[Frontiers in Bioscience 14, 59-71, January 1, 2009]

Genetic engineering of plant food with reduced allergenicity

Stephan Scheurer[1], Sophia Sonnewald[2]

[1]Paul-Ehrlich-Institut, Division Allergology, Paul-Ehrlich-Str. 51-59, Langen, Germany, [2]Friedrich-Alexander University Erlangen-Nueremberg, Staudtstr. 5, Erlangen, Germany

TABLE OF CONTENTS

1. ABSTRACT

Food allergies are a major health concern in industrialized countries. Since a specific immunotherapy for food allergies is not available in clinical routine praxis till now, reduction of allergens in foods, either by food processing or genetic engineering are strategies to minimize the risk of adverse reactions for food allergic patients. This review summarizes biotechnological approaches, especially the RNA interference (RNAi) technology, for the reduction of selected allergens in plant foods. So far, only a limited number of reports showing proof-of-concept of this methodology are available. Using RNAi an impressive reduction of allergen accumulation was obtained which was stable in the next generations of plants. Since threshold doses for most food allergens are not known, the beneficial effect has to be evaluated by oral challenge tests in the future. The article critically addresses the potential and limitations of genetic engineering, as well as of alternative strategies to generate "low allergic" foods.

2. INTRODUCTION

Food allergies are an increasing problem in industrialized countries (1). Currently 8% of the children and 2% of adults are allergic to at least one food source (2). Immediate type (IgE-mediated type I) food allergies are caused by an abnormal immunological response to natural occurring (glyco-) proteins in foods. Allergic reaction can be manifested by local or systemic reactions with symptoms ranging from mild Oral Allergy Syndrome to severe life-threatening anaphylactic shock reactions. Some food allergies are of certain clinical relevance because of their persistence after childhood (e.g. peanut) and as fundamental dietary products (e.g. soy, rice and wheat) in specific geographic regions or in certain developmental stages of life. Specific immunotherapy (SIT) is the only causative measure in treatment of type I-allergy to inhaled allergens and insect venom, but is currently not available for food allergy because stable extracts from plant foods standardized for the relevant allergens are missing

(reviewed in 3) and the risk of severe side effects. The only "therapeutic" approach in affected individuals with food allergy so far is avoidance of the respective food from the diet (4, 5). Elimination of a number of foods in the diet can cause deficiencies and nutritional disorders, especially for children, and may restrict social activities and contacts. Thus, there is a strong demand for the development of food with reduced allergenic potential.

Allergy prevention by reduction the content of allergens in foods comprises an elimination of allergens or a reduction of the IgE-binding properties during food processing, selection of low allergenic cultivars or genetic engineering of plants. Several research programs aiming at improved understanding of allergenic structures and at exploiting strategies to modulate their presence in processed and fresh food. Reduction of allergenic potency by food processing, e.g. by thermal treatment, is often limited by the high stability of certain allergenic proteins, e.g. of the non-specific lipid transfer protein (nsLTP) family (reviewed in 6). In addition, cross-reactive carbohydrate residues bound to proteins are known to be heat stable and may contribute to clinical relevance in several patients (7). Moreover, products obtained after thermal processing of foods by non-enzymatic browning, the so called Maillard reaction products, can enhance the allergenic activity (reviewed in 8).

The selection of cultivars with low amount of allergens is another strategy which was followed in fruits, e.g. in apple (9, 10). Whereas low allergenic cultivars could be identified for apple which would allow breeding of hypoallergenic cultivars, different sweet cherry cultivars revealed an overall similar content of major allergens (11). However, such low allergenic cultivars do not differ from allergenic cultivars by their genetic background, but rather by organ specific allergen accumulation and by allergen expression depending on cultivation or storage. Beside, this strategy is time-consuming and requires a large population of plants for selection. Therefore, genetic engineering is addressed as a promising approach to generate plant-derived foods with reduced amount of allergenic proteins. Since food allergies represent an increasing health issue, reduced allergenicity by means of genetic modification would provide an alternative to food avoidance and thereby improve the life quality of patients with food allergy.

3. REDUCTION OF PLANT ALLERGENS BY RNA INTERFERENCE

3.1. Genetic engineering / RNA silencing in plants

In comparison to conventional breeding, genetic engineering allows the specific and quick introduction of novel traits into the plant genome. Since the development of plant transformation techniques about 20 years ago a vast number of transgenic plants have been generated. Genetically modified (GM) plants were initially engineered to improve the agromonic performance and conferred e.g. resistance to herbicides or insectices or an enhanced tolerance to abiotic stress factors. These so called GM plants of first generation were primarily beneficial to producers, whereas GM plants in subsequent generations are intended to provide advantages for the consumers. For instance, GM plants have been created with increased nutritional value, higher amounts of health promoting substances like vitamins (12, 13) or lower amounts of allergenic proteins (see below). In addition plants have been exploited for the production of pharmaceutical or industrial valuable proteins by molecular farming (14, 15).

Plants are genetically modified by either non-directed mutagenesis, overexpressing heterologous proteins or by inhibiting expression of endogenous genes. Initial approaches of gene silencing were based on antisense or co-supression technology by introducing plasmid DNA constructs producing antisense or sense RNA, respectively. The co-suppression phenomenon was accidentally discovered in plants that were designed to overexpress an enzyme to increase flower pigmentation (16). Instead the inverse effect was observed and both the transgene and the endogenous gene were found to be silenced. These unexpected results were eventually explained by the formation of double-stranded (ds) RNA that trigger homology dependent RNA degradation. In fact, both the co-suppression and antisense phenomena are collectively refered to as post-transcriptional gene silencing (PGTS) that results in sequence-specific inhibition of gene expression (reviewed 17,18).

In recent years the RNA interference (RNAi) became a more efficient tool of gene silencing and is now widely used to engineer desired traits in crop plants (see recent reviews 19, 18). RNAi is a gene silencing mechanism that is mediated by endogenous dsRNA molecules. It is thought to be naturally involved in defence against invasive nucleic acids such as viruses or transposons as well as in post-translational regulation of gene expression and in epigenetic control of chromatin structure. Recent work revealed at least three natural RNA silencing pathways in plants (reviewed in 20): (I) Cytoplasmic RNA silencing by dsRNA results in mRNA cleavage and is known as PGTS. This pathway seems to be important in virus infected plants favouring viral spreading, or as a cellular induced antiviral response to inhibit the spread of infection. Moreover, RNA silencing comprises (II) gene regulation by silencing of endogenous messenger RNA (mRNA) by micro-RNA (miRNA) or (III) by suppression of transcription (transcriptional gene silencing) by sequence-specific DNA methylation. A common feature of all pathways is the formation of dsRNA which are processed into short 20-26 nucleotide (nt) fragments (sRNA) or miRNA triggering sequence-specific gene silencing. However, this is a flourishing research area with new pathways and components are still being discovered shedding more light on the mechanisms and biological roles of RNA silencing in plants (for recent review see 21).

In plants RNAi is achieved through hairpin (hp) constructs which produce ds RNA. Initially plants were seperately transformed with a sense and an antisense construct and dsRNA formation was induced by subsequent crossing (22). This study demonstrated that simultaneous expression of both polarities is more effective in gene silencing than either of the polarity alone. In a following

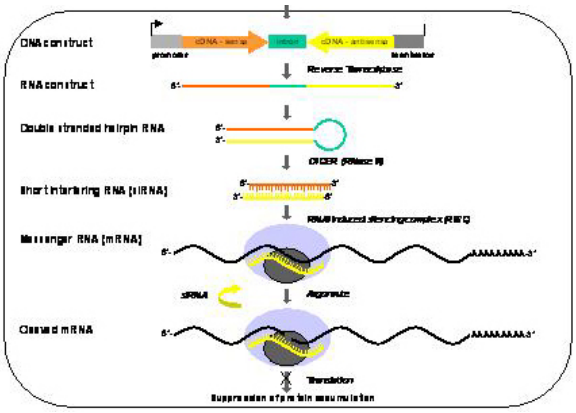

Figure 1. Cytoplasmatic RNA silencing of endogenous target proteins by introduction of double-stranded RNA into cells. Further details are explained in the text.

paper Smith NA *et al.* (23) showed that constructs containing both sense and antisense sequences separated by a spliceable intron can induce almost 100% efficiency in gene silencing and this is now the most widely used system for gene silencing in plants. Although the mechanism is not clearly understood (21), a general overview is depicted in Figure 1. In a typical construct the target gene (or parts of it) is cloned as an inverted repeat spaced with an unrelated spliceable intron under control of a strong promoter, e.g. the 35S promoter of the cauliflower mosaic virus (CaMV) for dicots or an ubiquitin promoter for monocots. In the host cell the RNA is transcribed and produces hp RNA with a dsRNA region. Subsequently, dsRNA molecules are cleaved into short fragments of 21nt termed short interfering (si) RNA by the enzyme dicer, a member of the RNase III family. One strand (guide or antisense strand) is then incorporated into the RNA-induced silencing complex (RISC) with Argonaute as a major component and is guided to the endogenous mRNA. The mRNA is then cleaved in sequence-specific manner resulting in gene silencing.

