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https://doi.org/10.1111/all.12684

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

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ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

First successful reduction of clinical allergenicity of food by genetic modification: *Mal d 1*-silenced apples cause fewer allergy symptoms than the wild-type cultivar

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To cite this article: Dubois AEJ, Pagliarani G, Brouwer RM, Kollen BJ, Dragsted LO, Eriksen FD, Callesen O, Gilissen LJWJ, Krens FA, Visser RGF, Smulders MJM, Vlieg-Boerstra BJ, Flokstra-de Blok BJ, van de Weg WE. First successful reduction of clinical allergenicity of food by genetic modification: *Mal d 1*-silenced apples cause fewer allergy symptoms than the wild-type cultivar. *Allergy* 2015; **70**: 1406–1412.

Keywords
apple allergy; food allergy; food challenge; genetically modified food; RNAi.

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Accepted for publication 23 June 2015

DOI:10.1111/all.12684

Edited by: Reto Crameri

Abstract

Background: Genetic modification of allergenic foods such as apple has the potential to reduce their clinical allergenicity, but this has never been studied by oral challenges in allergic individuals.

Methods: We performed oral food challenges in 21 apple-allergic individuals with Elstar apples which had undergone gene silencing of the major allergen of apple, Mal d 1, by RNA interference. Downregulation of *Mal d 1* gene expression in the apples was verified by qRT-PCR. Clinical responses to the genetically modified apples were compared to those seen with the wild-type Elstar using a visual analogue scale (VAS).

Results: Gene silencing produced two genetically modified apple lines expressing *Mal d 1.02* and other *Mal d 1* gene mRNA levels which were extensively downregulated, that is only 0.1–16.4% (e-DR1) and 0.2–9.9% (e-DR2) of those of the wild-type Elstar, respectively. Challenges with these downregulated apple lines produced significantly less intense maximal symptoms to the first dose (Vmax1) than with Elstar (Vmax1 Elstar 3.0 mm *vs* 0.0 mm for e-DR1, $P = 0.017$ and 0.0 mm for e-DR2, $P = 0.043$), as well as significantly less intense mean symptoms per dose (meanV/d) than with Elstar (meanV/d Elstar 2.2 mm *vs* 0.2 mm for e-DR1, $P = 0.017$ and 0.0 mm for e-DR2, $P = 0.043$). Only one subject (5%) remained symptom-free when challenged with the Elstar apple, whereas 43% did so with e-DR1 and 63% with e-DR2.

Conclusion: These data show that mRNA silencing of *Mal d 1* results in a marked reduction of *Mal d 1* gene expression in the fruit and reduction of symp-

Abbreviations
AU, arbitrary units; CI, confidence interval; e-DR1, extensively downregulated genetically modified apple line 1; e-DR2, extensively downregulated genetically modified apple line 2; FDR, false discovery rate; GM (line), genetically modified (line); GMO, genetically modified organism; IQR, interquartile range; meanV/d, mean visual analogue scale score per dose; p-DR, partially downregulated genetically modified apple line; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; RT-PCR, reverse transcriptase polymerase chain reaction; VAS, visual analogue scale; Vmax1, maximum visual analogue scale score to dose 1.

toms when these apples are ingested by allergic subjects. Approximately half of the subjects developed no symptoms whatsoever, and virtually all subjects wished to consume the apple again in the future.

Food allergies mediated by IgE antibodies are increasing in prevalence in westernized countries and are now a major health concern (1). Treatment of these disorders is currently experimental, so that the only measures available are strict avoidance and provision of emergency medication for those at risk for severe reactions (2). Apple allergy is one of the most common IgE-mediated forms of food allergy in areas where birch pollinosis is common (3). Cross-reactivity between the major allergen of birch (Bet v 1) and apple (Mal d 1) causes symptoms which, although often mild and transient, prevent allergic individuals from consuming apples and related fruits. Moreover, systemic allergic reactions to apple are not rare, may occasionally be severe, and are also associated in birch-endemic areas with sensitization to Mal d 1 rather than other apple allergens (4).

