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Production of hypoallergenic plant foods by selection, breeding and genetic modification

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Abstract

A set of plant-breeding technologies on the reduction of the allergenicity of food, i.c. the production of hypoallergenic apple cultivars by selection, breeding and genetic modification, is elaborated. The results of extended genomics and gene-mapping research on apple allergen genes (*Mal d 1*; *Mal d 2* (TLP); *Mal d 3* (nsLTP); *Mal d 4* (profilin)) are supporting to these techniques. The RNAi approach for allergen gene silencing is especially emphasized. The power of integrating medical, natural and agricultural research in the development of allergy prevention strategies is clearly demonstrated.

Keywords: food allergy; Mal d 1; Mal d 2 (TLP); Mal d 3 (nsLTP); Mal d 4 (profilin); skin prick test (SPT); allergen gene mapping; genetic markers; genetic modification; allergy prevention

Introduction

Three factors are relevant in the development of allergy: the genetic constitution of a (potential) patient; the presence of allergens in the air, in food or by contact; and the occurrence of adjuvant factors in the living environment that can affect the immune system and enhance the chance of allergy development. Allergies develop due to a continuous interaction of the environmental factors with the immune system. An allergy prevention strategy can be directed to the reduction to patients of the allergen load, for example in food. Two ways are open to produce such foods: (1) through the development of hypoallergenic primary material, and (2) through destruction or elimination of allergens or allergenic epitopes by food processing. Wichers et al. (2003) describe several processing technologies aiming at the reduction of allergenicity in food products. These technologies include chemical, biochemical (using proteases or oxidases) and physical (such as heating, extraction) methods. We will elaborate here on technologies to reduce the allergenicity in primary plant food

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products. The technologies of choice are (a) selection of low-allergenic cultivars from the existing biodiversity of a given crop; (b) breeding using characterized genotypes and genetic markers for low allergenicity; and (c) genetic modification to silence an allergen gene. This paper summarizes the relevant results from the EU-SAFE project (QLK1-CT-2000-01394), a project that aimed at the development of field-to-table strategies to reduce the incidence of plant food allergies in Europe. In this project, partners were involved from academic hospitals and medical science institutes, from agriculture and food research institutes, from plant-breeding companies, fruit-juice industry, and the European Asthma and Allergy Association (EFA). As a whole, the EU-SAFE project is a good example of an integrated and multidisciplinary approach aiming at allergy prevention. In a specific work package of this project, the above-mentioned breeding technologies have been applied to produce apple material with reduced allergenicity. Dutch partners from Wageningen University and Research Centre, University Medical Centre Utrecht and Sanquin Amsterdam cooperated closely on this subject.

Apple has been the crop of choice. Apple allergy is common in Europe, especially in the population of the northwestern part, in which the disease is strongly related to birch-pollen allergy due to cross-reactivity of the anti-Bet v 1 IgE antibodies in birch-pollen-allergic patients to the Mal d 1 allergen in apple (Van Ree 1997). Between 3 and 5% of this population suffers from hay fever, 50 to 70 % of whom become apple-allergic (Ebner et al. 1991). Mal d 1 and Bet v 1 are homologous proteins belonging to the so-called pathogenesis-related (PR) proteins of the PR-10 family (Van Loon and Van Strien 1999). Related fruits of the *Rosaceae* family such as pear, cherry and peach, as well as hazelnut, can also induce adverse reactions on the basis of the same IgE-mediated cross-reactivity to Bet v 1 (Van Ree 1997). Mal d 2 (taumatin-like protein, TLP) is another allergenic apple protein (a PR5 family member) structurally related to thaumatin (Krebitz et al. 2003). In the southern part of Europe, allergy to a different major apple allergen, Mal d 3, is more prevalent. Mal d 3 is a non-specific lipid-transfer protein (nsLTP), also a PR protein belonging to the PR14 family. IgE antibodies to nsLTP have also shown to be cross-reactive. Sensitization of patients for this allergen most likely occurs through eating of peach (Fernández-Rivas, Van Ree and Cuevas 1997), although sensitization by pollen from mugwort and *Parietaria* can not be excluded (Pastorello et al. 2002; Colombo et al. 2003). The last well-defined apple allergen is Mal d 4, a profilin (Wensing et al. 2002).