The selective and robust effect of RNAi on gene silencing makes it a valuable research tool, both in cell culture and in living organisms; synthetic dsRNA introduced into cells can induce suppression of specific genes of interest. RNAi is also used for large-scale screens to identify gene function. Here it is an easy, cost-effective approach to knock-down the expression of hundreds or thousands of genes and offers several advantages, e.g. its applicability to multigene families and polyploids, to the widely used insertional mutagenesis approaches (24, 18). The most commonly used techniques thereby are transposon mutagenesis and *Agrobacterium* mediated T-DNA insertions (25, 26). However, these techniques to generate non-directed mutations are only applicable to few model plant species such as Arabidopsis or Rice and due to the low frequency of mutation large numbers of plants in a mutagenized population need to be screened. A good overwiev over the different techniques is given by Alonso and Ecker (26). In the past few years a another strategy was developed called TILLING (Targeting Induced Local Lesions in Genomes) which combines chemical mutagenesis with high throughput screening for point mutation using a mismatch-specific endonuclease (27, 28). It is applicable to virtually any organism and promises to be an attractive strategy for both functional genomics and agricultural applications. Another approach to silence genes in plants is by virus induced gene silencing (VIGS). Here, target genes will be inactivated by infecting plants with a recombinant virus carrying a partial sequence of the endogenous host gene. This approach is only transient and is therefore very useful for studying function of essential genes, but it cannot change the genetic information for crop plant improvement (17).

Taken together, RNAi is an established tool to engineer desired characteristics in various plants species and is also promising for generation of plant foods with reduced allergenicity. However as discussed in the next chapters there are some "prove-of-principle" studies demonstrating the power of this technique, but there are also potential disadvantages which limit their commercial application and need attention.

3.2. Suppression of allergens in plant food by gene silencing

Two decades ago, the antisense technology (29) was applied to inhibit gene expression in tomatoes (30). In 1994 the FlavrSavr® tomato (*Lycopersicon esculentum*) received FDA approval and was introduced to the market of the US by Calgene Inc. (CA, US) as the first genetically modified plant food product (Campbell) under the trademark MacGregor™. Two years later Zeneca Plant Science (Syngenta) introduced a tomato puree derived from transgenic tomatoes to the market in Great Britain. In both cases a polygalacturonase (PG2A), an enzyme triggering the degradation of the cell wall and inducing ripening, was suppressed by the antisense strategy to slow down fruit maturation and to prevent fruit softening. Both products can be considered as first examples of hypoallergenic food, since PG2A later was described as a potential allergen in tomatoes (31, 32). Finally, the cultivation of the FlavrSavr® tomato was stopped, because of an increased susceptibility to pathogens and low yields in commercial production. Although initial marketing of the tomato puree by Sainsbury's and Safeway was very successful and shared up to 60% of the market, it was ceased in 1999 because of low public acceptance of GM food.

Today, approaches for the suppression of selected allergens by means of genetic engineering have been published for rice, soybean, peanut, *Rosaceae* fruits (cherry and apple) and tomatoes. Most commonly, transgenic plants are generated using *Agrobacterium tumefaciens*-mediated gene transfer. *Agrobacterium tumefaciens* possesses the natural ability to transform plant host cells by transferring and integrating a segment of bacterial DNA (referred to as T (transfer)-DNA) present on a Ti- (tumor inducing) plasmid into the plant genome. Thereby it modifies the host metabolism to its own benefit and causes grown call disease. The ability of *A. tumefaciens* to genetically modify plant cells has been exploited and was used to develop a vector-based transformation system (for

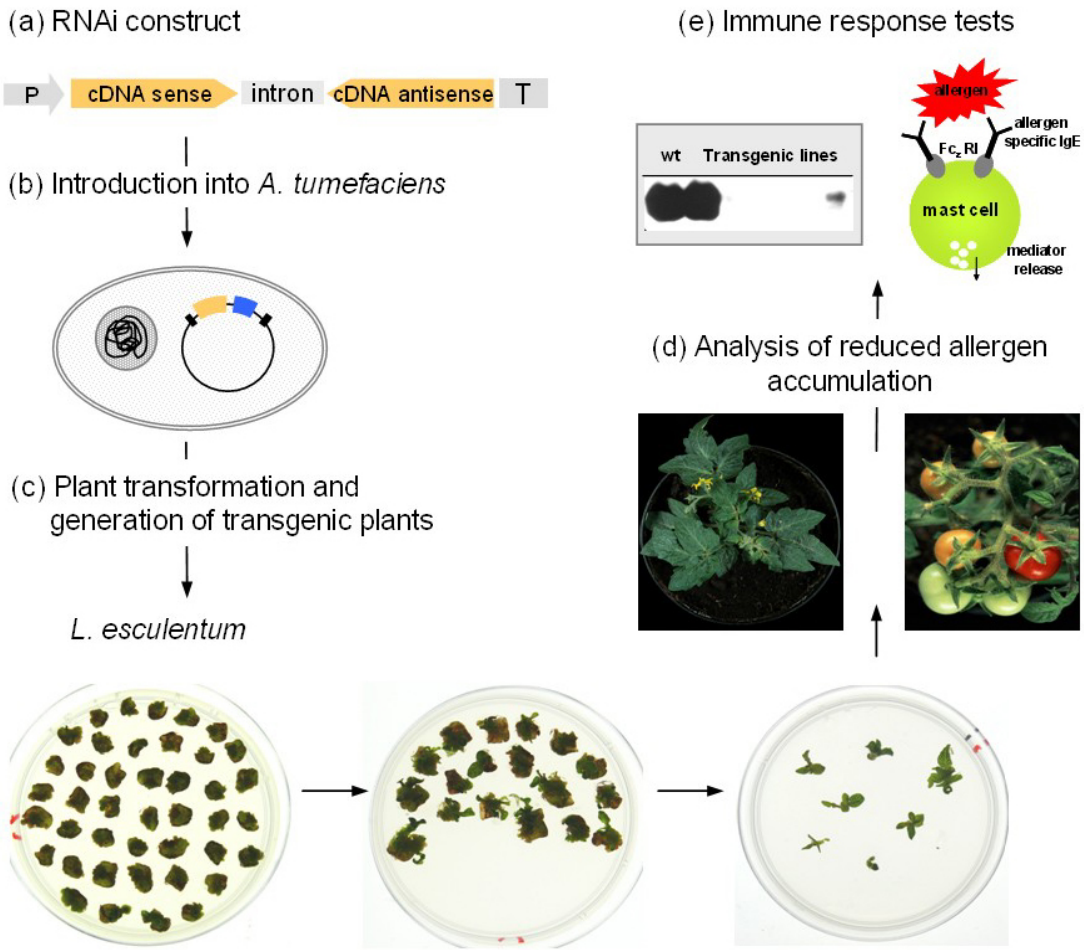

Figure 2. Generation of transgenic plants by *Agrobacterium tumefaciens* mediated gene transfer using tomato plant as an example. (a) Generation of an RNAi construct with target cDNA in inverted repeat orientation. (b) Transfer of the expression construct into *Agrobacterium tumefaciens*. The plant transformation vector carrying the RNAi construct (orange), a marker gene for selection of transgenic plants (blue) between left and right borders (black) being important for the integration into the plant cell genome. (c) Plant transformation and generation of transgenic plants under sterile conditions: Callus formation (left) is followed by differentiation of shoots (middle) and roots (right). Transformed plants can be selected using a marker gene conferring e.g. antibiotic resistance. Subsequently plants can be transferred into soil and allowed to form fruits. (d) Analysis of allergen accumulation in fruits can be performed by immunoblotting or by functional assays (histamine release triggered by allergen-induced crosslinking of FcεRI (high affinity receptor for IgE) -bound specific IgE on effector cells (e).