Genetic modification of plant foods such as apple is a possible method to create varieties tolerable to allergic individuals (5). RNA silencing technology has been applied to apple, where silenced varieties gave a reduction in the size of immediate skin tests to apple leaves in sensitized individuals (6). However, the lack of correlation between skin tests and the intensity of symptoms in patients with apple allergy has been well documented (7–10). Moreover, suppressing the expression of a single (iso)allergen in a food may not be sufficient to avoid symptoms, especially as other apple allergens such as lipid transfer protein may be of importance in some apple-allergic patients (11). Furthermore, suppression of one (iso) allergen may lead to compensatory overexpression of other (iso)allergenic proteins, as has been demonstrated for soy (12). In addition, there are numerous isoforms of Mal d 1 (13), such that complete suppression of all underlying genes is unlikely. Importantly, no studies to date on genetically modified foods included challenges with the putatively hypoallergenic foods themselves in allergic individuals, and it is therefore unclear whether this strategy would be successful in reducing or preventing allergic symptoms following ingestion of these products.

We therefore designed a proof of concept study and challenged apple-allergic individuals with Elstar apples which had undergone gene silencing of *Mal d 1* by the RNA interference method (6) and compared clinical responses in oral challenge tests to responses to wild-type Elstar apples.

Methods

Apples

The genetically modified (GM) apple lines were derived from the Elstar cultivar and harbour an RNAi construct for *Mal d 1.02* (6). They were produced by Plant Research International, Wageningen University and Research Centre, the Netherlands, as previously described (6). In 2005, they were transferred as rooted *in vitro* plantlets to the National Food Institute, Technical University of Denmark, where they were grown to potted plants under growth chamber conditions. In 2006, they were moved to GMO-approved greenhouse facilities of the Faculty of Agricultural Sciences, University of Aarhus, where they were grafted onto M9 rootstocks and raised to fruit-bearing trees according to Danish GMO regulations.

No reliable methods are available for quantification of protein content of (total) Mal d 1 or its isoforms and variants due to instability of the molecule (14). Therefore, gene expression and silencing were monitored by means of PCR-based gene expression studies during the vegetative phase of the apple trees as well as on the final fruit. The stability of the genetic modification was verified by repeated reverse transcriptase PCR evaluations of leaf tissue for *Mal d 1.02* (15). This analysis showed that in one GM line, there was consistent partial downregulation of *Mal d 1* (referred to hereafter as the 'p-DR' apple line), and in two other GM lines, downregulation of *Mal d 1* was consistently more extensive (referred to hereafter as the 'e-DR1' and 'e-DR2' apple lines).

In 2009, very few apples were produced. In 2010, more fruit was produced, but only limited quantities of e-DR2. All fruit was harvested at full maturity and stored at 3–4°C until shipping to Wageningen UR, in November of 2010, where fruit was similarly stored until the start of clinical testing. Comparisons of challenge responses of the GM apple lines were made with a wild-type (genetically unmodified) Elstar apple that was grown at the Wageningen UR Experimental Station at Randwijk, the Netherlands. Other apple cultivars used for inclusion of the patients in the study were provided by the study subjects themselves.

Assessment of Mal d 1 downregulation in fruit

Mal d 1 gene expression in fruit was examined by qRT-PCR for each of the 31 known *Mal d 1* genes (13) using available primers and protocols (16). For each single fruit, peel, pulp and core were separated and immediately transferred to liquid nitrogen, then stored at −80°C. Four individual fruits (biological replicates) were tested, except for e-DR2, where only three samples were tested due to limited availability. Each sample was evaluated by three technical replicates. Expression was estimated following a 'standard curve' approach (16), normalized with respect to *actin* and reported in arbitrary units (A.U.).

Patients

Adult subjects with a history of oral allergy to apple were recruited from those who had participated in earlier research on apple allergy in our centre and by means of advertising. Prior to the challenges, all subjects were given a questionnaire

about apple allergy and any other possible allergies. Individuals who had reacted systemically to apple in the past were excluded from enrolment in the study. Other exclusion criteria were possible pregnancy, use of beta-blocking agents, immunological or other severe disease or allergen immunotherapy. Physical examination to exclude major cardiopulmonary pathology was performed. Patients were included if they had a positive open challenge to the apple cultivar which they indicated had caused the most symptoms in the past.

