The use of RNAi and CRISPR Gene Editing Technology to decrease immunodominant Allergens in Foods: A Literature Review

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Abstract

Food allergies have become a critical health problem worldwide. This disease state can be associated with high morbidity and potential mortality from anaphylactic reactions. The economic cost of this disease state for individuals and their caregivers is significant and increasing. The common management of food allergy is avoidance and epinephrine use with only a limited number of other interventions available. A new field in food allergy treatment has emerged based upon gene editing (CRISPR) and gene silencing (RNAi) technology. These technologies could potentially reduce allergenicity of a plant food product such as a peanut, by alteration of a specific plant's major allergen genes or interference with the transcription of those genes. This review paper summarizes seven primary research papers that utilized either CRISPR or RNAi to reduce the allergenicity of a plant food product. All seven studies showed a significant decrease in known plant immunodominant allergen gene product compared to control. Some of the studies were able to compare the difference in allergenicity of the altered food product compared to control utilizing IgE binding; some investigations noted phenotypic similarities or differences in the transgenic plants; and one study performed an in vivo skin prick test showing decreased reactivity with the transgenic plant food compared to control. This review will additionally explain the function of the gene editing and silencing technology, food allergy, implication of the technologies on food allergies, and future steps for treating food allergy with gene editing/silencing technology.

Keywords: Anaphylaxis, Transgenic Crops, Gene Editing, Allergy

1.0 Introduction

Food allergies are reproducible adverse reactions to foods that are mediated by the immune system (Sicherer et al., 2020) and are a critical health problem in the United States. In a recent analysis of data from the 2021 National Health Interview Survey (NHIS), a nationally representative household survey of U.S. civilian noninstitutionalized population, the National Center for Health Statistics (NCHS) found that approximately 6% of children under the age of 17 have a food allergy (Zablotsky et al., 2023). Further analysis of the same 2021 NHIS data revealed a similar prevalence of food allergy in U.S. adults. Ng and colleagues reported that 6.2% of adults have a diagnosed food allergy (Ng and Boersma, 2023).

A population based survey study of roughly 40,000 U.S adults illustrated that the most common food allergies were shellfish, milk, peanut, tree nut and fin fish (Gupta et al., 2019). An allergic reaction from exposure of a food allergen to an allergen-sensitized individual can lead to one or more acute symptoms in multiple systems of the body, which can include hives, vomiting, wheezing, and anaphylaxis (Warren et al., 2021). Anaphylaxis is an IgE-mediated immune reaction to an allergen that can affect multiple organ systems associated with high populations of resident mast cells including the cardiovascular, respiratory, cutaneous, and gastrointestinal systems, and can be potentially fatal. (Sampson et al., 2005; Wod et al., 2014; Turner et al., 2017).

Epinephrine, the first line of defense for food allergy, is a drug administered by caregivers, parents, or children in the form of an autoinjector device to suppress anaphylaxis by blocking the release of immune mediators that act to upregulate the immune response (Gold et al., 2000)(Cardona et al., 2020; Patel et al., 2021). Epinephrine and other drugs are critical for the treatment of an anaphylactic reactions, costly, and only make up a small portion of the cost of having a food allergy. Enormous expenses exist for caregivers with a child who has a food allergy.

These expenses derive from long term outpatient visits to the clinic, medical drugs, emergency visits and the possibility of being hospitalized due to allergic reaction. (Gupta et al., 2013). According to a 3 month cross sectional study that included 1,643 caregivers of a child with at least one food allergy, the economic impact of food allergy for caregivers was estimated to be approximately $25 billion (Gupta et al., 2013). Due to the costs of food allergy and its prevalence, a growing need exists to create a variety of treatments for food allergies.

