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The impact of plant biotechnology on food allergy

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Concerns about food allergy and its societal growth are intertwined with the growing advances in plant biotechnology. The knowledge of plant genes and protein structures provides the key foundation to understanding biochemical processes that produce food allergy. Biotechnology offers the prospect of producing low-allergen or allergen null plants that could mitigate the allergic response. Modified low-IgE binding variants of allergens could be used as a vaccine to build immunotolerance in sensitive individuals. The potential to introduce new allergens into the food supply by biotechnology products is a regulatory concern.

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Introduction

Food allergies are a growing concern in the industrialized countries where the percentage of the population that exhibit clinical food allergies has increased rapidly over the past few decades [1]. Food allergies are often manifested with an escalating series of responses to allergen challenge, beginning with mild atopic reaction, then after repeated exposure the patient may exhibit more intense reactions culminating in a risk of fatal anaphylaxis. Among the major plant source foods wheat and soybean are often cited as major sources of food allergies owing to their inclusion as significant fractions of all foods especially processed foods so often used in industrialized countries. Many other plants are sources of allergens include seeds such as peanuts, tree nuts, sesame, and sunflower as well as fruit and vegetables including apple, tomato, kiwi, papaya, and carrots that can provoke severe and sometimes life-threatening anaphylactic responses. Food allergies develop from sensitization by proteins that could otherwise be safely consumed. Preventing possibly life-threatening allergic responses is a significant medical problem and a crucial question of liability and regulation for the food industry. Unfortunately there is no simple solution to the food allergy problem. Indeed, the food allergy problem has become progressively more difficult to manage as the rate of food allergic people continues to grow. The current primary treatment for food allergies is physical avoidance, that is to identify food risk and for the individual to take positive steps to avoid its consumption. To aid in avoidance the United States has the regulations of the Food Allergen Labeling Consumer Protection Act of 2004 [2] that requires plain language labeling of the 'big eight' allergens of wheat, soybean, peanut, tree nuts, fish, shellfish, dairy, and eggs that are the most common and formerly hidden ingredients of processed foods [3]. Although this appears to be a simple solution, its implementation in the real world is far more complex as labels are often separated from food especially in the growing tendency of industrialized countries to have a substantial fraction of meals away from the home. Many of the most at-risk individuals, infants, and children, either cannot read labels or cannot reliably take the warning seriously.

Plant biotechnology has had a major role in defining the problems of food allergy. Modifying food plants presents the potential to provide a means to address the problems of sensitization and management of food allergies. As plant biotechnology is used as production platforms to produce altered food and feed as well as industrial products there is potential that this will inadvertently produce potent food allergies is a risk, but how to define that risk is a continuing problem.

Using the plant biotechnology tool kit and its implications for food defining allergy

Biotechnology has revolutionized our understanding of which proteins are food allergens, how these proteins are related and often closely related to other members of the same family that are not known to be allergens. Using the sequence databases many food allergen proteins have had maps of antigenic sites produced and these antigenic sites have been placed on crystal structures.

Using bioinformatics tools for the analysis of the open reading frames from the sequence collections has resulted in identifying as of October 2009 11,912 protein families, Pfam, [4•]. For the 927 presently known clinical allergens there are only *222* Pfam domains present [5,6,7•]. The interpretation from this is that only a small fraction of proteins, perhaps *2%*, are allergens however this should be viewed with caution as the vast majority of proteins produced by any cell are accumulated at levels that are below the threshold for sensitization or hypersensitive response even if the protein was a known allergen. Until and unless a protein is used to challenge at levels consistent with inducing an allergenic response in a potentially sensitive person or animal model that protein has not truly been subjected to an *in vivo* evaluation. Further a large fraction of the proteins in Pfam are from the vast majority of organisms not part of the food supply under any circumstances.