This study was approved by the local medical ethics review commission. All subjects gave written informed consent before enrolment in the study.

Oral challenge tests

Subjects were asked to withdraw from antihistamines 48 h in advance of the challenges and not to eat or drink 1 h in advance. At inclusion, all subjects were challenged with the apple cultivar that gave the most symptoms during previous exposures. This was followed by challenges with Elstar and the GM apples in a random order in a single session. To minimize participant sensory perception without undue compromise of allergenicity of the apples, subjects were blind-folded and wore a nose clip during the challenges (9). Patients were asked to chew and swallow the apple in the usual way. A maximum of three consecutive doses of the same apple were given depending on the occurrence and intensity of allergic symptoms. If symptoms were considered tolerable by the patient or remained absent after a dose, a subsequent dose was given. The first dose consisted of a single bite (approximately 15 g) from a whole apple, the second dose consisted of 30 g of apple cut in a single piece, and the final dose consisted of 100 g of apple. All doses included the pulp and peel of the apple. Symptoms had to have resolved completely before the challenge with the next dose or apple could begin. Symptoms were recorded using a visual analogue scale (VAS) at baseline at the start of the challenge session and just prior to and 5, 10 and 15 min after each dose. Directly following the final oral challenge with each apple, subjects were asked whether they would be willing to eat that particular apple again. Challenges were first made in 17 subjects in 2009, the first year in which apples were available. However, quantities of all the GM apples were unexpectedly limited, and none of the subjects received all doses of any apple. The data presented here were obtained from challenges of 21 apple-allergic patients which all took place in December of 2010, before the commencement of the birch pollen season.

Statistical analysis

Statistical significance of differences in gene expression levels between the wild-type Elstar and the GM apple lines was evaluated using the Mann–Whitney *U*-test. A number of analyses were undertaken to assess the effect of the genetic modification of the Elstar on the occurrence and intensity of allergic symptoms by comparing the outcomes of the wild-type Elstar to those of the GM apple lines. The maximum VAS score at 5, 10 or 15 min after the first dose (Vmax1) was compared between the wild-type Elstar and the GM lines using the Wilcoxon test. In addition, the mean VAS score per dose (meanV/d) was calculated by dividing the sum of the VAS scores registered at 5, 10 or 15 min after each dose consumed by the number of times the VAS score was registered. The meanV/d was compared between the wild-type Elstar and the GM lines using the Wilcoxon test. The number of doses consumed for each of the different apples was compared between the wild-type Elstar and the GM lines using the Wilcoxon test. Percentages of subjects who remained symptom-free during the challenges were compared between the different apples using statistics of absolute risk reduction and 95% confidence intervals (95% CIs). To examine the distribution of 'time to effect' (eliciting dose during the challenge), Kaplan–Meier survival analysis was used. Finally, percentages of subjects who were willing to eat the different apples again in the future were compared using statistics of absolute risk reduction and 95% CIs.

Data were analysed using SPSS software for Windows (Version 20.0 IBM Corp., Armonk, NY, USA). The false discovery rate (FDR) due to multiple testing was controlled for by Benjamini–Hochberg's step-up procedure (17) to maintain an overall type I error rate of 5%. Adjusted *P*-values are reported.

Results

Assessment of Mal d 1 downregulation in fruit

Expression of *Mal d 1.02* was downregulated to 0.1–2.2% of the wild-type Elstar for the GM lines e-DR1 and e-DR2 (Table 1). This reduction was observed in all three tissues of the apple fruit: peel, pulp and core. In contrast, despite partial silencing in the leaves of the plants (15), the GM line p-DR showed no significant silencing in the peel and upregulation for the pulp and core (Table 1). The overall expression levels of the other *Mal d 1* genes were downregulated in GM apple lines e-DR1 and e-DR2, resulting in expression levels of 5–16% of the wild-type Elstar. In contrast, p-DR did not show significant changes in the overall expression of the other *Mal d 1* genes (Table 1).

