protein sequence. Finally, AlgPred facilitated a BLAST search against 2890 antigen-related proteins (ARPs) obtained and assign a protein allergen if it has a BLAST hit [10,11].

Allermatch is a FASTA package (version 3.4.t21) available at <ftp://ftp.virginia.edu/pub/fasta/>. The default parameters (ktup = 2, matrix = Blosum50, Gap open = -10, Gap extend =

-2). According to the current FAO/WHO Codex Alimentarius guidelines, the analysis tool provides the search methods where the alignment of 80-amino-acid sub-sequences of the input sequence was done using a sliding window of 80-amino-acid size (Fiers et al., 2004).

Homology modeling and visualization

Homology models of proteins are of great interest for better appreciating biological properties experiments when no experimental three-dimensional structures are available, especially if these are novel proteins. The 3D protein structure was modeled on the SWISS-MODEL workspace using the Alignment Mode [12], and was visualized using the program PyMol [13].

Results and discussion

Following our preliminary screening of the 50 rice proteins with AlgPred and Allermatch, we found five proteins that were considered potentially allergenic (Table I). A cysteine proteinase precursor (Q7F3A8) was predicted to be the most allergenic. It contains the IgE epitope, VKNSWGTAWGEGGYI, which is homologous to known allergens from short ragweed (*Ambrosia artemisiifolia*), kiwi (*Actinidia deliciosa*), papaya (*Carica papaya*), pineapple (*Ananas comosus*), and soybean (*Glycine max*). A homology model of the protein is shown in Figure 1, and the IgE cross-reactive epitope is highlighted in red. Cysteine proteinase, one of the significant groups of plant proteases, has been thoroughly studied due to its crucial role in senescence and programmed cell death [14]. Interestingly, the potential allergen is a member of the structurally-related protein superfamilies - the prolamins [15]. These protein families have structural domains of plant food allergens known to trigger a reaction via the gastrointestinal tract [16].

Based on the SVM method, due to dipeptide composition, a putative germin A protein (Q6YZA4) was predicted to be allergenic by AlgPred. It should be noted that the sensitivity, specificity, accuracy, and Mathew's correlation coefficient for SVM for dipeptide composition for AlgPred are 88.8%, 88.2%, 88.5%, and 0.770, respectively [10,17]. Germins and the related

germin-like proteins (GLPs) are glycoproteins expressed in many plants in response to biotic and abiotic stress. In an immunoblotting assay, 24 out of 82 tested sera (29.26%) from allergic patients showed IgE-binding to germins. Germins can bind to IgE antibodies likely via their carbohydrate moieties [18].

Similar to germins, subtilisin-like serine proteinase (Q0E256) are also involved in the protective signaling mechanisms. *ALE1*, a gene homologous to subtilisin-like serine proteases, was found to be expressed within specific endosperm cells adjacent to the embryo and regulates the formation of cuticle on embryos and juvenile plants [19]. Strangely, its biological role in modifying proteins for plants is also mirrored by the *C. elegans* (nematode). A homolog subtilisin-like serine proteinase is involved in cuticle formation and essential for early development and adult morphology [20]. Subtilisin-like serine proteinase (Q0E256) in rice is homologous to the allergen Cuc m 1 from muskmelon. The thermally-stable Cuc m 1 is the only plant food allergen belonging to the family of serine proteases. Most allergens from this family are fungal allergens from the subfamilies of alkaline or vacuolar serine proteases [21].

The glucan endo-1,3-beta-glucosidase 14 (Q0JDD4), a poly-

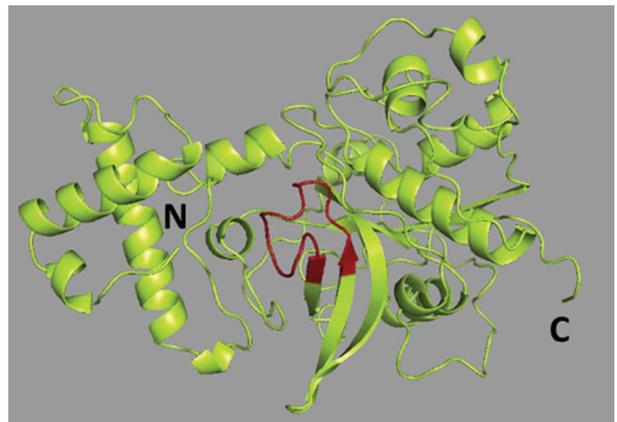


Figure 1: Homology model for *O. sativa* cysteine endopeptidase (Q7F3A8) showing the putative IgE epitope (shaded red).

Table 1: Up-regulated genes in GM rice that have allergenic potentials based on the differences in the microarray profile differences between T1 NipponBare GM vs. control. The GM rice is a T1 generation of an Agrobacterium-transformed transgenic line [7]. Allergens which have similarity within an 80-amino acid window of the up-regulated protein are shown in bold letters.