Most apple-allergic patients avoid eating the fruit, abstaining themselves from a food source of high nutritional and health value: “An apple a day keeps the doctor away” is a common saying. It is worthwhile, therefore, to develop strategies to make apple also a healthy fruit for apple-allergic patients. Several technologies aiming at this goal are elaborated below.

Selection

Apple-allergic patients sometimes report differences in the allergenic reaction from different cultivars. This phenomenon was confirmed by DNA cloning and immunological analysis (Son et al. 1999).

In EU-SAFE, differences in allergenicity among apple cultivars were also tested by prick-to-prick skin prick test (SPT) and double-blind placebo-controlled food challenges (DBPCFC) in well documented birch-pollen-related apple-allergic patients. For selection, a broad diversity of apple cultivars and genotypes was available at Plant Research International, Wageningen, from which over twenty apple

cultivars have been analysed. The fruits were harvested at their usual degree of ripeness and were stored for several months at 2°C. The responses in nine patients revealed Golden Delicious as one of the highest-, and Santana as one of the lowest-allergenic cultivars. The statistically significant difference in allergenicity between these two cultivars was confirmed in a DBPCFC in five patients and proved to be a factor 30 (Bolhaar et al. 2004). These differences in allergenicity were reproducible in fruits from several harvest years (Van de Weg et al., unpublished results).

The identification of Santana as a low-allergenic cultivar may permit the consumption of this cultivar by patients suffering from birch-pollen-related apple allergy. Confirmation of the result in a larger patient population is under way. This research shows the usefulness of prick-to-prick SPT (combined with DBPCFC for confirmation) as a rapid and quantitative test for allergenicity in cultivar screening (Bolhaar et al. 2004). The selection strategy described here for the production of hypoallergenic cultivars is not restricted to apple but can be applied to any crop in which a diversity of genotypes is available. A reliable test system is, however, a basic requirement. In case of apple, further testing among the wide range of existing apple cultivars is a realistic option to find more cultivars and breeding lines with low Mal d 1 allergenicity.

Before sale, most apple fruits are stored for several months at low temperatures. Fruit growers have considerably optimized the storage conditions during the last decades. Especially storage at low temperatures under reduced oxygen and increased carbon-dioxide concentrations appears to be favourable. These conditions (3°C, 2.5% oxygen, 1% carbon dioxide) also proved to have a reducing influence on allergenicity in comparison to cold storage under normal air conditions. In five cultivars tested, including Golden Delicious and Santana, a significantly 15% mean lower allergenicity (calculated from prick-to-prick SPT reactions in 5 birch-pollen-related apple-allergic patients) was observed (Bolhaar et al. 2004). This observation suggests that it makes sense to manipulate storage and transport conditions further as a method to control Mal d 1 levels in apple fruits.

Since in the southern part of Europe patients who suffer from LTP-related allergy to apple and related *Rosaceae* fruits have been identified and well documented (Sánchez-Monge et al. 1999; Van Ree 2002), a similar selection procedure among the existing diversity might result in low-LTP allergenic apple cultivars.

Genomics for breeding

As described above, apple cultivars are known to be different in their allergenicity. Knowledge of the genetic basis of such differences would allow breeding of hypoallergenic cultivars at a broader scale. In view of this, the genetics and genomics of the four presently known apple allergens have been analysed.

The *Mal d 1* isoallergen gene family has been identified by genomic PCR cloning and gene localization in the apple genome. The results indicated that the *Mal d 1* family consists of 18 gene members, which have been mapped as multiple gene clusters on the two homoeologous linkage groups (chromosomes) 13 and 16. One single *Mal d 1* locus was identified on a different chromosome; one gene remained unmapped (Gao et al. in press-c). In eight genetically unrelated cultivars of known allergenicity, the allelic diversity of these genes has been analysed. At the amino-acid level, one to several isoforms per individual gene were found among these cultivars. Further analysis of the allergenicity of the individual genes and their expression in the fruit has been performed in the progenies of high- and low-allergenic cultivars. In four

independent skin prick tests on Dutch birch-pollen-related apple-allergic patients, significantly different allergenicity was found between Santana (low) and its grandparent Golden Delicious (high) and twelve other cultivars of known allelic diversity of *Mal d 1* genes. It appeared that the two haplotypes (allelic compositions of a haploid set of chromosomes) in Golden Delicious of linkage group 16 were completely replaced in Santana, whereas the haplotypes of linkage groups 6 and 13 remained unchanged. These data strongly suggest a correlation of the Mal d 1 allergenicity to expressed genes on linkage group 16 (Gao et al., unpublished data). In addition, comparing the haplotypes of all fourteen cultivars to their allergenicity (as the result of SPT) showed the presence of the genetic marker *Mal d 1*.06A-ssr-154 in homozygous condition to be correlated to low Mal d 1 allergenicity (Gao et al. in press-a).