Patients

A total of 21 consecutive patients were included in the study, as no patients needed to be excluded for any reason. The demographic characteristics of these patients are shown in Table 2. In all but one patient, the inclusion challenge resulted in oral allergy symptoms which were significantly more intense than those produced by the Elstar apple (results not shown). All patients underwent a challenge with Elstar, p-DR and e-DR1. The e-DR2 was challenged in only eight patients because of limited quantities of this apple.

Oral challenge outcomes

Maximum VAS of dose 1 (Vmax1)

There was no significant difference between the Vmax1 with Elstar and p-DR ($P = 0.446$), but there was a highly

Table 1 *Mal d 1* expression levels in apple tissue (peel, pulp and core) of three GM apple lines and wild-type Elstar. Expression levels as determined by qPCR are normalized with respect to *actin* and reported in arbitrary units (A.U.) based on medians and 95% confidence intervals

			Mal d 1 gene									
			1.02					Other *Mal d 1* genes				
Cultivar/GM apple line	Tissue	*N*	AU Median	95%CI	U†	*P*	% relative to Elstar	AU Median	95%CI	U†	*P*	% relative to Elstar
Elstar	Peel	4	51.64	42.51–61.82				49.77	42.86–56.68			
	Pulp	4	4.53	4.19–9.68				10.76	3.12–22.11			
	Core	4	6.92	4.06–9.24				11.00	7.80–18.32			
e-DR1	Peel	4	0.05	0.04–0.19	0	0.043*	0.1	2.79	1.83–5.41	0	0.043*	5.6
	Pulp	4	0.07	0.00–0.25	0	0.043*	1.5	1.76	0.93–4.80	1.00	0.057	16.4
	Core	4	0.04	0.01–0.07	0	0.043*	0.6	1.25	0.93–2.96	0	0.043*	11.4
e-DR2	Peel	3	0.08	0.02–0.37	0	0.046*	0.2	2.49	1.93–3.53	0	0.046*	5.0
	Pulp	3	0.10	0.03–0.29	0	0.046*	2.2	1.07	0.94–2.11	0	0.046*	9.9
	Core	3	0.07	0.07–0.51	0	0.046*	1.0	0.95	0.57–1.74	0	0.046*	8.6
p-DR	Peel	4	77.42	45.14–142.74	2.00	0.105	150	45.42	24.49–53.27	5.00	0.442	91.3
	Pulp	4	85.81	77.50–130.82	0	0.043*	1894	11.80	4.16–16.69	8.00	1.000	109.7
	Core	4	54.06	28.15–91.19	0	0.043*	781	15.67	6.71–33.15	7.00	0.809	142.5

*Values that are significant at $\alpha = 0.05$ (two-tailed).
†*U*-values from the Mann–Whitney *U*-test following from pairwise comparisons between each of the GM lines to wild-type Elstar.

Table 2 Descriptive characteristics of study subjects

Sex, *n* (m/f)	6/15
Age in years, mean (SD)	24.2 (4.5)
Reported allergies, *n* (%)	
Pollen	19 (90)
Fruits (other than apple)	6 (29)
Nuts	8 (38)
Vegetables	4 (19)
Animal dander	6 (29)
House dust mites	7 (33)
Inclusion apple challenge, *n* (%)	
Golden delicious	17 (81)
Granny smith	2 (10)
Jonagold	1 (5)
Elstar	1 (5)

significant difference between Vmax1 elicited by Elstar (median 3.0 mm) and e-DR1 (0.0 mm, $P = 0.017$) and a significant difference between Elstar and e-DR2 (0.0 mm, $P = 0.043$). The Vmax1 seen with the p-DR (median 3.0 mm) was significantly higher than that of e-DR1 ($P = 0.026$) or e-DR2 ($P = 0.043$). There was no difference between the Vmax1 of e-DR1 and e-DR2 ($P = 0.809$) (Table 3 and Fig. 1).