Sequence and bioinformatics has shown that some food allergens cross broad phylogenetic lines. For instance, members of the papain superfamily of cysteine proteases have a number of examples that are allergenic either as respiratory or contact allergens such as the dust mite Der1p or as food allergens (Gly m Bd 30k/P34 (soybean), bromelian (pineapple), actinidin (kiwi fruit), and papain (papaya). Papain superfamily members are conserved in Eukaryotes with thousands of gene family members now known but only a few known as clinically relevant allergens. IgE epitopes have been determined for some of the allergenic papain superfamily members and these do not align to produce a common set of IgE binding sites for the allergenic subset of this gene family. A further complexity is that comparing IgE binding site maps in sensitive but unrelated individuals show that while all are sensitive to the same protein the sites that their IgEs bind have both commonalities and differences (e.g. [8–10]).

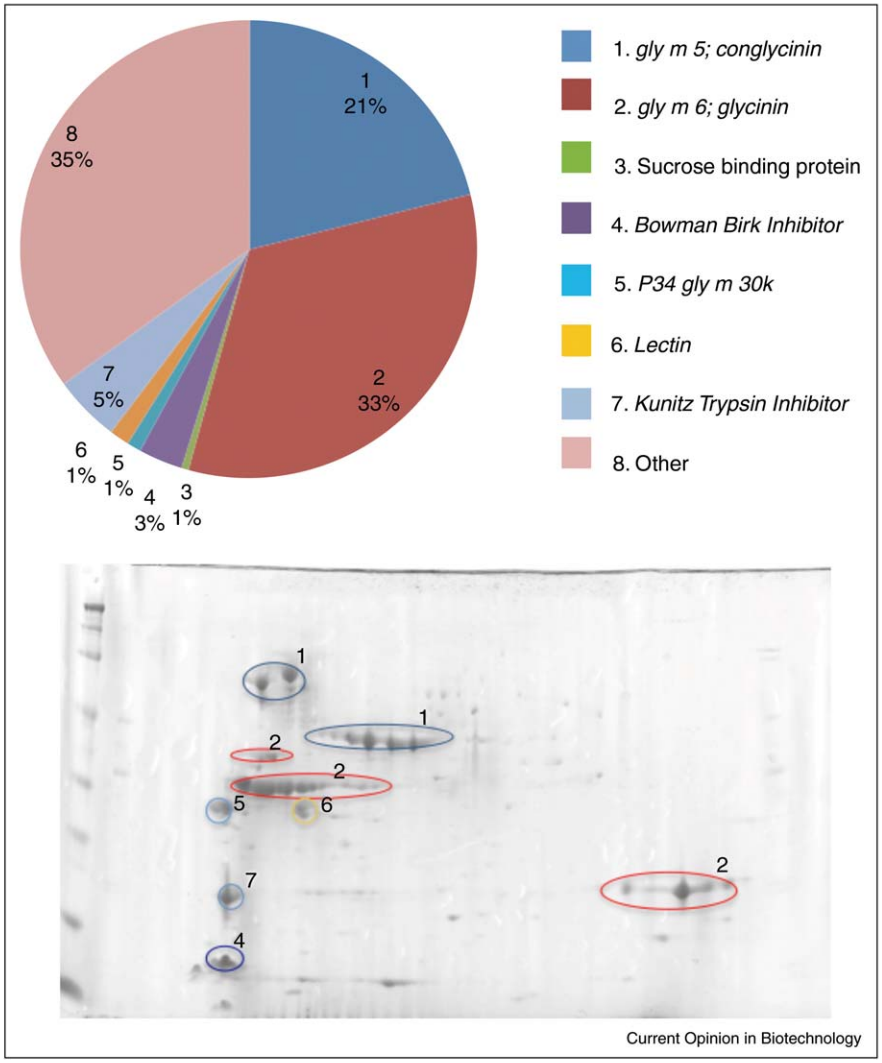
Seed storage proteins including 2S albumins, 7 S vicilins, and 11 S legumin family of proteins include the most potent of the plant allergens responsible for most plant-source induced anaphylaxis deaths. The 2S storage proteins of tree nuts as well as the 2S proteins of sunflower and peanut result in instances of anaphylaxis death. Other seeds have abundant 2S proteins including the *Brassicas* and the Cucurbit squashes are much more rarely allergenic. Similar broad allergenic responses have demonstrated with the lipid transfer proteins (LTP) with many examples from seed and from vegetative parts of the plant such as tap-roots and fruit being dominant allergens [11•]. LTP examples include carrot, peach, apple, beet-root as well as seeds including tree nuts and peanuts with some sensitive people broadly reactive to the LTPs of diverse species. Even in closely related plants such legume seeds where the 7S proteins of peanut and soybean are significant allergens while the homologous 7S proteins of the common green bean appears to be rarely allergenic.