recent review see 33 and 34). Crucial experiments towards this purpose were the finding that (i) foreign DNA inserted into the T-DNA is also transferred to the nucleus and (ii) that removal of bacterial genes does not impede the ability to transfer DNA but rather prevents tumour formation. Nowadays used binary vectors retained only the T-DNA borders of the original T-DNA, which are important for the integration into plant genome flanking the gene construct to be transferred. Often the insert also contains a marker gene for selection of transformed plants. Furthermore the vectors are provided with a replicon allowing replication in *Agrobacterium* and *E. coli* and selection markers. All functions necessary for transfer of the T-DNA are present on a separate, "disarmed" Ti-plasmid (helper plasmid) usually residing in the *Agrobacterium* strain. Thus, a target gene (e.g. a cDNA coding for an allergenic protein) is cloned into a binary plasmid vector under control of a suitable promoter. Subsequently the plasmid is transformed into *A. tumefaciens* cells. For plant transformation, *A. tumefaciens* cells carrying the gene of interest are incubated with wounded plant explants in sterile tissue culture. Transformation of plant cells occurs by mobilisation of the T-DNA and transfer to the plant cell nucleus via the *Agrobacterium* encoded type IV secretion system, where the T-DNA integrates into the genome of the cell. The transformation of explants is followed by regeneration of whole plantlets and selection of transgenic plants using marker genes conferring antibiotic or herbicide resistance. When shoots and roots are well developed plantlets can be transferred to soil and plants can be screened for the desired trait (see Figure 2).

First reports for a targeted suppression of an allergen by genetic engineering came from **rice** (*Oryza*

sativa) (35, 36). In rice a 14-16 kDa alpha-amylase/trypsin inhibitor has been identified as an IgE-reactive protein in patients allergic to rice. Gene expression was subsequently repressed by applying an antisense construct in tandem repeat orientation. The antisense construct was cloned under the control of various rice-seed specific promoters and introduced into rice cells by electroporation. Transgenic seeds were analyzed by immuno- and northern blotting. ELISA analysis with a monoclonal antibody showed reduced protein accumulation of the alpha-amylase/trypsin inhibitor in seeds from 300 µg to 60-70 µg. The reduction was only moderate with approximately 20% remaining allergenic protein, but was shown to be stable for three generations. However, the IgE reactivity of transgenic lines was not investigated (35, 36). Until now only two rice allergens, namely the 35 kDa Ory s 1 and the 14 kDa Ory s 12, are listed in the IUIS allergen data base. The clinical relevance of the 14-16 kDa allergenic alpha-amylase/trypsin inhibitor has not been proven yet. Apparently, suppression of rice allergens was not preceded in subsequent studies.

Soy provides essential nutrition values for many infants and the Asian population. Soy is ubiquitous used in processed food because of its high protein content and the good physicochemical properties desired in food processing. **Soybean** (*Glycine max*) possesses at least 15 IgE-reactive proteins, including the alpha-subunit of conglycinin and a member of cysteine proteases termed P34/Gly m Bd 30k, which accounts for the majority of IgE-reactivity (37). In soybean, both genetic and reversed genetic approaches have been followed to reduce accumulation of major allergens. Using a genetically mutated soybean cultivar lacking the alpha-subunit of conglycinin, Samoto M *et al.* (38) described the additional removal of Gly m Bd 30K (P34) by purification using a simple centrifugation step in order to obtain an allergen reduced soybean extract. Attempts to identify P34/Gly m Bd 30k negative soybean varieties failed, probably due to the high number of distinct linear epitopes which should have been altered simultaneously in a hypoallergenic variant (39). Following a co-suppression approach using a part of the Gly m 30K cDNA Herman EM *et al.* inhibited expression of Gly m Bd 30K (P34) in seeds of transgenic soybean plants (39, 40). The authors observed no developmental changes of the plant or seeds as well as no changes in seed protein composition as indicated by 2D-SDS PAGE. In addition, suppression was stable over three years. Transgenic soybeans revealed no IgG and IgE reactivity using sera from soybean-sensitive patients. Functional tests, such as the stimulation of human effector cells to demonstrate the reduction of allergenic potency have currently not been reported.

First reports of using the RNAi technology for suppression of allergen gene expression were described for Ara h 2 (41), a major **peanut** (*Arachis hypogea*) allergen, at the annual meeting of the American Academy of Allergy & Immunology 2002 (42). Ara h 2 (conglutin) is one of the most potent peanut allergenic proteins recognized by more than 81% of peanut allergic individuals (43). Unlike other food allergies, peanut allergy does not become lost during childhood and frequently persists in adults. Moreover, accidental ingestion of peanut is very likely because peanuts are added to a wide number of processed foods and can therefore be present as a hidden allergen contaminant. Accidental ingestion of peanut in allergic subjects can elicit severe symptoms depending on the threshold doses (44). Hence, a genetic engineering strategy was proposed to reduce allergens in peanut seeds. In a first approach a 430 bp genomic fragment of Ara h 2 was cloned in sense and antisense orientation driven by the CaMV 35S promoter and introduced in embryogenic peanut tissue by particle bombardment (45). The Ara h 2 fragment was produced from a cDNA stretch of a highly conserved region between known isoforms. Microprojectile-mediated co-transformation of the *htp* marker gene allowed the selection of positive transformants by hygromycin. Southern blots with genomic DNA from callus showed a stable integration of the Ara h 2 cDNA fragment into the genome, and PCR screening revealed up to 75-85% transgenic lines. Following northern blot analysis of vegetative tissues confirmed reduction of Ara h 2-mRNA for several transgenic lines. Remarkably, the transgenic plants developed no seeds. Unfortunately, the findings presented in the paper were not substantiated by experimental data. Very recently the same authors provide promising results showing the production of transgenic peanuts seeds with a reduced accumulation of Ara h 2 by *Agrobacterium tumefaciens* mediated gene transfer using a 265 bp genomic DNA cloned as inverted repeat (46). In contrast to the ballistic approach this protocol enables the production of transgenic peanut plants within 5 to 7 months, instead of at least 10 months. PCR analysis of leaves and subsequent probing of the PCR product by Southern blot hybridization, in order to verify the PCR band, were performed to screen kanamycin-resistant plants for the presence of the transgene in T_0 plants. A sandwich-ELISA showed a suppression of Ara h 2 from 27.73% and 57.17% Ara h 2 content in peanut protein extract derived from wild-type and negative controls plants, respectively to less than 13.26% in transgenic seeds. This assay was more sensitive than an immunoblot, which could only detect an Ara h 2 content higher than 10%. Using sera from five peanut allergic patients in an indirect ELISA the overall IgE-reactivity for most transgenic seeds was significantly lower than for the control samples. Since the Ara h 2 content was reduced to comparable values in the tested seeds, the authors conclude that patients were obviously sensitized to additional peanut proteins. An interesting observation was the difference in the suppression level of two seeds derived from the same pod, which was regarded to the heterozygosity of the T_0 transformants. Although the transgene did not adversely affect the agronomic performance and the phenotype (growth, morphology, reproduction, flowering, and number of pods) the authors noted different protein profiles of some transgenic plants in comparison to the wild-type controls. Further molecular and immunological analyses including *in vivo* assays are necessary for a detailed characterization of the transgenic plants. Noteworthy, silencing of allergen genes in transgenic peanut seeds was applied in an US patent (No.: 6.943.010, September 13, 2005). The patent is addressed to suppress expression of a set of peanut allergens (single or multiple genes), using antisense and/or

sense technology, and using both tissue-specific constitutive and inducible promoters, and different transformation strategies.