In a similar way, the genes, their loci and allelic diversity have been analysed for the other allergen genes in apple. Of Mal d 2 (taumatin-like protein), two gene copies were identified at the same position on linkage group 9. We still expect the presence of other *Mal d 2* genes on the homoeologous linkage group 17 (Gao et al. in press-b).

Mal d 3 (non-specific lipid-transfer protein, nsLTP) genes were found on the homoeologous linkage groups 4 and 12. Assessment of the deduced nsLTP amino-acid sequences in 10 genetically unrelated apple cultivars gave a total of two variants for the one, and three variants for the other gene. This indicates that the variations in the expressed proteins are very minor and that differences in Mal d 3 allergenicity among apple cultivars will mainly depend on the content of Mal d 3 (Gao et al. 2005).

Genomic characterization of *Mal d 4* (profilin) revealed the existence of four genes of which two gene copies were found on linkage group 9 and two other single genes on linkage groups 2 and 8 (Gao et al. in press-b). Also here, more genes on the homoeologous chromosomes 17, 7 and 15, respectively, are expected to exist.

These results have relevance for breeding. If the genomic-map position of the expressed allergen gene is identified, breeding strategies can be designed to replace the gene by a low-allergenic allele (if identified) or by a gene with reduced expression. Especially in the case of extended gene families, like pathogenesis-related (PR) proteins which often have allergenic representatives, knowledge of the genomics of the allergen genes (their number in the genome, their arrangement in gene clusters and the sequence of the individual gene members) is useful to identify the individual member that has come to expression. In the case of the presence of multiple genes in gene clusters, proteomics approaches like QTOF and HPLC might reveal peptide sequences that can be traced back to the original gene (Helsper et al. 2002). In addition, genomics data are useful to predict biochemical and physicochemical characteristics of the protein regarding its molecular weight, PI value, secondary and tertiary structure, thermal stability and resistances to proteolysis. Although the allergenicity of a given protein cannot be predicted yet, many molecular properties have been identified that might predispose such a protein to become an allergen (Breiteneder and Mills 2005).

Breeding

In fruits from an arbitrarily selected set of genotypes from a progeny population of a cross between the apple cultivars Fiesta and Discovery, the allergenicity has been analysed by SPT in two birch-pollen-related apple-allergic patients. Fiesta was relatively high-allergenic compared to Discovery, which was moderately allergenic. The tests revealed a broad range of variation in allergenicity between the fruits from

the individual progeny genotypes. Three of these genotypes showed a very low allergenicity and one genotype a very high allergenicity as compared to the allergenicity of the parental cultivars. In general, the allergenicity of the fruits from these progeny genotypes was similar to both patients. The results are promising for breeding in such a way that, probably because of the complex genetic nature of the allergenicity of apple, crossing of apple cultivars opens possibilities for the production of hypoallergenic cultivars. The aid of genetic markers will be advantageous in this matter to speed up breeding for the production of market-valuable hypoallergenic cultivars (Van de Weg et al., unpublished results).

The data were reproduced with fruits from the progeny population of Fiesta and Discovery from a next year's harvest in a larger patient group. Locus-specific markers for all four allergen genes and their alleles were used to identify the allergen-specific sensitivity of patient groups. Preliminary SPT data demonstrated the existence of a low- and high-allergic patient group among a Dutch population of clinically defined birch-pollen-related apple-allergic patients. According to genetic marker trace-back and statistic data correlation, the high-allergic patient group appeared also to respond to Mal d 4 (profilin) (Van de Weg et al., unpublished results).

Genetic modification (GM)

The technology

In comparison to conventional plant breeding, genetic modification offers a quicker way to introduce novel traits into the genome of a host plant. Several techniques for genetic modification have been developed during the last thirty years. Most commonly used is the technique applying *Agrobacterium tumefaciens* as the vector organism to transfer the new DNA to the host genome.