Altering plants and their allergens to mitigate food allegenicity

Attempts have been made to reduce allergenicity by producing allergen-reduced or allergen-null plants by biotechnology or by selection as a proof of concept. These experiments have demonstrated that it is feasible to completely eliminate specific allergens from food plants. Beginning with the first attempts to partially silence the rice allergen [12,13], to completely eliminating a major allergen of soybean [14,15] as well as the subsequent suppression of major peanut [16•], tomato [17–19], and apple allergens [20] induced genetic modification has proven effective at silencing allergens. Parallel approaches searching germplasm collections of soybean has identified nulls of storage proteins [21], Gly m Bd 30k/P34 [22], trypsin inhibitor [23], and lectin [24] that could be stacked through breeding to produce seeds null for several allergens. Induced mutation populations and germplasm searches in peanut have yielded peanut lines deficient in allergenic proteins [25•,26•,27•]. This approach has been used for peanut and has resulted in identifying peanuts lines null for Ara h 2 the major demonstrated allergen [28,29•]. One of the difficulties in using genetic modification or nulls to create low-allergen or hypoallergenic seeds is that for many seeds the allergenic proteins account for the dominant portion of the seed proteome (Figure 1). This is complicated by different populations being sensitive to different allergenic proteins, for example, soybean Gly m Bd 30k/P34 in a US neonatal population [8,9,30] while soybean storage proteins as allergens in European children [31•]. In addition, different cultivated varieties and their ancestral breeding lines vary greatly in IgE binding proteins [30] complicating breeding and varietal development. With demonstration that one major allergen is sufficient to sensitize [32•], seeds with as many ten or more distinct allergens will be difficult to alter to render them low-allergen or hyperallergenic. This indicates to stack nulls to produce low-allergen content seeds it will be necessary to alter most of the seed's protein content and this will vary by species, variety, and target-sensitive population that presents a difficult management and regulatory problem. If this approach is used it raises the question of what is left of the seed protein and would such a seed be viable or useful. Further even with the suppression of one or more allergens will the remaining allergenic proteins in its matrix be sufficient to induce a hypersensitive response at some threshold that is perhaps higher than the 10 mg that is a peanut threshold [33•].

A further complication in silencing part of a seed's protein content is that seeds generally appear to compensate for a shortfall of a major protein by accumulating other seed proteins to maintain a relatively constant protein content. Soybeans with silenced β-conglycinin storage protein the protein content was compensated by increased accumulation of glycinin storage protein that maintains the normal 39% protein level [14]. β-conglycinin is an established IgE binding protein so silencing removes one allergen replaced by glycinin also an IgE binding protein. Whether this is a net loss or gain of allergenicity has not been tested on sensitized people. This observation was one of the first of what is emerging to be a broader potential problem and opportunity with strategies to alter seed allergenicity by producing nulls of major seed allergens. Because each event of silencing a major protein,

Figure 1



The IgE binding proteins of many allergenic seeds comprise the large majority of the seed's protein content. Soybean has 16 described allergens of which 7 (italics) are illustrated on the two dimensional gel of the total seed proteins. The pie chart shows the relative abundance of the 7 allergens determined by spot volume analysis that together are in excess of 60% of the total proteins. This illustrates the problem of modifying seeds to create allergen nulls with most of the protein content and valued nutritional composition being the seed allergens.

allergen or not, leads to significant rebalancing of the protein content of the seed its capacity to sensitize naïve individuals is potentially altered with a changed mixture and abundance of possibly allergenic proteins. This leads to questions of whether silencing an allergen or allergens will prove to be advantageous. Beyond IgE binding tests the allergen-content modified plants have not been tested in key aspects of food allergy including animal tests on the potential of this material to sensitize and once sensitized how is the allergy is manifested when challenged with allergen-modified as well as conventional samples.