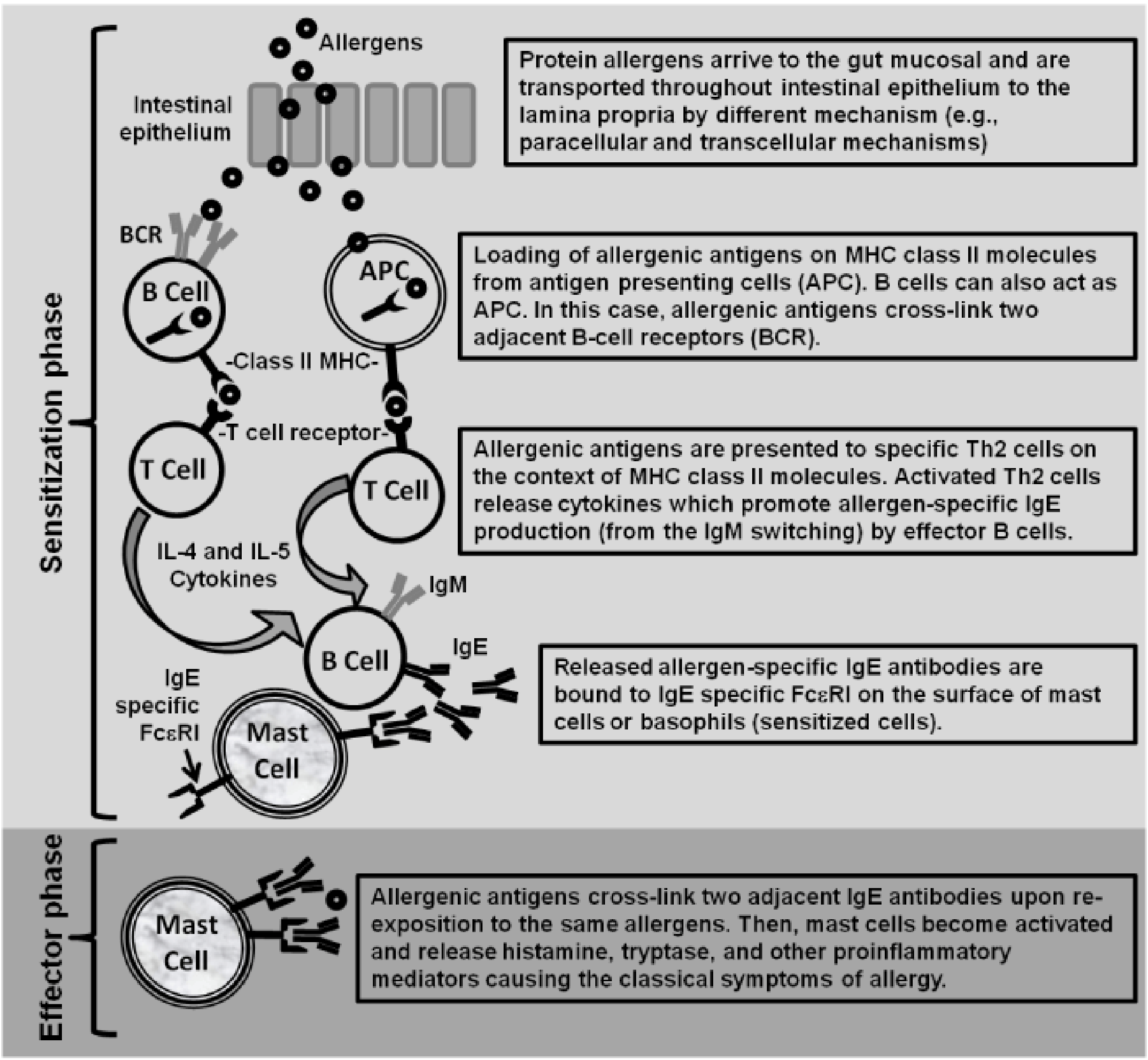
Researchers have looked in the direction of gene editing and silencing technology to potentially reduce allergenicity from the allergen itself as a method to cure food allergy. The objective of this review is to explain the function of the gene editing and silencing technology, food allergy, implication of the technologies on food allergies, and future steps for treating food allergy with gene editing/silencing technology.