Affymetrix ID	Protein Name	ACCN #	Mass (Da)	Fold Change Expression	AlgPred	Allermatch
Os.4181.1.S1_at	Cysteine endopeptidase	Q7F3A8	40,720	14.59	Contains an IgE epitope	Amb a 11 (<i>Ambrosia artemisiifolia</i>) = 68.80% Act d 1 (<i>Actinidia deliciosa</i>) = 61.20% Cari p Papain (<i>Carica papaya</i>) = 57.50% Ana c 2.0101 (<i>Ananas comosus</i>) = 53.80% Gly m Bd30K (<i>Glycine max</i>) = 52.54
Os.23557.1.A1_at	Germin-like protein 8-7	Q6YZA4	24,335	8.25	Potential allergen	GL82_ORYSJ** (<i>Oryza sativa</i>) = 100%
Os.47778.1.A1_s_at	Glucan endo-1,3-beta-glucosidase 14	Q0JDD4	42,041	8.08	Non-Allergen	Hev b 2 (<i>Hevea brasiliensis</i>) = 51.29% Mus a 5 (<i>Musa acuminata</i>) = 50.00%
Os.57054.1.S1_at	Subtilisin-like serine proteinase	Q0E256	53,783	8.02	Non-Allergen	Cuc m 1 (<i>Cucumis melo</i>) = 57.50%
Os.45902.1.A1_at	Os04g0317400 protein	Q7XVQ2	12,373	19.03	Non-Allergen	Sor h 2 (<i>Sorghum halepense</i>) = 53.80%

* Information on allergen name, species, and % identical amino acid

** No allergen name, allergen ID provided



galacturonase, showed significant identity with allergen Hev b 2 (1,3-glucanase) of *Hevea brasiliensis* (Para rubber tree) and Mus a 5 of *Musa acuminata* (banana). Hev b 2 is one of the allergens that cause latex allergy, an IgE-mediated hypersensitivity disorder in which patients are sensitized to natural rubber latex [22]. Recently, a novel allergen, PR-1a was cloned. PR-1a is a causative agent of peach tree pollen sensitization and is similar to glucan endo-1,3-beta-glucosidase 14. IgE of subjects who developed peach tree pollen allergies living in areas where this tree is widely cultivated was found to recognize a glucan endo-1,3-beta-glucosidase-like protein [23].

Lastly, we found potentially allergenic an unnamed protein (Q7XVQ2) that was overly expressed up to 19-fold in the transcriptome of the transgenic rice analyzed. A PFAM analysis indicated that it has an expansin C-terminal domain, a component of a plant cell wall protein involved in the non-enzymatic rearrangement of cell walls during cell growth. The expansin domain is associated with the allergens lol PI, PII, and PIII from *Lolium perenne* or ryegrass [24]. These grass pollens are widely implicated in the cross-sensitivity of people to house dust mites triggering severe asthma or allergic rhinitis [25].

From 1992–2017, more than 1,300 separate assessments by regulatory agencies around the world have reviewed the safety data on various GM crops, with every report concluding that the GM crop is as safe as conventionally-developed crops. For the 20 years of accumulated data, it has been concluded by far that the application of transgenic methods does not affect the levels of allergenic proteins native to a particular crop [6]. While a motif, sequence homology-based screening, is a part of a pipeline for allergenicity assessment of novel foods, we emphasize that *in silico* analysis should be backed up or validated by empirical research. A significant limitation of this study is that the potential allergens were data mined from transcriptome data. The food allergens Ara h 1 (peanut), Pru p 3 (peach) and Gly m 5 (soybean) are usually present at relatively high amounts from 1,000 to 10,000 ppm [6,26]. By contrast, the putative allergens proteins identified in this study were associated with only 8–15 fold increased in mRNA expression as compared to the non-GMO counterpart. Thus, it may be orders of magnitude lower than the sensitizing level of typical food allergens. There is also a possibility that post-translational modifications may happen downstream and that the potentially allergenic proteins may susceptible to gastric digestion. Of note, the ability of a protein's epitopes to survive gastric digestion is an essential characteristic of food allergens, while *in vitro* pepsin resistance tests remain an integral part of the weight-of-evidence approach to assessing the allergenic potential of any novel protein [27]. Further research can focus on studying these potential allergens in the food matrix since the context of the food ingestion can modify the conformational epitopes. This technical issue assumes significance and needs to be given some thought concerning novel foods' safety assessment.

Conclusions and perspectives

In conclusion, an *in silico* method may be a useful tool for the initial prediction of potentially allergenic proteins provided

gene expression data is available. Five potential allergens have been identified for a transgenic rice variety. As recent studies recommend, modern bioinformatics tools could serve as a preliminary yet robust approach to identifying allergenic proteins in food [28–32]. Since this is a preliminary study, further tests should be done to conclude the likelihood of allergenicity of the proteins.

Authors' contribution

RGR and AMG collected and analyzed the data and wrote the initial draft of the manuscript; CCD and MVA validated the initial bioinformatics results, performed homology modeling, contributed to writing, and finalized the paper.

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