The biology of protein rebalancing in seeds presents an opportunity to engineer an exchange of known allergenic proteins for alternate proteins with low allergenicity. The enhancement of maize's protein content and amino acid composition was demonstrated by expressing the 11S Amaranth storage protein in parallel with studies showing that the protein has low-allergenic risk [34,35]. The compensatory protein rebalancing shown with β-conglycinin nulls can be exploited to exchange for green fluorescent protein (GFP). By mimicking the glycinin gene whose gene product compensates for the absent β-conglycinin [36•]. These experiments are among the first steps in what could be a series of technical developments for engineering the seed protein content to silence allergens and exchange the allergens for other less allergenic proteins.

Immunotherapy is a promising treatment approach for pre-existing food allergy where small and increasing doses of an antigen is given to a sensitive individual to build up immunotolerance ([37•], for review). While many clinical tests of immunotherapy have been conducted with extracts of the whole allergenic source, peanut, for example, there is a biotechnological variant of this approach that may prove effective and suitable to standardize as a vaccine. With comprehensive transcript and genomic sequences the entire gene families encoding allergenic proteins have been determined. These sequences are used to produce comprehensive peptide maps of the allergen then the IgE binding sites for the gene family members can be determined (e.g. [8–10]). Sequence information for the dominant conserved IgE binding sites can be used to create epitope variants not recognized by the IgEs [38] that when assembled form an IgE binding site disarmed mutant variant of the protein. The IgE binding 'disarmed' allergen can be used as an immunotherapy vaccine to build immunotolerance to an allergen without exposure to allergenic version of the protein.

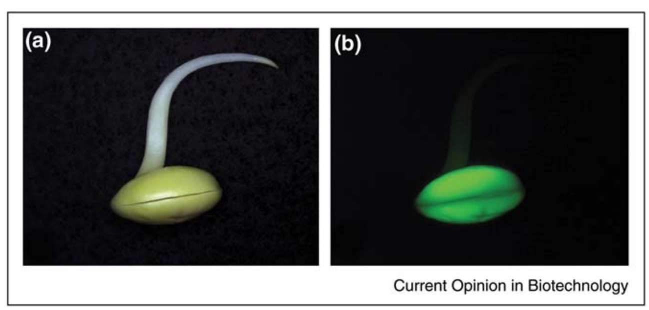
Good gut health may have an important role in impeding the acquisition of food allergy. Gut health and general health can be improved by micronutrients and there many projects underway to produce functional foods with enhanced nutrient content ([37•] for review). Among these micronutrients β-carotene produced in plants will on demand be cleaved to produce vitamin A. There are many projects underway to improve β-carotene content in plant foods of which the 'golden rice' project [39,40] is the most prominent. Recent observations suggest high levels of β-carotene may impede the development and manifestation of food allergy [41,42]. Additional attention to gut health may prove one means to lower the rate of neonates and young children acquiring food allergies.

Food allergy and the deployment of plant biotechnology

Two biotechnology-generated events catapulted the awareness of the potential for biotechnology to increased allergenic risk as a consequence of introducing new traits into plants. Most seeds do not possess an optimum balance of amino acids, fatty acids, and other constituents for use as food and feed. Among the earliest goals of plant biotechnology were efforts to rebalance essential amino acid content to be more aligned with food and feed needs. Among the strategies tested was the expression of high-sulfur content 2S storage protein genes derived from the Brazil nut in soybean. The use of this strategy was aborted when it was recognized that the 2S storage proteins from tree nuts are potent allergens and correlated with potentially lethal anaphylaxis [43]. No product containing the Brazil nut 2S protein was ever approved or released and no one was harmed but this event galvanized the awareness of the potential to introduce allergenicity by transgenic techniques. Concerns of biotechnology-introduced allergens was further raised with the Starlink episode in which a variant of *Bacillus thuringiensis* toxin (Bt) approved only for animal feed and expressed in field maize contaminated human processed food such as taco shells. This resulted in an expensive recall of products suspected containing unapproved materials, adverse press, government hearings to evaluate the event and its consequences, and there were a number of claims of allergic reaction to products that might have contained some of the Starlink maize. Subsequent analysis showed that the claims of allergic reaction were inaccurate and could not be supported by IgE binding studies [44].