2.0 Food Allergy Immunological Mechanisms

Allergens are generally recognized as a foreign substance within the body by allergen-specific immune cells. Food allergies are categorized into three main immunological mechanisms: IgE-mediated, non-IgE mediated, and mixed food allergies (Yu et al., 2016).

2.1 IgE-mediated Food Allergies

One must first be sensitized to have an IgE-mediated allergy reaction. When consuming the allergen, the allergen travels through the lining of the small intestine and is exposed to antigen presenting cells (APC's) or in some cases, B-cells. The antigens are then loaded onto cell surface molecules on the APC's called MHC Class II, which hands off the antigen to T-cells. The T-cells then release cytokines which signal B-cells to create IgE antibodies, which are a type of antibody produced by the immune system (Ontiveros et al., 2014). When in contact with the body (Oral, Cutaneous), the allergen will bind adjacently to the IgE antibodies bound to the Fc receptor on a mast cell, releasing inflammatory mediators known as cytokines that start and then upregulate an immune response leading to symptoms such as hives and the anaphylaxis response (Ontiveros et al., 2014).



Stages of IgE-Mediated food allergy. (Ontiveros et al., 2014)

2.2 Non-IgE-mediated Food Allergies

Non-IgE mediated food allergies mainly affect the gastrointestinal (GI) tract (Nowak-Wegrzyn et al., 2015). One of the most prevalent and most studied non-IgE GI allergy is food protein-induced enterocolitis syndrome (FPIES) (Nowak-Wegrzyn et al., 2015).

FPIES is a non-IgE mediated food allergy that causes delayed reactions to the gastrointestinal tract. FPIES can occur at any age but usually emerges in the first three months from birth, and is usually outgrown by around five years of age. Common foods associated with FPIES include oats, cows milk, and soy (Nowak-Wegrzyn et al., 2015).

Other non-IgE mediated food allergies include food protein enteropathy (FPE), which mainly affects the small bowel, food protein induced allergic proctocolitis (FPIAP), which affects the rectum and colon, and eosinophilic gastrointestinal disorders (EGIDs), which include a variety of gastrointestinal symptoms, including eosinophilic infiltration of the GI tract (Calvani et al,, 2021)

2.3 Mixed IgE- and Non-IgE-mediated Food Allergies

Mixed food allergies include both IgE-mediated and cell mediated immunological reactions. Non-IgE mediated reactions and mixed allergy reactions are not understood very well. Most forms of mixed food allergies and non-IgE allergies affect the gastrointestinal tract and they do not cause anaphylaxis. (Calvani et al., 2021)

2.4 Management of Food Allergies

The most common management for food allergy is food avoidance and the use of epinephrine- which is used to suppress anaphylaxis should it occur (Warren et al., 2021). However, many recent advances in the production of food allergy treatments have been discovered, with the most successful being Oral ImmunoTherapy (OIT). OIT works by administering small oral doses of an allergen to an individual and sequentially increasing the dosage over time to achieve a maintenance dose for which a patient will become desensitized to the particular allergen. OIT has been proven to be an effective method to desensitize patients with food allergy (Epstein-Rigby et al., 2022).

In 2018, an international team of researchers conducted a double-blind placebo phase 3 trial to evaluate the effectiveness and viability of the peanut derived oral immunotherapy (OIT) drug named AR101 in peanut allergic patients. Enrolled participants were 4-17 years of age and had a previous history of peanut allergy with extensive testing to prove the allergy was severe. Participants were randomly assigned to receive the AR101 or placebo in the form of capsules. Both placebo and AR101 capsules were administered daily. The trial lasted 24 weeks and resulted in the active drug group experiencing much milder symptoms than the placebo group at 24 weeks (Vickery et al., 2018)(Pouessel and Lezmi 2023).