RNA interference has been also used to decrease the accumulation of the major allergens Pru av 1 and Mal d 1 in transgenic **cherry** (*Prunus avium*) (11) and **apple** (*Malus domesticus*) (47), respectively. Both allergens are members of the pathogenesis-related protein 10 family (PRP-10) and share homology with the major birch pollen allergen Bet v 1. Primary sensitisation to Bet v 1 and subsequent IgE-crossreactivity with the homologous food allergens is the cause for clinical manifestation of birch pollen associated food allergy (reviewed in 48). For either silencing approach intron-containing hp constructs of Pru av 1 or Mal d 1 were designed. For cherry only the transformation was achieved but no experimental data concerning reduced allergenic potential were obtained. A more comprehensive study was presented for apple. Due to the long regeneration time of apple only leaves were tested for a reduction in allergen accumulation. Six of nine transgenic apple lines were positively screened by RT-PCR, and five out of six transgenic lines showed reduced skin reactivity by prick to prick testing with leaves (47). Three patients were tested each with three transgenic lines resulting in a score of <0.25 in at least one tested line. Judged by the results of skin prick tests and immunoblotting, Mal d 1 expression in leaves was substantially reduced. An approximately 10-fold reduction of Mal d 1 was extrapolated by immunoblotting with a Mal d 1-reactive monoclonal antibody and patient sera. The transgenic plantlets exhibited a normal phenotype and growth rate. However, these promising results need to be confirmed in fruits of transgenic apple trees to support the feasibility of producing hypoallergenic Mal d 1 deficient apples by gene silencing. Although low IgE-binding isoforms have been described in the same organism, IgE-binding frequently is not restricted to certain isoforms of a protein family. Therefore, gene silencing approaches should not discriminate between isoforms and targ*et al*most all allergenic isoforms of the respective protein family. In the study by Gilissen et. al. (47) a siRNA construct derived from Mal d 1 cDNA from cv. Gala was able to suppress Mal d 1 mRNA in cv. Elstar, because of a stringent sequence identity. It is likely to speculate that, in the same manner as for homologous proteins, the high sequence identity between isoform is sufficient for an overall silencing of known allergenic Mal d 1 isoforms in a certain apple strain.

The most comprehensive studies applying RNAi for gene silencing of allergens were performed using **tomato** *(Lycopersicon esculentum*). Tomato (cv. Microtom) was selected for proof-of-principle studies since they belong to a well characterized plant species with established transformation and regeneration protocols. In addition, suitable promoters for organ specific and systemic expression are available, and the development of fruits is very fast. Moreover, the prevalence of tomato food allergy is calculated with 1.5 – 16 % among food allergenic populations (49, 50). Le QL & Lorenz Y *et al.* (51) showed for the first time a substantial suppression of an allergen in fruits. Transgenic tomato plants constitutively expressing an hp RNAi construct targeting Lyc e 3, an allergenic non-specific lipid-transfer-protein (nsLTPs), showed an decreased accumulation of nsLTP in the peel of tomato fruits which was less than 0.5% compared to wild-type plants. Interestingly, both Lyc e 3 isoforms Lyc e 3.01 and Lyc e 3.02 showing 76.5% base pair identity were efficiently silenced using one RNAi construct (51). Here, the presence of five stretches with identities of more than 15 nucleotides might be sufficient to direct the RISC complex to both target mRNAs. The hypothesis is supported by Thomas CL *et al.* (52) who described that a lower size limit of 23 nt of complete identity an will enable an effective suppression. Functional *in vitro* histamine release assays using human basophils were performed to prove whether the decrease of allergen expression can be correlated with a decrease of allergenic potential. Using two patient sera with reactivity to Lyc e 3 revealed that the allergenic potency of nsLTP Lyc e 3-deficient tomatoes was reduced 10 – 100-fold, meaning that in comparison to wild-type fruits a 10 - 100 times higher amount of protein extract derived from transgenic tomato fruits were necessary to trigger similar amount of histamine release (51). The same authors were able to show that allergen suppression is stable in tomatoes of the T_1 (53) and T_2 generation (Le et. al, unpublished). Lorenz Y *et al.* (53), for the first time tested genetically modified foods with reduced allergenic potential in humans. Prick-to-prick tests were performed with transgenic tomato fruits after removing the seeds with five tomato allergic patients with sensitization to LTPs (Table 1). Skin reactivity showed a significant and remarkable reduction in four out of five patients investigated, whereas three patients were negative (wheal diameter <3 mm). The reduced skin reactivity confirmed the diminished allergenic potency estimated previously *in vitro* histamine release assays. Residual allergenic activity of transgenic fruits might be contributed by (I) additional tomato allergens the patients are concomitantly sensitized to, (II) residual LTP accumulation, or (III) up-regulation of additional IgE-binding proteins. So far no induction of other IgE-binding proteins could be detected by 1D SDS immunoblotting in the peel of tomato fruits, whereas the pulp was not investigated. Although residual Lyc e 3 can not be detected by means of immunoblotting, competitive ELISA revealed a residual LTP amount of 0.02% (content in fresh weight).

In addition to nsLTPs, tomato profilin (Lyc e 1) (54) has been targeted for gene silencing by RNAi in transgenic tomato plants (55). In tomato two Lyc e 1 isoforms (Lyc e 1.01 and Lyc e 1.02) have been identified. Profilins are panallergens with a strong IgE-cross-reactivity between unrelated species. The clinical relevance seems to be more pronounced in exotic fruits, where they had been described as major allergens (prevalence of sensitization >50%) (56). As a consequence of a less efficient suppression of profilin accumulation in the peel using a fruit specific promoter (Sonnewald U. *et al.* unpublished), the constitutive CaMV 35S promoter was used to knock-down the profilin expression. To this end, a 300 bp cDNA fragment of Lyc e 1.02 was inserted in sense and antisense orientation separated by an intron into a plant expression

Table 1. Transgenic tomatoes with reduced Lyc e 3 expression reveal a diminished allergenic potency

Prick-to-prick	P1	P2	P3	P4	P5
Wt (cv. Microtom)	10.3 (100%)	8.0 (100%)	12.3 (100%)	12.5 (100%)	6.0 (100%)
Lyc e 3_RNAi (cv Microtom)	2.0 (19.4%)	2.3 (28.8%)	5.8 (47.2%)	10.0 (80%)	1.8 (30%)
Wt (commercial)	7.5	7.5	11.0	13.0	7.5
Histamine	8.0	8.0	7.0	10.0	8.5

Skin reactivity after prick-to-prick testing of transgenic tomatoes (cv. microtom) with reduced Lyc e 3 (nsLTP) accumulation in comparison to wild type (wt) tomatoes (microtom and commercial tomatoes from the market). Histamine dihydrochloride 10 mg/ml served as positive control. The skin reactivity with a wheal diameter >3 mm was considered positive. Skin testing was performed with tomato allergic patients with positive IgE-response to tomato (CAP testing) and confirmed sensitization to Lyc e 3. Prick-to-prick tests were performed in duplicate or triplicate, Data kindly provided by E. Ernesto, Castellon, Spain (53).

vector. Since both isoforms share 88% cDNA identity and approximately 84% identity within the selected region, the RNAi construct was sufficient to suppress both isoforms. RNAi-mediated gene suppression resulted in a 10-fold reduction of profiling protein accumulation and reduced skin reactivity. Consistent with the essential cellular function of profilin, transgenic lines exhibited severe growth retardations such as late flowering, reduced biomass accumulation and impaired fruit and seed development. Profilins are ubiquitous proteins involved in actin-binding and regulation of the cytoskeleton function as well as in signal transduction. To overcome the growth phenotype, approaches were currently initiated to prove whether a non-allergenic variant can substitute the biological function of profilin in these transgenic tomato plants without introducing a novel allergen. Yeast profilin showing only 33% amino acid identity, but a conserved tertiary structure seems to be a promising candidate since it revealed no obvious IgE-binding capacity (Sonnewald U. *et al.* unpublished).