Biotechnology offers the prospect to express and accumulate essentially any protein from any source in plants. Laboratory-level studies have produced antibodies, vaccines, enzymes, food/feed proteins, and many other potential products in diverse plants. Plant-based production offers the economic advantages of mass protein production using the efficient multiplier of agricultural production. The continuing use, and need, for conventional crop plants as production platforms will require protocols to evaluate the potential for novel proteins including synthetic proteins to be food allergens. Figure 2 shows a mock industrial product producing GFP at 8% protein level in soybeans by exchanging the β-conglycinin seed storage protein for GFP [36•]. GFP has been tested as potential allergen and in rat model tests it does not induce an allergenic response when produced in Canola seeds [45]. However would GFP still not be an allergen if expressed in the more allergenic soybean matrix? This is one of the significant

Figure 2



A mock industrial product test is shown with germinated transgenic soybean seeds using GFP accumulated at 8% of the total seed protein [36•]. To be economically viable transgene products will need to be accumulated as major proteins so that its potential exposure will exceed the threshold. This test illustrates the type of biotechnology outputs that will need to be evaluated for allergenic risk. The soybean seeds appear overtly normal and only when closely examined for a pale green color or fluorescent **(b)** is it obvious that they are producing and sequestering a foreign protein. GFP has been evaluated for allergenic potential and the results of these tests indicate that GFP poses little risk of inducing allergies [45]. However crucial tests of assaying GFP in the context of a food allergen crop such as soybean remain to be undertaken.

questions of plant biotechnology is whether there are any consequences of expressing even previously demonstrated safe protein in transgenic plants. The concern of introduced food allergens has broad ramifications for the deployment of biotechnology products. Will a novel protein exhibit adjuvant properties resulting in sensitization by proteins of the non-allergenic host plant. Or will the reciprocal occur with the matrix of the host plant inducing an allergic response to biotechnology products. There are no examples of this being tested although model vaccines such as plants expressing proteins with adjuvant properties such bacterial enterotoxins as vaccine prototypes [46] expressed in a wide variety of plants would be a good model for animal tests of potentially forcing allergenic sensitization.

Conclusions

The complexity of the allergy issue will influence the approval process of any transgenic that might enter the human food chain. In Europe, for instance, where the precautionary principal of guilty until proved innocent essentially requires extreme levels of proof of lack of adverse potential for approval that may make the approval of novel transgenic products almost impossible. People already consume a large fraction of the Pfam members at some level among the tens of thousand of proteins present in food. The evidence is very few are actually allergenic and moreover very few examples within a Pfam are actually allergenic. But threshold, exposure, and context still limit the assessment of potential allergenicity to probability. From a probability perspective it is then likely that few if any plant biotechnology products will prove to be potent allergens unless a potent allergen is the transgene product but still some testing is necessary for the approval process even in nations where deploying biotechnology is accepted and well developed [47•,48•].

Acknowledgement

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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The on-line interactive compilation of all protein families known derived from the genome database in its current 2010 version that is the starting place to examine protein sequence and interrelationships that provides the foundation to create descriptions of the set of proteins that are allergens.

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An on-line allergen atlas provides resource information for a comprehensive list of allergens arranged by allergen name. Key word searches and BLAST searches can be performed.