Recently, in 2020 the AR101 drug was FDA approved (Patrawala et al., 2022). However, due to the intense commitment required to complete OIT and the risk of allergic reaction occurring while performing OIT, this treatment may not be for everybody. For Non-IgE food allergy, treatment usually relies on diet and elimination of the trigger foods for either EoE, FPIES and

FPIAP. Treatment for Non-EoE and EGID non-IgE mediated food allergy involves the use of steroids and PPI (Cianferoni 2020),(Nowak Wegrzyn et al., 2015)

3.0 Gene Editing Technologies

Researchers have come a long way in the treatment of allergies using OIT, however, there have been new discoveries in the treatment of food allergies using gene editing technologies. This review will focus on two revolutionary methods, known as CRISPR and RNAi, and how they have been utilized in the treatment of food allergy.

3.1 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

CRISPR, which is a natural molecular biological process in bacteria, utilizes a guide RNA and the Cas9 protein. The guide RNA directs the Cas9 protein to a targeted gene site where the Cas9 protein can cleave the gene. After the gene is cleaved, repair mechanisms come in and repair the DNA. In bacteria, CRISPR is used to defend against invading viruses or plasmids by cleaving parts of the viral DNA (Jinek et al., 2018). For molecular engineering purposes, CRISPR has been used for a variety of purposes, including the use of CRISPR on plant and animal genomes and the editing of major allergen genes to reduce allergenicity (Knott and Doudna 2019)

3.2 RNA Interference (RNAi)

A heavily researched method to prevent proteins from being produced is called RNAi. RNAi is an invaluable genetic knockdown technology that uses a double-stranded RNA molecule that silences the targeted mRNA by preventing proper translation, and thus, preventing the protein from being produced (Fire et al., 1998). RNAi has been used on many targets, such as reducing the allergenicity of a peanut (Dodo et al., 2007).

4.0 Dominant allergen genes from each food

4.1 Peanut Allergens

The seeds from the peanut plant *Archis hypogaea* have 16 allergenic proteins that have the ability to cause the production of IgE antibodies in sensitized individuals. Of the 16 allergenic proteins in peanuts the most prominent for inducing an allergic reaction are Ara h 1, Ara h 2, and Ara h 3 (Palladino and Breiteneder 2018).

4.2 Wheat Allergens

Wheat contains a group of proteins named alpha-Amylase/trypsin inhibitors (ATI), which are present in all cereal crops including, rye, maize, and barley. ATI contains several polypeptides named 0.28, CM3 and CM16, that are identified as the major allergen polypeptides, and play a role in many wheat sensitivities such as Celiac disease. (Tundo et al., 2018); (Geisslitz et al., 2022)

4.3 Soybean Allergens

The prominent proteins in soybeans are called seed storage proteins, and are a source of soybean allergy. The allergenicity of a soybean is mainly due to the seed storage proteins Gly m Bd 30 k, which is the most allergenic, and Gly m Bd 28 k. Many patients with soybean allergy have been identified to have IgE antibodies correlated with the Gly m Bd 30 k protein (Mulalapele and Xi 2021).

4.4 Brown Mustard Allergens

The major brown mustard allergen, *Brassica Juncea* (Bra j 1) , is a part of a group of seed storage proteins entitled 2S albumins, which are used by the plant as nutrients during seed growth. 2S albumins are found in an abundance of foods including tree nuts, spices, legumes, and cereals. These proteins have been shown to bind to IgE to start the allergic immune cascade in allergic patients' sera (Moreno and Clemente 2008).

4.5 Apple Allergens

The major allergen found in apples is called *Malus Domestica* (Mal d 1). Mal d 1 belongs to a group of proteins named pathogenesis related (PR) proteins. The IgE epitope structure is very similar across all of the PR proteins in plants allowing for cross-reactivity. Sensitized individuals to birch tree pollen can develop an immunologic cross reactivity to apples from apple IgE antibodies that have structurally homologous PR proteins. (Ahammer et al., 2017)

Table 1: Summary of Immunodominant Allergens in Plants of Interest

Plant	**Immunodominant Allergen**	**References**
Soy	Gly m Bd 30k, Gly m Bd 28k	Mulalapele and Xi 2021
Wheat	CM3, CM16, 0.28	Tundo et al., 2018; Geisslitz et al., 2022
Apple	Mal d 1	Ahammer et al., 2017
Brown Mustard	Bra j 1	Moreno and Clemente 2008
Peanut	Ara h 2	Palladino and Breiteneder 2018