Besides molecular approaches to inhibit accumulation of food allergens, silencing of a major pollen allergen, Lol p 5, has been reported for **ryegrass** (57). Lol p 5 showing an IgE prevalence of 90 % in ryegrass pollen allergic patients contributed to 66% of the overall IgE reactivity to ryegrass. Silencing of Lol p 5a was performed by antisense strategy after microprojectile bombardment, under the control of the pollen specific promoter Ory s1 (58). No remaining Lol p 5 was detectable using specific monoclonal and polyclonal antibodies in pollen extracts and pollen development was not effected. The authors observed a reduced IgE binding capacity in immunoblots in a study with 12 patient sera. Moreover, the authors showed that the antisense RNA construct induced suppression of the isoforms Lol p 5a and b, both with a cDNA sequence identity of 70% (reviewed in 59).

3.3. Potential and limitations of RNAi mediated suppression of allergens in plant food

As shown above, the RNAi technology has been used in several studies to provide proof of principle and to demonstrate its potential to reduce allergens in several plants severing as important source of foods. However, the technology also bears some limitations (regarding technical and clinical issues) for the production of foods with low allergenicity which have to be considered very critically, before bringing them on the market.

Allergic patients are frequently sensitized to multiple proteins in one food source. So far, four allergens from soybean, apple and cherry, each, are listed in the Allergen Nomenclature Sub-committee of the International Union of Immunological Societies (IUIS) (www.allergen.org). Today, three allergens from tomato and eight allergens from peanut are included in the official allergen database, but there is strong evidence for a larger number of allergens (31, 32). Although the clinical relevance of individual allergens is different and patients showed different sensitization profiles in regard to the number of allergens sensitized, gene silencing of a single allergen, even suppression is almost complete, will not automatically abolish the overall IgE-reactivity of the food. RNAi offers the possibility to inhibit expression of multi-gene families by a single siRNA or because of the small size of the fragments required it allows also silencing of multiple target genes using one RNAi construct. Simultaneous inhibition of gene family members can be achieved by selecting nucleotide regions of high homology often found within coding regions. Selecting less conserved 5´- or 3´-untranslated regions theoretically allows inhibition of individual members of gene families. Multi-target silencing can be accomplished by construction of chimeric hp RNA molecules composed of synthetic oligonucleotide stretches specific for the selected target genes (chimeric RNAi).

Possible off-target effects might be however a limitation. Thus, it has been reported for animal systems that siRNA might silence non-target genes caused by limited sequence-specificity (e.g. 60, 61). However, no off-targeting has been observed in plants so far (18). A possible explanation for that could be a greater sequence specificity of RNAi in plants compared to animals. Hence, the lowest size required for silencing a reporter gene was found to be 23nt complete identity in *Nicotiana benthamiana* plants (52) compared to 7nt sequence identity between siRNA and mRNA in human cells which can cause inhibition of gene expression (61). Later authors showed that off-targeting is associated with partial homology of siRNA to 3' untranslated regions. Nevertheless the possibility of off-targeting in plants cannot be excluded, since no systematic studies have been performed yet. Another promising approach of gene silencing might be by using artificial miRNA (amiRNA) (62). The authors showed that miRNA can specifically and efficiently silence target genes in a predictable manner.

Allergens targeted for gene silencing might fulfil essential cellular function as shown for the profilin family. As far as structural features of proteins distinguishing between allergenic and non-allergenic polypeptides can be

determined, strategies to substitute their biological function can be applied, e.g. by over-expressing a hypo-allergenic variant in the silenced background. Another possibility is to restrict the genetic manipulation temporally and/ or spatially by using either tissue-specific or inducible promoters. Thereby the risk of unwanted side effects for the growth of the plants should be minimized as well as possible off-target effects. Thus, fruit-, seed-, or pollen-specific silencing of target genes can be envisaged by selecting appropriate promoter sequences. In contrast, by conventional breeding methods, tissue-specific silencing of gene families cannot easily be achieved. Gene silencing has been shown to spread systemically for non-plant target genes (e.g., viruses) (20), however, silencing of endogenous genes seems to be cell autonomous making tissue-specific silencing feasible. In fact, a seed-specific promoter has been shown to be effective in suppression of an endogenous photomorphogenesis regulatory gene leading to a significant increase in carotinoid and flavonoid content; both compounds being beneficial for human health (63). Another example is the disruption of gossypol toxin production in cotton seed by tissue-specific RNAi (64). In addition, chemically-inducible RNAi vectors have been developed using e.g. dexamethasone (65) or ethanol (66) to achieve temporally restricted RNAi-silencing.

Studies addressing the stability and efficacy of RNA interference strategies under varying environmental conditions, e.g. by abiotic and biotic factors, are also necessary. Environmental factors that might negatively effect gene silencing include low temperatures and fungal, bacterial and viral infections. Using *N. benthamiana* as model system Szittya G *et al.* (67) demonstrated that siRNA accumulation and efficient silencing of viral and transgenes were negatively affected by low temperatures. In addition several lines of evidences showed that RNA silencing is an antiviral defence mechanism. Therefore, it is not surprising that many plant viruses encode suppressors of RNA silencing (for review see 68) which do have the potential to jeopardize RNAi approaches in transgenic plants. In the same way it will be also possible that plants circumvent gene silencing of endogenous proteins to compensate negative effects. It is known that several allergens belong to the family of pathogen-related proteins (PRPs). PRP silencing will probably have an impact on the susceptibility to pathogen infection and maybe be counter-regulated by the induction of other PRPs compensating the suppressed proteins. Studies of compensatory mechanisms and investigation of consequences possibly induced by silencing of defence-related proteins recently have been started.

Threshold doses, defined as the lowest observed adverse effect level (LOAEL), an amount of a specific food that would elicit mild, objective symptoms in highly sensitive individuals are not available for most of the foods. In order to define threshold doses for a specific allergen which is targeted by gene suppression, food challenges of patients monosensitized to an individual allergen or oral provocation tests with purified allergens will be necessary. Therefore, the question remains open whether e.g. a 100-fold reduction of a certain allergen will protect the patients from allergic reactions after ingestion of food. This points to another drawback of the RNAi technology namely that RNAi often leads only to partial suppression and no null-mutants can be obtained. This is in contrary advantageous in case of lethal knock-down mutants and in the sense that a range of phenotype can be observed allowing studying function of essential genes.

A 90%−99% decrease in allergenicity, which was achieved in the study by Le LQ and Lorenz Y *et al.* (51) for Lyc e 3, was considered to have a strong effect for certain patients. However, whether Lyc e 3-suppressed tomato fruits after ingestion are still capable of causing allergic symptoms has to be assessed in clinical trials by oral food challenge tests. In future, the implementation of provocation tests (e.g. skin prick testing and oral challenge tests) is mandatory to verify the reduced allergenicity of transgenic lines in a representative patient population. Oral challenge tests have not been performed with transgenic fruits, yet and will be a prerequisite the production of GM-foods under Good-manufacturing practise (GMP). Instead, a double-blind, placebo-controlled food challenge with up to 1 mg of a hypoallergenic mutant of the apple allergen Mal d 1, with an 2 to 7.8–fold reduced IgE binding capacity in comparison to the wild type allergen was performed by Bolhaar ST *et al.* (69) and did not result in allergic reactions after ingestion in two patients tested. The study indicates that a moderate decrease in IgE-binding potency was translated in a strong inhibition of biological activity, as shown by a 10-200-fold reduced SPT and basophil histamine release.