Mean VAS per dose (meanV/d)
There was no significant difference in the meanV/d obtained with the Elstar and p-DR apples ($P = 0.216$). The Elstar showed significantly higher meanV/d (median 2.2 mm) than e-DR1 (0.2 mm, $P < 0.017$) and the e-DR2 (0.0 mm, $P = 0.043$). Similarly, the p-DR showed a significantly higher meanV/d (1.2 mm) than did e-DR1 ($P = 0.026$) or e-DR2 ($P = 0.043$). There was no difference between the meanV/d of e-DR1 and e-DR2 ($P = 0.159$) (Table 3 and Fig. 2).

Number of doses consumed
In comparison with the Elstar, of which only 57% of patients consumed all three doses, significantly more doses were consumed of the e-DR1 apple, where all patients consumed all three doses ($P = 0.034$). Although all patients also consumed all three doses of the e-DR2 apple, this did not reach significance in comparison with Elstar ($P = 0.086$). In contrast, in comparison with the p-DR, where 76% of patients consumed all three doses, the number of doses that were consumed of the e-DR1 ($P = 0.050$) and of the e-DR2 ($P = 0.105$) was not significantly greater (Table 3 and Fig. 3).

Number of subjects remaining symptom-free
Only one of 21 subjects remained symptom-free after consuming all challenge doses with Elstar, and only 2 of 21 did so with the p-DR. In contrast, 9 of 21 (43%) patients challenged with e-DR1 and 5 of 8 (63%) patients challenged with e-DR2 remained completely symptom-free during the entire challenge. This indicates a significant absolute risk reduction of 0.38 (95% CI; 0.12–0.59) comparing Elstar with e-DR1 and a significant absolute risk reduction of 0.58 (95% CI; 0.21–0.82) comparing Elstar with e-DR2 (Table 3).

Number of subjects willing to eat the apple again in the future
Six of 21 subjects indicated they would be willing to eat the Elstar apple again (29%). In contrast, 15 of 21 stated they would be willing to eat the p-DR apple again (71%). This approval increased to 20 of 21 patients for e-DR1 (95%) and 8 of 8 subjects for e-DR2 (100%). This indicates a significant absolute risk reduction of 0.46 (95% CI; 0.16–0.67)

Table 3 Outcomes of the challenges with Elstar, and three GM apple lines (p-DR, e-DR1 and e-DR2)

Apple	Elstar	p-DR	e-DR1	e-DR2
Type	Wild	GM, partially downregulated	GM, extensively downregulated	GM, extensively downregulated
Patients challenged, *n*	21	21	21	8
Vmax1 in mm, median (IQR)	3.0 (2.0–9.0)	3.0 (2.0–6.0)	0.0 (0.0–1.0)	0.0 (0.0–1.5)
MeanV/d in mm, median (IQR)	2.2 (0.8–6.0)	1.2 (0.4–2.7)	0.2 (0.0–1.3)	0.0 (0.0–0.7)
Doses consumed, *n* (%)				
One dose	7 (33%)	1 (5%)	0	0
Two doses	2 (10%)	4 (19%)	0	0
Three doses	12 (57%)	16 (76%)	21 (100%)	8 (100%)
Patients remaining symptom-free, *n* (%)	1 (5%)	2 (10%)	9 (43%)	5 (63%)
Patients willing to eat apple again, *n* (%)	6 (29%)	15 (71%)	20 (95%)	8 (100%)

IQR, interquartile range.

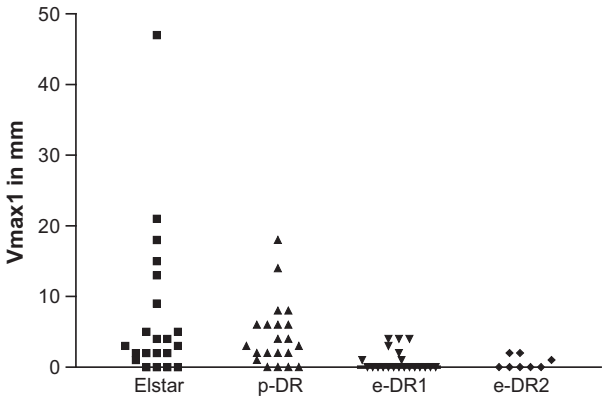

Figure 1 Maximum intensity of symptoms to the first challenge dose to the different apple lines (number of totally negative; $n = 4$, $n = 4$, $n = 14$, $n = 5$, respectively).