Excised pieces of plant leaves (explants) are incubated for one day in a liquid medium containing *A. tumefaciens* cells carrying the gene or DNA of interest on a plasmid, a circular DNA molecule present in the bacterium next to its bacterial chromosome. Linked to the gene of interest, the plasmid also contains a selection gene conferring resistance e.g. against an antibiotic or a herbicide. During the incubation step, cells of *A. tumefaciens* attach to the wall of the explant cells and inject a part of the plasmid DNA into the host cell. This DNA is transferred to the plant cell nucleus and becomes integrated into the plant cell genome. After the incubation, the explants are transferred to a solid growth medium with the antibiotic, enabling only those plant cells to grow that have taken up the new DNA. Once built in, the transferred DNA will act the same as the host DNA. Its genetic information will be transcribed into mRNA that is transferred to the ribosomes and translated into protein. The transformed cells first produce a callus, a clump of undifferentiated cells, from which, due to specific changes of the medium composition, shoots will develop. These shoots can be harvested and cultured into plants that can be transferred to the soil.

The culture area of GM crops

In 2004, the area of GM crops represented 5% (about 80 million hectares) of the agricultural area worldwide. Major countries culturing GM crops are the USA (60%) and Argentina (20%). Rising countries are China, Canada, Brazil, South Africa and India. Major crops are soybean (60%), maize (23%), cotton (11%) and rapeseed (6%), involving a sum of 40 billion Euros (Runge and Ryan 2004). Potato, tomato and rice are rising. The most important GM traits are agricultural traits (input traits) like herbicide and insect resistance or a combination of both. New traits of interest are

resistances against drought and salt, to enable crop plants to grow on low-quality soils. Other categories of GM traits aim at improvement of the plant product (product or output traits). These include better nutritional value, longer shelf life, production of cheap diagnostics (e.g. antibodies) and reduction of allergenicity.

Genetic modification and allergy

GM is surrounded by fears and concerns. Major concerns relate to the potential of GM plants to become uncontrollable weeds, and to the unwanted flow of the transgenes into wild relatives of the crop in the natural environment and making them uncontrollable weeds. However, till today, GM crops in the field do not behave differently in these aspects compared with traditionally bred crops. Other concerns deal with freedom of choice or with ethics or ideology. Here, emotional and rational aspects and a diversity of stakeholders' interests touch each other (Gremmen et al. 2004). There is also the fear of introducing a toxic or allergenic compound by GM.

For our purpose it is relevant to inventory the possible relationship between GM and allergenicity. This relationship is twofold: (1) GM may introduce a new allergenic protein in the food chain or may increase the allergenicity of a known allergenic product; and (2) through GM, allergen genes may be knocked out. Concerning the first possibility, it has been demonstrated that an allergen in its original organism remains an allergen in the host. This has resulted in the termination in an early stage of a research programme aiming at the introduction of a sulphur-rich protein from the Brazil nut into soybean because the nut protein proved to be an allergen. Although this GM soybean cultivar was developed for improvement of fodder quality, possible mixing up of this GM material with soybeans intended for human food was prevented in this way (Lehrer, Horner and Reese 1996). The risk of introducing an allergen through GM into novel foods is negligibly low because of the use of decision-tree models (FAO and WHO 2003) to test the potential allergenicity of transgenic proteins. These decision trees focus on the origin of the gene (whether or not from a known allergenic source), sequence similarities with known allergens, immunological in-vitro and in-vivo reactivity, stability during digestion experiments, and immune-reactivity in animal tests (Crevel and Madsen 2004; Fiers et al. 2005). Until now, no reports are published on allergenic effects of GM foods. In addition, comparison of allergenicity of traditional and GM soybean did not reveal any difference in allergenicity (Burks and Fuchs 1995). However, in contrast to the fear of introduction of potential allergens through the GM route, non-GM novel foods such as exotic products like kiwi, sesame seeds, Sharon fruits, etc. with proven allergenicity have easily been accepted by the consumer (Bolhaar et al. 2005; Gremmen et al. 2004).