Probably the introduction of GM-food into the market will be hindered by the low acceptance of the population. The reason is that GM-plants frequently were generated by introduction of new potential toxic or allergenic proteins in order to improve the agronomical performance and enhance the nutrition values. This meets with a refusal and apprehensiveness in the public. In contrast to the public concerns of introducing of GM food with potential allergens into the market, several novel non-GM foods with proven allergenicity, e.g. kiwi or banana, have been entered the market and subsequently accepted by the consumers or are now under investigation for allergenicity assessment (70, reviewed in 71). However, using an RNAi strategy for the production of GM-food new genetic information (if marker free) is not introduced, since endogenous genes are targeted for gene silencing. Beside, RNAi is a natural and widespread mechanism of gene regulation in living organisms. Recently a study evaluating the attitude towards low-allergenic food was performed by Miles S *et al.* (72) using a questionnaire. Overall 60 food-allergic patients from Austria, Spain and The Netherlands (20 patients each) were included. Seventy to ninety five percent of the patients approve the production of low-allergenic food in general, the acceptance for low-allergenic food produced by genetic engineering was likewise high (55-85%), but usually participants prefer the generation of low-allergenic food by conventional methods. In addition to safety, price and quality are factors of major concern. Therefore, the issue of GM-food with improved nutritional values, especially low-allergenic foods, needs

further extensive discussion with stakeholder groups and the public.

3.4. Risk assessment of genetically modified foods

In general genetic engineering is aimed to improve the agronomical performance and nutritional value by introduction of proteins with the desired properties. But genetic engineering has also the potential to introduce new toxic and allergenic proteins into foods. In contrast to silencing of allergens by RNAi, other studies aimed to introduce novel proteins, which are known as allergens, e.g. chitinase and nsLTPs into carrots (73) or alpha-amylase inhibitor from bean into peas (74) to enhance the resistance to pathogens, or a sulphur-rich allergenic protein from brazil nut into soybean to improve the nutritional value (75). Therefore, decision-tree approaches have been proposed to facilitate the risk assessment of genetically modified foods (76). As no single criterion is sufficiently predictive of allergenicity, it was recommended that the risk assessment process should adopt an integrated step-wise, case-by-case approach, taking different information into account. Such an integrative approach includes information on (I) relationships between novel proteins and known allergens (origin from known allergenic source) defined by bioinformatics tools (sequence similarity with known allergens), (II) cross-reactivity defined using patient allergic sera, (III) *in vitro* measures of protein digestibility and (IV) determination of *in vivo* sensitisation potency using animal models. Until now, no reports have been published on increased allergenicity of GM foods available on the market. The described risk assessment will also be valid for transgenic food generated by the RNAi. Although the introduction of selection marker genes and promoter sequences into the recipient plant is likely, the RNAi method is applied to remove existing allergens by introduction of genetic information (allergen cDNA in inverted repeats) being already present in the plants. Therefore, the existing risk assessment has to be considered critically. The advantages of recombinant DNA technology over conventional breeding are (1) that a more precise information about the transferred gene is provided and (2) the reduced time to obtain the plant with the desired trait.

3.5. Alternative strategies by non-GM techniques

Considering the public concerns against GM-plants and –food, alternative strategies can be envisaged for crop improvement after providing prove-of-principle by e.g. RNAi technology. One possibility remains still conventional breeding, which is achieved through sexual crossing, particularly of cultivated lines (77). Parents with certain characteristics (in this case low amount of allergenic protein) will be intercrossed and subsequent generations will be examined with respect to the desired trait. This process is based on segregation of alleles from the parents and the offspring contains a novel combination of the alleles. Hence, conventional breeding is less restricted to the transfer of a certain gene, and results in the transfer of unknown genes. That also means that selection of the parental lines is critical for the success. The breeder uses natural variation within a certain species, which however limits the potential for improving specific traits. Intraspecific variation is exploited by using ancestral, wild relatives as well as by inducing variation through mutagenesis or molecular breeding (77). In contrast to conventional breeding genetic engineering offers the possibility that proteins from any species can be interchanged, a feature that makes the technology controversial.

TILLING is another technique which might be preferred to RNAi since it allows reducing or eliminating the expression of target genes without genetic transformation (for recent reviews see 28 and 78). TILLING provides the possibility to obtain allelic series of induced point mutants in any gene of interest (27, 78). TILLING is based on chemical mutagenesis by ethylmethansulfonate (EMS) followed by high-throughput screening for point mutations. Briefly, the mutagenized population (M1) will allow to self-fertilized and the resulting M2 individuals are used to prepare DNA samples. Pools of DNAs are amplified by PCR using fluorescently labelled, gene-specific primers. The products were heat-denatured and allow re-annealing randomly. Heteroduplexes between wild type and mutated DNA were cleaved by single strand-specific endonuclease CEL1. Fragments were subsequently separated by electrophoresis using the LI-COR gel system (78) to identify pools carrying the mutation. Individual DNA samples are subsequently screened to search for individuals. If several genes (members of gene families or different genes) are targeted simultaneously, inhibition of independent genes requires several rounds of selection followed by crossing of the individual mutants finally yielding crop plants carrying the desired traits. This approach on one hand is time consuming on the other hand once successful it provides stable inheritance of the incorporated traits. Thus, TILLING would allow obtaining null-mutants and furthermore represents a non-GM strategy to produce hypoallergenic food.

4. SUMMARY AND PERSPECTIVE

Plant genetic engineering has the potential to remove or reduce established allergens. The applicability of creating low allergenic food by means of dsRNAi has been clearly demonstrated and, several studies provided a proof of principle. RNAi technology was found to be an effective tool to generate foods with reduced allergenicity *in vivo*. Moreover, suppression of allergen expression was stable in the next generation plants.

Advantages and disadvantages of the RNAi technology for the production of foods with low allergenicity have to be critically evaluated. Genetically modified foods with low allergen expression will neither have a beneficial effect on the causal treatment of allergic diseases nor prevent patients from allergic symptoms after consumption of foods with homologous and cross-reactive allergens. But, fruits with reduced allergen expression may, in the future, permit their consumption by patients with

food allergy and thereby leading to an improvement of the life quality.

Although promising, plant biotechnology has not been used widely to decrease the allergenic potential of plant-derived food. Main reasons for this may be the low acceptance of GMO-food in the public. Moreover, the efficacy of the method depends on sufficient degree of silencing. Furthermore, allergenic proteins may fulfil essential cellular functions and thus may be indispensable in plant cells. These limitations could be overcome by simultaneous silencing of multiple target genes, gene silencing in a tissue- specific or temporal manner, or by creation of stable mutants using hypoallergenic variants of the selected allergenic protein. However, before introduction of low-allergenic GM-food to the market, agronomical performance should be assessed and safety has to be evaluated by clinical studies, considering all ethical and legal requirements.

5. ACKNOWLEDGEMENTS

The authors wish to thank Stefan Vieths and Uwe Sonnewald for stimulating discussions as well as Enrique Ernesto and Vera Mahler for excellent collaborations. Work in our laboratory is funded by DFG.

6. REFERENCES

1. Gupta R., A. Sheikh, D. Strachan & H. R. Anderson: Increasing hospital admissions for systemic allergic disorders in England: analysis of national admissions data. *BMJ* 327, 1142-1143 (2003)

2. Sampson H. A.: Update on food allergy. *J Allergy Clin Immunol* 113, 805-819 (2004)

3. Becker W. M., L. Vogel & S. Vieths: Standardization of allergen extracts for immunotherapy: where do we stand? *Curr Opin Allergy Clin Immunol* 6, 470-475 (2006)

4. Chehade M.: IgE and non-IgE-mediated food allergy: treatment in 2007. *Curr Opin Allergy Clin Immunol* 7, 264-268 (2007)

5. Bruijnzeel-Koomen C., C. Ortolani, K. Aas, C. Bindslev-Jensen, B. Björksten, D. Moneret-Vautrin & B. Wüthrich: Adverse reaction to food. Position paper. *Allergy* 50, 623-635 (1995)

6. Breiteneder H. & C. Mills: Nonspecific lipid-transfer proteins in plant foods and pollens: an important allergen class. *Curr Opin Allergy Clin Immunol* 5, 275-279 (2005)

7. Ballmer-Weber B. K., A. Hoffmann, B. Wüthrich, D. Lüttkopf, C. Pompei, A. Wangorsch, M. Kastner & S. Vieths: Influence of food processing on the allergenicity of celery: DBPCFC with celery spice and cooked celery in patients with celery allergy. *Allergy* 57, 228-235 (2002)