5.0 Application of Gene Editing to Reduce Food Allergenicity

5.1 RNA Interference

5.1.1 Peanut

By silencing specific proteins from being produced, RNAi can be used for a variety of health related challenges such as food allergy. Researchers Hortense W. Dodo and colleagues used RNAi to silence the Ara h 2 gene in the peanut (Dodo et al., 2007). They accomplished this by first generating a complementary RNAi fragment from the Ara h 2 genomic DNA. They then cloned the fragment into a plant transformation vector used to transfer genes into cells. They inserted the vector containing the RNAi fragment into the *A. Tumefaciens* EHA 105 bacterium, and infected the peanut hypocotyls with the bacterium. They used multiple methods, including PCR, SDS- PAGE, and western immunoblotting to detect the presence of the Ara h 2 protein. The authors noted a significant reduction in Ara h 2 content in transgenic seeds. They noticed that both the transgenic plants and wildtype were similar phenotypically and exhibited similar growth rates. Using an antibody measurement system named ELISA on the sera of patients with peanut allergy, they found significantly less IgE binding with the transgenic peanut compared to the Wild Type control peanut. This marks a successful reduction in allergenicity from the peanut by targeting the major peanut allergen gene Ara h 2 (Dodo et al., 2007).

5.1.2 Apple

Another case of RNAi to reduce allergenicity, is the use of RNAi on the apple allergen Mal d 1. Researchers from the Netherlands created an intron spliced RNA containing the Mal d 1 inverted repeat sequence to transform in-vitro apple plantlets. They used the Elstar apple cultivar for transformation and leaflets divided into explants for observation of Mal d 1 presence. The 2 fragments used for the hairpin DNA construct were obtained through a PCR of genomic DNA isolated from the cultivar gala. The amplified fragments from PCR were cloned into an expression cassette. The expression cassette was cloned into a binary expression vector creating the vector pBihpMald1. In three independent transformation experiments, leaf explants were placed in a liquid medium containing a culture of *A. tumefaciens* strain carrying the pBihpMald1 vector. Next, the explants were placed in a medium with kanamycin, some mediums but not all, the kanamycin was absent, indicating the successful implantation of the vector. The authors also checked for presence of the construct using PCR analysis. The results showed a significant reduction of Mal d 1 expression in transformants by Western blot analysis. No phenotypic or growth rate differences were noted between the transformants and the control unmodified plants. The in vitro plants were used for a skin prick test on human volunteers with apple allergy including a control allergen of Mal d 1. They found a significant decrease in wheal size of the in vitro plantlets compared to the control allergen (Gilissen et al., 2005).

5.1.3 Wheat

An international study conducted by researchers in 2020 discovered a method to reduce the allergenicity of wheat using RNAi. They achieved this incredible feat by co-transforming 1,669 embryos of *Triticum aestivum L. cv*. Bobwhite with three vectors for each of the ATI allergen genes (0.28, CM16, and CM3) with the plasmid pUBI::BAR. The presence of the transgene was verified using PCR analysis. Using qRT-PCR, the researchers found a significant decrease in the expression of the allergen genes, 0.28, CM16, and CM3 compared to the untransformed plants. They noted that both the transgenic and wildtype plants were similar phenotypically and exhibited similar growth rates. After the confirmed reduction of the major wheat allergen genes, they tested the IgE binding of the bobwhite compared to the RNAi silenced genes of bobwhite. They found a significant reduction of IgE binding in the silenced genes, declaring the success of the study. (Kalunke et al., 2020)

5.1.4 Soybean

In another study, researchers from China successfully decreased the amount of the Gly m Bd 30k major allergen protein in soybeans. The vector pMD19-I was created from an amplified PCR product from a plasmid and cloned into another vector. Then a 396-bp fragment of Gly m Bd 30k was inserted into the vector, which resulted in the vector pCAMBIA3301-30k-RNAi and inserted into the *A. Tumefaciens* EHA105 bacteria. The explants were obtained from the seedlings which were cultivated with the *A. Tumefaciens* EHA 105 bacterium. After co-cultivation the seedlings were cultured with bialaphos. The remaining seedlings that survived the cultivation with bialaphos were thought to be transgenic because *A. Tumefaciens* has a bialaphos resistance gene. The authors verified the presence of the Gly m Bd 30k transgene through PCR and southern blotting. Western blotting analysis illustrated a significant reduction in Gly m Bd 30k mRNA the 3rd transgenic line compared to wildtype (Liu et al., 2013).