Hypoallergenic apple

The other side of the coin shows the possibility to apply GM for the silencing of undesired genes. The acceptance by allergic consumers in Austria, Spain and The Netherlands of low-allergen food produced using GM was reasonably high (with a mean of 77%), as measured from a questionnaire (Miles et al. 2004). Allergen genes in rice and soybean have been knocked out successfully (Herman 2003; Tada et al. 1996). Recently, the anti-sense approach has been optimized in the RNAi method (Kusaba 2004). This method for post-transcriptional gene silencing is especially efficient when the gene construct used consists of an inverted repeat of a fragment of the targeted gene sequence separated by an intron sequence. Such construct results in the formation of a so-called intron-spliced hairpin RNA. Gene silencing results from sequence-specific RNA degradation. Endogenous mRNA seems to be a target of

double-stranded-RNA-mediated genetic interference. It is proposed that RNAi works by double-stranded-RNA-directed enzymatic RNA degradation. In this way, the endogenous mRNA is prevented from passing from the nucleus to the ribosomes where it normally directs protein production.

In the framework of the EU-SAFE project, the silencing of *Mal d 1* has now been carried out successfully. In apple, representatives of the *Mal d 1* gene family contain a single intron or are intronless (Gao et al. in press-c). On the basis of an isolated intron-containing *Mal d 1* gene sequence, a gene construct was designed coding for an intron-spiced-hairpin RNA and transferred to the apple cultivar Elstar through *A. tumefaciens*. Resulting shoots were selected on the basis of having a normal phenotype and growth rate. With PCR, in 6 of 9 selected plantlets, the presence of the construct was demonstrated. Analysis with SPT (prick-to-prick) in three apple-allergic patients showed that the wild-type plantlet had significantly ($P<.05$) higher allergenicity than 5 of the transformants. Reduction of expression of *Mal d 1* was confirmed by immunoblotting. In wild-type and unsuccessful transformants, a strong band was detected with Mal d 1-reactive mAB 5H8 at the expected apparent molecular weight of 17 kDa. This band was virtually absent in the transformants that carried the gene-silencing construct. With human IgE antibodies, the same observations were made. It is concluded that *Mal d 1* expression was successfully reduced by RNA interference. This translated into significantly reduced in-vivo allergenicity. These observations support the feasibility of the production by gene silencing of apples hypoallergenic for Mal d 1. The production of an apple plant with a significant reduction of the overall expression of *Mal d 1* from an existing economically successful cultivar using RNAi seems an attractive time-saving (by a factor 2) and simpler alternative than crossing strategies where each new genotype has to be tested for its market value, including tests for taste and texture, production and storage, consumer acceptability and economic viability. Such tests will at least take 15 to 20 years.

Conclusions

Several breeding technologies and their potentials for the production of hypoallergenic foods have been shown using apple as a model. Cultivar selection, genomics of allergen genes, breeding and genetic modification, all have shown to be applicable for the purpose of reducing the allergen load to allergic patients. The knockout strategy for the introduction of hypo-allergenicity is expected to become a common procedure towards the production of hypoallergenic raw materials (Gilissen et al. 2005a). Based on the techniques describes here, strategies can be developed to contribute to allergy prevention, making use of the knowledge framework in an integrated and multidisciplinary approach (Gilissen et al. in press; 2005b). This work also clearly demonstrates the power of integrating medical, natural and agricultural research.

With regard to the *Mal d 1*-silenced plantlets, these will be grown now to fruit-bearing trees to study the phenotype of the adult plants and the consolidated absence of the allergen in the fruits. Before introduction on the market and labelled as such, the hypoallergenic products should first be validated by reliable medical and/or immunological testing. Also ethical and legal questions, especially related to GM products, have to be considered. Adequate communication on these issues to different stakeholder groups is a relevant prerequisite that needs further establishment and exploration (Miles et al. 2005).

Currently, a new generation of GM crops is under development. More and more, plant-own DNA is used to introduce a desired trait or to silence unwanted genes. The GM RNAi approach is a good example, also because RNA interference is a natural and widespread mechanism of gene regulation in living organisms. In addition, selection and reporter marker genes are applied that are flanked by sequences that allow specific recombinases to excise these genes from the host DNA after they fulfilled their task during the early stage of the modification process. These new developments enable to produce 'clean' GM crop cultivars that are hardly distinguishable from their parent (except for the new phenotype) and that do no longer contain 'unwanted alien' genes. A more relaxed application of European legislation on such new-generation GM crops is ahead.

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References

Bolhaar, S.T., Van Ree, R., Ma, Y., et al., 2005. Severe allergy to sharon fruit caused by birch pollen. *International Archives of Allergy and Immunology,* 136 (1), 45-52.