8. Maleki S. J.: Food processing: effects on allergenicity. *Curr Opin Allergy Clin Immunol* 4, 241-245 (2004)

9. Bolhaar S.T., W. E. van de Weg, R. van Ree, E. Gonzalez-Mancebo, Z. Zuidmeer, C. A. Bruijnzeel-Koomen, M. Fernandez-Rivas, J. Jansen, K. Hoffmann-Sommergruber, A. C. Knulst & L. J. Gilissen: *In vivo* assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity of apple cultivars. *J Allergy Clin Immunol* 116, 1080-1086 (2005)

10. Zuidmeer L., W. A. van Leeuwen, I. Kleine Budde, H. Breiteneder, Y. Ma, C. Mills, A. Sancho, E. J. Meulenbroek, E. van de Weg, L. Gilissen, F. Ferreira, K. Hoffmann-Sommergruber & R. van Ree: Allergenicity assessment of apple cultivars: hurdles in quantifying labile fruit allergens. *Int Arch Allergy Immunol* 141, 230-240 (2006)

11. Reuter A., A. Matt, S. Scheurer, J. Jehle & S. Vieths: Development of an *in vitro* model to reduce the allergenicity of sweet cherry (Prunus avium L.) by RNA interference (RNAi). *J Allergy Clin Immunol* 111(2), 730 (2003)

12. Sonnewald U.: Plant Biotechnology: From basic plant science to industrial applications. *J Plant Physiol* 160, 723-725 (2003)

13. Yonekura-Sakakibara K. & K. Saito: Review: Genetically modified plants for the promotion of human health. *Biotechnol Lett* 28, 1983-1991 (2006)

14. Biemelt S. & U. Sonnewald: „Molecular farming in plants." Nature Encyclopedia of Life Sciences. London: Nature Publishing Group. http://www.els.net/. (2005)

15. Twyman R. M., E. Stoger, S. Schillberg, P. Christou & R. Fischer: Molecular farming in plants: host systems and expression technology. *TRENDS in Biotech* 21, 570-578 (2003)

16. Napoli C., C. Lemieux & R. Jorgensen: Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* 2, 279-289 (1990)

17. Tang G. & G. Galili: Using RNAi to improve plant nutritional value: from mechanism to application.*TRENDS in Biotech* 22, 463-469 (2004)

18. Small I.: RNAi for revealing and engineering plant gene functions. *Curr Opin Biotechnol* 18, 148-153 (2007)

19. Mansoor S., I. Amin, M. Hussain, Y. Zafar & R. W. Briddon: Engineering novel traits in plants through RNA interference. *Trends Plant Sci* 11, 559-565 (2006)

20. Baulcombe D.: RNA silencing in plants. *Nature* 431, 356-363 (2004)

21. Brodersen P. & O. Voinnet: The diversity of RNA silencing pathways in plants. *Trends Genet* 22, 268-280 (2006)

22. Waterhouse P. M., M. W. Graham & M. B. Wang: Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci* 95, 13959-13964 (1998)

23. Smith N. A., S. P. Singh, M. B. Wang, P. A.. Stoutjesdijk, A.. G. Green & P. M. Waterhouse: Total silencing by intron-spliced hairpin RNAs. *Nature* 407, 319-320 (2000)

24. Kusaba M.: RNA interference in crop plants. *Curr Opin Biotechnol* 15, 139-143 (2004)

25. Parinov S. & V. Sundaresan: Functional genomics in Arabidopsis: large-scale insertional mutagenesis complements the genome sequencing project. *Curr Opin Biotechnol*, 11, 157-61 (2000)

26. Alonso J. M. & J. R. Ecker: Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis. *Nat Rev Genet* 7, 524-536 (2006)

27. Colbert T., B. J. Till, R. Tompa, S. Reynolds, M. N. Steine, A. T. Yeung, C. M. McCallum, L. Comai & S. Henikoff: High-throughput screening for induced point mutations. *Plant Physiol* 126, 480-484 (2001)

28. Henikoff S., B. J. Till & L. Comai: TILLING. Traditional mutagenesis meets functional genomics. *Plant Physiol* 135, 630-636 (2004)

29. Green P. J., O. Pines & M. Inouye: The role of antisense RNA in gene regulation. *Annu Rev Biochem* 55, 569-597 (1988)

30. Smith C. J. S., C. F. Watson, J. Ray, C. R. Bird, P. C. Morris, W. Schuch & G. Grierson: Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* 334, 724-726 (1988)

31. Foetisch K., D. Y. Son, F. Altmann, H. Aulepp, A. Conti, D. Haustein & S. Vieths: Tomato (Lycopersicon exculentum) allergens in pollen-allergic patients. *Eur Food Reseach Technol* 213, 259-266 (2001)

32. Kondo Y., A. Urisu & R. Tokuda: Identification and characterization of the allergens in the tomato fruit by immunoblotting. *Int Arch Allergy Immunol* 126, 294-299 (2001)

33. Gelvin S. B.: *Agrobacterium*-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. *Microbiol and Molec Biology Rev* 67, 16-37 (2003)

34. Zupan J., T. R. Muth, O. Draper & P. Zambryski: The transfer of DNA from *Agrobacterium tumefaciens* in plants: a feast of fundamental insights. *Plant J* 23, 11-28 (2000)

35. Tada Y., M. Nakase, T. Adachi, R. Nakamura, H. Shimada, M. Takahashi, T. Fujimura & T. Matsuda: Reduction of 14-16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Letter* 391, 341-345 (1996)

36. Nakamura R. & T. Matsuda: Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. *Biosci Biotechnol Biochem* 60, 1215-1221 (1996)

37. Burks A. W. Jr, J. R. Brooks & H. A. Sampson: Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J Allergy Clin Immunol* 81, 1135-1142 (1988)

38. Samoto M., K. Takahashi, Y. Fukuda, S. Nakamura & Y. Kawamura: Substantially complete removal of three major allergenic soybean proteins (Gly m Bd 30K, Gly m Bd 28K, and the alpha-subunit of conglycinin) from soy protein by using a mutant soybean, Tohoku 124. *Biosci Biotechnol Biochem* 60, 1911-1913 (1998)

39. Herman E. M.: Genetically modified soybeans and food allergies. *J Exp Bot* 54, 1317-1319 (2003)

40. Herman E. M., R. M. Helm, R. Jung & A. J. Kinney: Genetic modification removes an immunodominant allergen from soybean. *Plant Physiology* 132, 36-43 (2003)

41. Burks A. W., L. W. Williams, C. Connaughton, G. Cockrell, T. J. O´Brien & R. M.Helm: Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 90, 962-969 (1992)

42. Konan K. N., O. M. Viquez & H. W. Dodo: Downregulation of Arah2, a Major Peanut Allergen in Transgenic Peanut Plants. *J Allergy Clin Immunol* 109, 482 (2002)

43. Koppelmann S. J., M. Wensing, M. Ertmann, A. C. Knulst & E. F. Knol: Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. *Clin Exp Allergy* 4, 583-590 (2004)

44. Wensing M., A. H. Penninks, S. L. Hefle, S. J. Koppelman, C. A. Bruijnzeel-Koomen & Knulst. The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. *J Allergy Clin Immunol* 110, 915-920 (2002)

45. Dodo H., K. Konan and O. Viquez: A genetic engineering strategy to eliminate peanut allergy. *Curr Allergy Asthma Rep* 5, 67-73 (2005)

46. Dodo H. W., K. N. Konan, F. C. Chen & O. Viquez: Alleviating peanut allergy using genetic engineering: the silencing of the immunodominant allergen Ara h 2 leads to

its significant reduction and a decrease in peanut allergenicity. *Plant Biotechnol J.* 2007 Sep 3; [Epub ahead of print] (2007)