Table 2: Summary of Studies Using RNAi to Reduce Food Allergenicity

Plant	Phenotype	IgE Binding	Skin Prick Test	References
Soy	No major differences between the WT and RNAi lines were noticed	N/A	N/A	(Liu et al., 2013)
Wheat	No significant differences were noticed in growth or morphology between the untransformed plants and the plants that had lost the transgene.	In all cases IgE binding of the WT (Bobwhite) was higher than the RNAi silenced lines.	N/A	(Kalunke et al., 2020)

Apple	Phenotypes of trasformed plantlets were "Indistinguishable" from WT plantlets.	N/A	Transformants showed smaller SPT response than control plantlets.	(Gilissen et al., 2005)
Peanut	No phenotypical differences observed between transgenic, non transgenic, and WT plants using aspects of growth, mophology, and reproduction.	IgE binding capacities of all transgenic peanut samples were lower than the WT.		(Dodo et al., 2007)

WT= Wild type, SPT = Skin Prick Test

5.2 CRISPR

5.2.1 Brown Mustard

This study's aim was to edit and remove the Bra J I allergen gene in brown mustard seeds. Seeds of two brown mustard lines were used. Binary vectors, pEGFP, pBrj1256 and pBrj3477 were constructed containing complementary sequences to the Bra j I sequence in the form of sgRNA expression cassettes.

The cotyledon brown mustard explants were co-cultivated with a strain of *A.tumefaciens* LBA4404 harboring the binary vectors. Then the explants were placed in a regeneration medium with antibiotic, kanamycin. The surviving shoots (The shoots that implemented the vector containing the kanamycin resistance gene as well as the sgRNA's) were subcultured for 56 days and then placed in pots to be grown for both the wild type and transgenic plants. Once at maturity the seeds were harvested for the first generation. The researchers then used PCR on the DNA from the two lines of brown mustard and discovered a 695-bp deletion of the Bra J I allergen gene in one line and a 790-bp deletion in the other. Western blot analysis on the transgenic seeds showed an absence of Bra j I protein in all mutant lines. Phenotypically, in some transgenic lines, the seed formation was reduced, seed viability was reduced and seeds showed precocious development compared to the wild type lines. (Assou et al., 2021).

5.2.2 Wheat

In this study, researchers were able to knock out the Alpha-Amalyse/Trypsin inhibitor proteins (ATI allergen genes) from a durum wheat cultivar, svevo. Guide RNA targets were created on the coding sequence of CM16 and CM3 genes. These Guide RNAs were synthesized and cloned into multiple vectors. The Plasmid vectors were then co-bombarded with the durum wheat cultivar. Regenerating plantlets were transformed into a regeneration medium and grown until maturity.

Gene amplification was carried out to detect the presence of the allergen genes. The researchers found a significant reduction of the genes, while there were some shorter base pairs present in the DNA, however much weaker than the control. Guide RNA is designed to cause large mutations in the CM16 and CM3 genes, the editing events were visible in gel electrophoresis by PCR. No off-targets were detected by in-silico analysis. ELISA demonstrated no reactivity for alpha-amylase/Trypsin inhibitor CM3 confirming mutations caused a gene knockout. Alpha-amylase/Trypsin inhibitor CM16 ELISA reactivity could not be accessed since no monoclonal antibody against CM16 was available at the time of the study. (Camerlengo et al., 2020).