Bolhaar, S.T.H.P., Van de Weg, W.E., Van Ree, R., et al., 2004. In vivo assessment by prick-to-prick testing and double-blind placebo-controlled food challenge of allergenicity of apple cultivars. *In:* Bolhaar, S.T.H.P. ed. *Fruit allergy: from sensitization and symptoms to prevention and treatment*. University Medical Centre Utrecht, Utrecht.

Breiteneder, H. and Mills, E.N., 2005. Molecular properties of food allergens. *Journal of Allergy and Clinical Immunology,* 115 (1), 14-23; quiz 24.

Burks, A.W. and Fuchs, R.L., 1995. Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. *Journal of Allergy and Clinical Immunology,* 96 (6 Pt. 1), 1008-1010.

Colombo, P., Bonura, A., Costa, M., et al., 2003. The allergens of Parietaria. *International Archives of Allergy and Immunology*, 130 (3), 173-179.

Crevel, R. and Madsen, C., 2004. Assessing the allergenicity of novel and GM foods. *In:* Mills, E.N.C. and Shewry, P.R. eds. *Plant food allergens*. Blackwell science, Oxford, 171-195.

Ebner, C., Birkner, T., Valenta, R., et al., 1991. Common epitopes of birch pollen and apples: studies by western and northern blot. *Journal of Allergy and Clinical Immunology,* 88 (4), 588-594.

FAO and WHO, 2003. *Codex principles and guidelines on foods derived from biotechnology*. FAO, Rome. [ftp://ftp.fao.org/codex/standard/en/CodexTextsBiotechFoods.pdf]

Fernández-Rivas, M., Van Ree, R. and Cuevas, M., 1997. Allergy to *Rosaceae* fruits without related pollinosis. *Journal of Allergy and Clinical Immunology,* 100 (6 Pt. 1), 728-733.

Fiers, M.W.E.J., Kleter, G.A., Peijnenburg, A.A.C.M., et al., 2005. *In silico* prediction of potential allergenicity of proteins according to the FAO/WHO guidelines with the help of Allermatch. *In:* Gilissen, L.J.W.J., Wichers, H.J., Savelkoul, H.F.J., et al. eds. *Allergy matters: new approaches to allergy prevention and management: the international conference on allergy prevention, Wageningen, The Netherlands 4-6 February 2004*. Springer, this volume, chapter 12.

Gao, S.Z., Van de Weg, W.E., Matos, C., et al., in press-a. Allelic constitution of *Mal d 1* genes on linkage group 16 is related to the differences in allergenicity among apple cultivars. *In:* Gao, S.Z. ed. *Localization of candidate allergen genes on the apple (Malus domestica) genome and their putative allergenicity*. Wageningen University and Research Centre, Wageningen.

Gao, S.Z., Van de Weg, W.E., Schaart, J.G., et al., in press-b. Genomic characterization and linkage mapping of the apple allergen genes *Mal d 2* (thaumatin-like protein) and *Mal d 4* (profilin). *In:* Gao, S.Z. ed. *Localization of candidate allergen genes on the apple (Malus domestica) genome and their putative allergenicity*. Wageningen University and Research Centre, Wageningen.

Gao, Z.S., Van de Weg, W.E., Schaart, J.G., et al., in press-c. Genomic cloning and linkage mapping of the *Mal d 1* (PR-10) gene family in apple (*Malus domestica*). *Theoretical and Applied Genetics*.

Gao, Z.S., Van de Weg, W.E., Schaart, J.G., et al., 2005. Linkage map positions and allelic diversity of two *Mal d 3* (non-specific lipid transfer protein) genes in the cultivated apple (*Malus domestica*). *Theoretical and Applied Genetics,* 110 (3), 479-491.

Gilissen, L J., Bolhaar, S.T., Matos, C.I., et al., 2005a. Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *Journal of Allergy and Clinical Immunology,* 115 (2), 364-369.

Gilissen, L.J.W.J., Van Heteren, G., Van der Meer, I.M., et al., in press. Towards prevention of allergy through an integrated multidisciplinary approach. *NJAS Wageningen Journal of Life Sciences*.