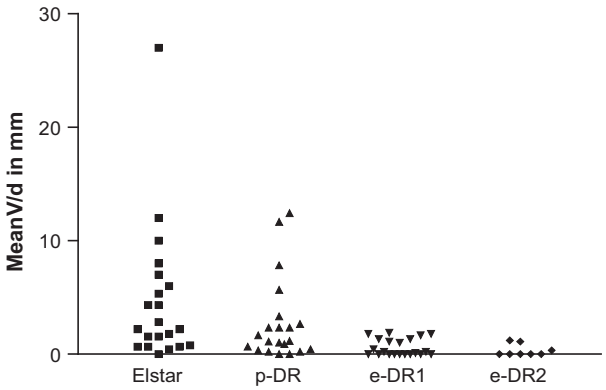

Figure 2 The VAS mean per dose for the different apple lines (number of totally negative; $n = 1$, $n = 2$, $n = 9$, $n = 5$, respectively).

comparing Elstar with p-DR, a significant absolute risk reduction of 0.67 (95% CI; 0.39–0.82) comparing Elstar with e-DR1 and a significant absolute risk reduction of 0.71 (95% CI; 0.33–0.86) comparing Elstar with e-DR2 (Table 3).

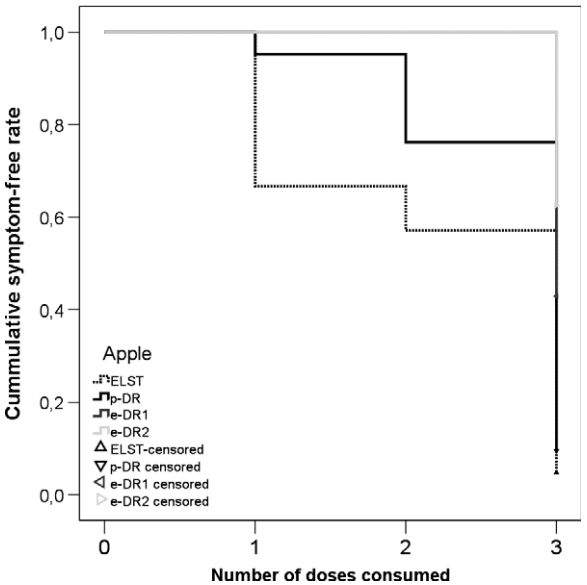

Figure 3 Eliciting dose for the different apple lines (Kaplan–Meier survival analysis).

Discussion

In this study, we have shown that gene silencing with RNAi technology aimed at reducing the major allergen content of apple is capable of producing apples for which many apple-allergic subjects are completely tolerant. To our knowledge, this is the first time that this has been demonstrated for an allergenic food. This finding is a proof of concept that genetically modified foods may be of value in allowing patients to eat foods to which they are otherwise allergic.

Another important aspect of the therapeutic potential of genetically modified foods is the possibility that prolonged exposure to such foods may eventually induce tolerance to these foods in their native form. Oral tolerance induction has been widely studied in recent years (18, 19). A limitation of this treatment form is the frequency of side-effects because of the allergenicity of foods in their native form which thus

requires medical supervision and is difficult to maintain for long periods of time (18, 19). Studies on immunotherapy with inhalant allergens using peptides and recombinant allergens demonstrate the efficacy of such preparations despite the fact that many allergens found in the source material are absent from the therapeutic extract (20). Although this approach has not been used in the treatment of food allergy, these findings raise the possibility that foods in which allergens have been silenced by genetic modification may be useful in promoting oral tolerance induction to the native food.

The acceptance of genetically modified foods is contingent upon societal acceptance of these products. Factors determining such acceptance have been studied in apple allergy, where it has been found that the relative importance of 'benefits' is greater than that of 'rejection factors' (21). This is in keeping with our results, where significant numbers of participants indicated they would eat the genetically modified study apples again in the future. This suggests that genetically modified foods would be accepted by food-allergic patients themselves despite concerns about such products at a societal level.