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Non-specific lipid binding proteins are probably ubiquitous in plants and the LBPs are among the major allergens of many plants ranging from seeds to fruit. Why some species' LBPs are highly and potentially dangerously allergenic while others are not remains an open question. This paper has examined a few of the most allergenic LBPs to look for common features in the subset of LBPs that are significant allergens.

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This paper shows results on reducing the major peanut allergen using transgene silencing. The silencing was significant but not complete leaving some Ara h 2 in the peanut that depending on the threshold for a response may or may not be significant in a theoretical accidental exposure. The paper also shows that the reduction of Ara h 2 leads to changes in abundance other peanut seed proteins that are also known

IgE binding proteins leading to the question of how a seed with a rebalanced protein/allergen compensation may yield altered sensitization and hypersensitive response outcomes.

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This is a significant attempt to stack allergen nulls in an effort to design a less allergenic peanut. However like the Dodo *et al.* results this also leads to rebalancing the protein composition with other proteins many if not all being known allergens replacing the absent proteins. This variant of a peanut needs to be tested for its capacity to sensitize in addition to it being tested on the binding of IgE proteins from already hypersensitive individuals.

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The authors have searched a peanut collection and isolated a variation of the immunodominant Ara h 2 that has much reduced binding of IgE from peanut sensitive patients. This is an important paper showing that conventional plant breeding approaches could be used to alter the immunoreactive characteristics of peanut. There is the potential to introgress this gene into cultivated peanut exchanging the allergenic version of Ara h 2 for this version. Ara h 2 is a dominant peanut allergen and its absence either by suppression or by alternate less allergenic version would hypothetically reduce the allergenic risk of peanut. Whether this would indeed lower the risk to sensitized individuals remains to be investigated. How this Ara h 2 variant will be perceived by a naïve immune system for sensitization also remains a question. But the potential to engineer less allergenic versions of peanut and other plant allergens is an approach that should continue to be explored and supported.

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This paper shows an approach that could be easily adapted for higher through-put searches of protein composition that provides a technical means to search through large collections of either naturally occurring nulls or in TILLING mutation collections generated to identify lines with altered protein composition such as the null of allergen(s).

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This paper indicates that the reduction of one of the peanut allergens Ara h 1 is not sufficient to impact the overall allergenicity of peanuts. Almost the entire protein content constitutes the allergome of peanut so the altering of one of the many abundant allergens is not sufficient to impact the peanut's allergenic risk.

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This paper shows that in contrast to the neonatal allergenic response to soy in which Gly m Bd 30k/P34 has a dominant role for US resident/tested infants a test group of European children exhibited strong responses to the two major soybean storage proteins conglycinin and glycinin both of which have been subjects of successful experiments to either silence the proteins by biotechnology or to select varieties that are null. This paper shows that it is possible to design test situations to evaluate plant science based approaches to modify allergen content with specific allergenic responses shared by large populations of sensitive people.

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This paper shows that one of the major soybean allergens the storage protein alpha subunit of beta conglycinin can when produced in a prokaryotic expression system that would not be expect to correctly fold the protein still can by itself separated from the rest of the soybean matrix produce IgE-mediated hypersensitive response to sensitized rats. The ability to conduct animal assays together with the plant biotechnology capacity to alter expression of allergens in plants such as the seeds of soybean could be used synergistically to examine a wide variety of questions of sensitization and response.

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This paper illustrates how small a quantity (approx. 10 mg) of peanuts can provoke anaphylaxis reaction that significantly impacts strategies to produce low or hyperallergenic foods such as peanut. If breeding or creating low-allergen/hyperallergenic foods its threshold for response could be much higher but even two orders of magnitude is only 1 g showing just how difficult and problematic modifying foods to lower the risk for potent dangerous allergens will be.

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This paper describes a means to engineer seed protein composition using a predictable system of exploiting the rebalancing that occurs when one storage protein is silenced and other seed proteins increase in abundance to compensate. The results show that a foreign protein allele can be incorporated into the rebalancing process leading to a quantitatively reproducible change in the seed's protein composition leading to establishing a means to engineer an altered composition that could include replacement of allergenic with nonallergenic proteins.

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