Gilissen, L.J.W.J., Wichers, H.J., Savelkoul, H.F.J., et al., 2005b. Future developments in allergy prevention: a matter of integrating medical, natural and social sciences. *In:* Gilissen, L.J.W.J., Wichers, H.J., Savelkoul, H.F.J., et al. eds. *Allergy matters: new approaches to allergy prevention and management: the international conference on allergy prevention, Wageningen, The Netherlands 4-6 February 2004*. Springer, this volume, chapter 1.

Gremmen, B., Gilissen, L.J.W.J., Jacobs, J.G.M., et al., 2004. *Zorgen over veiligheid*. Wageningen Universiteit en Research Centrum, Wageningen. META Reeks no. 4.

Helsper, J.P., Gilissen, L.J., Van Ree, R., et al., 2002. Quadrupole time-of-flight mass spectrometry: a method to study the actual expression of allergen isoforms identified by PCR cloning. *Journal of Allergy and Clinical Immunology,* 110 (1), 131-138.

Herman, E.M., 2003. Genetically modified soybeans and food allergies. *Journal of Experimental Botany,* 54 (386), 1317-1319.

Krebitz, M., Wagner, B., Ferreira, F., et al., 2003. Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. *Journal of Molecular Biology,* 329 (4), 721-730.

Kusaba, M., 2004. RNA interference in crop plants. *Current Opinion in Biotechnology,* 15 (2), 139-143.

Lehrer, S.B., Horner, W.E. and Reese, G., 1996. Why are some proteins allergenic? Implications for biotechnology. *Critical Reviews in Food Science and Nutrition,* 36 (6), 553-564.

Miles, S., Bolhaar, S.T.H.P., González-Mancebo, E., et al., 2004. Attitudes towards low allergen food in food allergic consumers. *In:* Bolhaar, S.T.H.P. ed. *Fruit allergy: from sensitization and symptoms to prevention and treatment*. University Medical Centre Utrecht, Utrecht, 91-105.

Miles, S., Crevel, R., Chryssochoidis, G., et al., 2005. Communication needs and food allergy: an analysis of stakeholder views. *In:* Gilissen, L.J.W.J., Wichers, H.J., Savelkoul, H.F.J., et al. eds. *Allergy matters: new approaches to allergy prevention and management: the international conference on allergy prevention, Wageningen, The Netherlands 4-6 February 2004*. Springer, this volume, chapter 18.

Pastorello, E.A., Pravettoni, V., Farioli, L., et al., 2002. Hypersensitivity to mugwort (*Artemisia vulgaris*) in patients with peach allergy is due to a common lipid transfer protein allergen and is often without clinical expression. *Journal of Allergy and Clinical Immunology,* 110 (2), 310-317.

Runge, C.F. and Ryan, B., 2004. *The global diffusion of plant biotechnology: international adaptation and research in 2004*. Council of Biotechnology Information, Washington. [http://www.apec.umn.edu/faculty/frunge/globalbiotech04.pdf]

Sánchez-Monge, R., Lombardero, M., García-Sellés, F.J., et al., 1999. Lipid-transfer proteins are relevant allergens in fruit allergy. *Journal of Allergy and Clinical Immunology,* 103 (3 Pt. 1), 514-519.

Son, D.Y., Scheurer, S., Hoffmann, A., et al., 1999. Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. *European Journal of Nutrition,* 38 (4), 201-215.

Tada, Y., Nakase, M., Adachi, T., et al., 1996. Reduction of 14-16 kDa allergenic proteins in transgenic rice plants by antisense gene. *FEBS Letters,* 391 (3), 341-345.

Van Loon, L.C. and Van Strien, E.A., 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology,* 55 (2), 85-97.

Van Ree, R., 1997. The oral allergy syndrome. *In:* Amin, S., Lahti, A., Maibach, H.I. ed. *Contact Urticaria Syndrome*. CRC Press, Boca Raton, 289-299.

Van Ree, R., 2002. Clinical importance of non-specific lipid transfer proteins as food allergens. *Biochemical Society Transactions,* 30 (Pt. 6), 910-913.

Wensing, M., Akkerdaas, J.H., van Leeuwen, W.A., et al., 2002. IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance. *Journal of Allergy and Clinical Immunology,* 110 (3), 435-442.

Wichers, H., Matser, A., Van Amerongen, A., et al., 2003. Monitoring of and technological effects on allergenicity of proteins in the food industry. *In:* Mills, E.N.C. and Shewry, P.R. eds. *Plant food allergens*. Blackwell, Oxford, 196-212.