The p-DR line showed clinical effects which were intermediate between the wild-type Elstar and the e-DR apple lines. Despite the fact that silencing of *Mal d 1.02* in leaves was moderate, upregulation of *Mal d 1.02* in fruit was observed (Table 1). The finding of upregulation of certain Mal d 1 isoforms following a genetic modification procedure such as RNA interference is not unusual, as upregulation has been reported as being a response to various kinds of stress, including infection, storage or the genetic modification procedure itself (8, 22–25). However, the observation that upregulation of *Mal d 1.02* is accompanied by decreased clinical symptoms is unexplained, and suggests that Mal d 1.02 may not be uniquely important as a cause of clinical allergenicity in comparison with other Mal d 1 isoallergens. Differences in allergenicity have been demonstrated in previous studies in relation to Bet v 1 isoforms and IgE responses (26) as well as *Mal d 1* genes and skin test results (27). Here, we extend that observation and speculate that different Mal d 1 isoforms may have differential effects on clinical symptom elicitation. Further examination of the expression and clinical allergenicity of individual Mal d 1 proteins may clarify this.

There are some limitations to this study. All oral challenges took place outside the birch pollen season. As it has been reported that symptoms of pollen–fruit syndrome may be more severe during the birch pollen season (28), our data may underestimate the clinical allergenicity of the genetically modified apples. A second possible limitation is that single-blind challenges with sensory limitation were used to assess the clinical reactions instead of double-blind, placebo-controlled challenges. However, preparing materials from apple for double-blind use is notoriously difficult due to instability of the apple allergen, Mal d 1 (29). Consequently, the double-blind challenge procedure runs the risk of underestimating clinical allergenicity. Mal d 1 instability is also the reason that we were unable to measure Mal d 1 directly in a reliable fashion. No tools exist to individually assess the content of the multiple isoforms and variants of Mal d 1 that are highly similar in amino acid sequence and that are likely to differ considerably in quantity considering that expression levels of the underlying genes vary by several orders of magnitude (13, 25, 30; Table 1). Indeed, usually only the most abundant isoforms such as Mal d 1.01 and Mal d 1.02 were traced whereby other isoforms remained unidentified and/or unnoticed (31). Therefore, gene expression and silencing were monitored by means of PCR-based gene expression studies during the vegetative phase of the apple trees as well as on the final fruit. Another possible limitation is that all the apples were challenged in a single session. Although this raises the possibility of a desensitization effect for apples challenged last, the order of the challenges was randomized for each patient, so that such effects would have been distributed equally over all apple cultivars. Finally, the growing and storage conditions which pertained to the wild-type Elstar apple were different from those pertaining to the GM apples. Although no consistent significant differences have been shown to result, for example, from storage (10), other studies have found such differences and this may have influenced our study outcome. However, we also found significant differences between the effects of the e-DR and p-DR lines, including lower VAS scores of the first dose, lower mean VAS scores per dose, and greater numbers of patients remaining symptom-free during the entire challenge with the e-DR line. As all the GM lines were grown and stored under identical conditions, we feel that the influence of factors such as growth and storage conditions on the study outcomes we observed was likely to be limited.

In summary, we have shown for the first time that mRNA silencing of Mal d 1 results in apples causing very few symptoms when ingested by apple-allergic subjects. Approximately half of the subjects developed no symptoms whatsoever, and almost all subjects wished to consume the GM apple again in the future. These findings suggest that genetically modified foods may allow for long-term consumption of these products by food-allergic individuals without intensive medical supervision. Such exposure may have the potential to induce lasting tolerance to these foods in their native form.

Acknowledgments

This study was carried out with financial support from the Ministry of Economic Affairs, Agriculture and Innovation of the Netherlands (KB-05-001-019, KB-15-001-007) and from the Commission of the European Communities for the project ISAFRUIT, which is funded under the Thematic Priority 5-Food Quality and Safety of the 6th Framework Programme of RTD (Contract no. FP6-FOOD-CT-2006-016279). We thank Dr. T. Warkentin and Dr. G.H. Koppelman for critically reviewing this manuscript. We thank Dr. M. Kerkhof for statistical assistance and advice.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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