5.2.3 Soybean

A study conducted by researchers in Japan discovered the possibility of editing the Enrei and kariyutaka soybean allergens using CRISPR/Cas9 technology. The aim was to edit the major soybean allergen Gly m Bd 28 K and Gly m Bd 30 k. They first constructed a guide RNA expression vector, which contained 2 guide RNA expression cassettes. A new vector was made which was constructed by inserting the guide RNA expressions cassette vector into a cas-9 binary vector containing the glufosinate resistant gene (Bar gene). They used an agrobacterium-mediated transformation using *Agrobacterium Tumefaciens* EHA105 harboring the plasmid with the guide RNA's. To observe the presence of the allergen genes they extracted total RNA from the 3rd generation seeds and put it through RT-PCR analysis. Mutant seeds showed significantly lower levels of the major soybean allergen Gly m Bd 28 K and Gly m Bd 30 k compared to wildtype. (Sugano et al., 2020)

Table 3: Summary of Studies Using CRISPR to Reduce Food Allergenicity

Plant	Phenotype	IgE Binding	Skin Prick Test	References
Soy	N/A	N/A	N/A	(Sugano et al., 2020)
Wheat	N/A	N/A	N/A	(Camerlengo et al., 2020)
Brown Mustard	Seed production notably reduced in transgenic lines and the	N/A	N/A	(Assou 2021)

	seeds of some transgenic lines had seeds which were heavier or lighter than the WT.			

WT = Wild type

5.3 Limitations in research (Results)

The current review illustrates the existence of technologies, RNAi and CRISPR/Cas9, to genetically alter a plant food source to decrease or eliminate its allergenic potential to humans. Existing studies show that these technologies can be used to significantly decrease a dominant allergen gene's expression in a number of plant species. To date, the amount of information on this subject is limited. The studies that do exist only answer the basic questions concerning the success of gene editing/interference in a small number of plant species. More investigation is warranted to fill in the large knowledge gaps that exist.

Of the seven studies presented in this review paper, all have shown that CRISPR and RNAi technologies can reduce the amount of allergic gene product in the plants studied, but only three investigations looked into human serum IgE binding of the plant's products, a measure of allergenicity, and then compared them to control and only one study used in vivo skin prick testing with a transgenic plant product compared to control. Only five of the seven studies investigated basic phenotypic and growth rate differences between a genetically altered plant and the control. Only one investigation detailed that seed formation and viability was reduced in transgenic plants.

Researchers in many of the studies utilized test gene editingor silencing technology on only one type of cultivar of the allergen, for example; the enrei and karitutaka soybeans that were used in the experiments with CRISPR/Cas 9 technology. But hundreds of soybean variants exist and non-modified versions of this plant would still be harmful to a soybean allergic individual.

6.0 Future Directions

There exists promising but limited data concerning the ability to genetically alter food crops to be less allogeneic for consumers. More studies are required on the same and different food crops with specific universal standards on what questions are required to be answered to allow a genetically manipulated crop to become a food source for humans.

Investigations will need to be performed to uncover the population's acceptance of a genetically modified food introduced into our environment and a willingness to actually ingest it. And what health risks, short and long term these modified foods may have on humans and our ecosystems.

Transgenic plants and the foods they deliver to us would need to be carefully studied to see their effect in nature on their own wild type plant and their other related variants. Additional investigation should be performed into the effects of mutant species on other related and non-related plant species from cross-fertilization and competition. A genetic change in a plant can affect other plant system functions such as resistance to natural pathogens, growth rates, competition in the environment the new plant would cause and taste of the food product.

Hopefully, the fund of knowledge from the current research will continue to expand and eventually include the editing of many variations of a particular crops that can be grown in different climates around the world.

7.0 Conclusion

With the increased prevalence of food allergy in the U.S. population, there is a dire need for a variety of long term, effective treatments. Manipulation of the genes responsible for an allergic reaction or of the genes encoding for antigens responsible for inciting an immune response represent pathways to address this need. This review found that teams of scientists have already genetically modified foods to reduce or eliminate their predominant allergens with RNAi and CRISPR/Cas9. This approach appears promising but limited at this time because this method is plant specific.

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