47. Gilissen L. J., S. T. Bolhaar, C. I. Matos, G. J. Rouwendal, M. J. Boone, F. A. Krens, L. Zuidmeer, A. Van Leeuwen, K. Hoffmann-Sommergruber, A. C. Knulst, D. Bosch, W. E. Van de Weg & R. Van Ree: Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *J Allergy Clin Immunol* 115, 364-369 (2005)

48. Vieths S., S. Scheurer & B. Ballmer-Weber: Current understanding of cross-reactivity of food allergens and pollen. *Ann N Y Acad Sci* 964, 47-68 (2002)

49. Ortolani C., M. Ispano, E. A. Pastorello, R. Ansaloni & G. C. Magri: Comparison of results of skin prick tests (with fresh foods and commercial food extracts) and RAST in 100 patients with oral allergy syndrome. *J Allergy Clin Immunol* 83, 683-690 (1989)

50. Ortolani C., M. Ispano, E. Pastorello, A. Bigi & R. Ansaloni: The oral allergy syndrome. *Ann Allergy* 61, 47-52 (1988)

51. Le Q. L., Y. Lorenz, S. Scheurer, K. Fötisch, E. Enrique, J. Bartra, S. Biemelt, S. Vieths & U. Sonnewald: Design of tomato fruits with reduced allergenicity by ds-RNAi mediated inhibition of ns-LTP (*Lyc e* 3) expression. *Plant Biotechnology Journal* 4, 231-242 (2006)

52. Thomas C. L., L. Jones, D. C. Baulcombe & A. J. Maule: Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in Nicotiana benthamiana using a potato virus X vector. *Plant J*, 25, 417-425 (2001)

53. Lorenz Y., E. Enrique, L. Lequynh, K. Fötisch, M. Retzek, S. Biemelt, U. Sonnewald, S. Vieths & S. Scheurer: Skin prick tests reveal stable and heritable reduction of allergenic potency of gene-silenced tomato fruits. *J Allergy Clin Immunol* 118, 711-718 (2006)

54. Westphal S., W. Kempf, K. Fötisch, M, Retzek, S. Vieths & S. Scheurer: Tomato profilin Lyc e 1: IgE cross-reactivity and allergenic potency. *Allergy* 59, 526-532 (2004)

55. Le Q. L., V. Mahler, Y. Lorenz, S. Scheurer, S. Biemelt, S. Vieths & U. Sonnewald: Reduced allergenicity of tomato fruits harvested from Lyc e 1-silenced transgenic tomato plants. *J Allergy Clin Immunol* 118, 1176-1183 (2006)

56. Crespo J. F., M. Retzek, K. Foetisch, E. Sierra-Maestro, A. B. Cid-Sanchez, C. Y. Pascual, A. Conti, A. Feliu, J. Rodriguez, S. Vieths & S. Scheurer: Germin-like protein Cit s 1 and profilin Cit s 2 are major allergens in orange (Citrus sinensis) fruits. *Mol Nutr Food Res* 50, 282-290 (2006)

57. Bhalla P. L., I. Swoboda & M. B. Singh: Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. *Proc Natl Acad Sci* 96, 11676-11680 (1999)

58. Bhalla P. L., I. Swoboda & M. B. Singh: Reduction in allergenicity of grass pollen by genetic engineering. *Int. Arch. Allergy Immunol* 124, 51-54 (2001)

59. Bhalla P. L. & M. Singh: Knocking out expression of plant allergen genes. *Methods* 32, 340-345 (2004)

60. Fedorov Y., E. M. Anderson, A. Birmingham, A. Reynolds, J. Karpilow, K. Robinson, D. Leake, W. S. Marshall & A. Khvorova: Off-target effects by siRNA can induce toxic phenotype. *R*NA 12, 1188-1196 (2006)

61. Birmingham A., E. M. Anderson, A. Reynolds, D. Ilsley-Tyree, D. Leake, Y. Fedorov, S. Baskerville, E. Maksimova, K. Robinson, J. Karpilow, W. S. Marshall & A. Khvorova: 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods* 3, 199-204 (2006)

62. Schwab, R., S. Ossowski, M. Riester, N. Warthmann & D. Weigel: Highly specific gene silencing by artificial microRNAs in Arabidopsis. *Plant Cell* 18, 1121-33 (2006)

63. Davuluri G. R., A. van Tuinen, P. D. Fraser, A. Manfredonia, R. Newman, D. Burgess, D. A. Brummell, S. R. King, J. Palys, J. Uhlig, P. M. Bramley, H. M. Pennings & C. Bowler: Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat Biotechnol* 23, 890-895 (2005)

64. Sunilkumar G., L. M. Campbell, L. Puckhaber, R. D. Stipanovic & K. S. Rathore: Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. *Proc Natl Acad Sci* 103, 18054-18059 (2006)

65. Wielopolska, A., H. Townley, I. Moore, P. Waterhouse & C. Helliwell: A high-throughput inducible RNAi vector for plants. *Plant Biotechnol J* 3, 583-590 (2005)

66. Chen S., D. Hofius, U. Sonnewald & F. Bornke: Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. *Plant J* 36, 731-740 (2003)

67. Szittya G., D. Silhavy, A. Molnar, Z. Havelda, L. Lakatos, Z. Banfalvi & J. Burgyan: Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO J* 22, 633-640 (2003)

68. Roth B. M., G. J. Pruss & V. B. Vance: Plant viral suppressors of RNA silencing. *Virus Res* 102, 97-108 (2004)

69. Bolhaar S. T., L. Zuidmeer, Y. Ma, F. Ferreira, C. A. Bruijnzeel-Koomen, K. Hoffmann-Sommergruber, R. van Ree & A. C. Knulst: A mutant of the major apple allergen,

Mal d 1, demonstrating hypo-allergenicity in the target organ by double-blind placebo-controlled food challenge. *Clin Exp Allergy* 35, 1638-1644 (2005)

70. Gubesch M., B. Theler, M. Dutta, B. Baumer, A. Mathis, T. Holzhauser, S. Vieths & B. K. Ballmer-Weber: Strategy for allergenicity assessment of 'natural novel foods': clinical and molecular investigation of exotic vegetables (water spinach, hyacinth bean and Ethiopian eggplant). *Allergy* 62, 1243-1250 (2007)

71. Meredith C.: Allergenic potential of novel foods. *Proc Nutr Soc* 64, 487-490 (2005)

72. Miles S., S. Bolhaar, E. Gonzales-Mancebo, C. Hafner, K. Hoffmann-Sommergruber, M. Fernades-Rivas & A. Knulst: Attitudes towards low-allergen food in food allergic consumers. *Nutrition & Food Science* 35, 220-228 (2005)

73. Jayaraj J. & Z. K. Punja: Combined expression of chitinase and lipid transfer protein genes in transgenic carrot plants enhances resistance to foliar fungal pathogens. *Plant Cell Rep* 26, 1539-1546 (2007)

74. Prescott V. E., P. M. Campbell, A. Moore, J. Mattes, M. E. Rothenberg, P. S. Foster, T. J. Higgins & S. P. Hogan: Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *J Agric Food Chem* 53, 9023-30 (2005)

75. Nordlee J. A., S. L. Taylor, J. A. Townsend, L. A. Thomas and R. K. Bush: Identification of a Brazil-nut allergen in transgenic soybeans. *N Engl J Med* 334, 688-692 (1996)

76. Davies H. V.: GM organisms and the EU regulatory environment: allergenicity as a risk component. *Proc Nutr Soc* 64, 48148-6 (2005)

77. Caligari P. D. S: Plant breeding and crop improvement. Nature Encyclopedia of Life Sciences. London: Nature Publishing Group. http://www.els.net/. (2001)

78. Gilchrist E. J. & G. W. Haughn: TILLING without a plough: a new method with applications for reverse genetics. *Curr Opin Plant Biol* 8, 211-215 (2005)

Key Words: Food Allergen, RNA Interrference, RNAi, short interfering RNA, siRNA, Review

Send correspondense to: Stephan Scheurer, Paul-Ehrlich-Institut, Division Allergology, Paul-Ehrlich-Str. 51-59, Langen, Germany; Tel: 49-6103-775310, Fax: 49-6103-771258, E-mail: schst@pei.de