

Silencing the major apple allergen Mal d 1 by using the RNA interference approach

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Background: Apple allergy is dominated by IgE antibodies against Mal d 1 in areas where birch pollen is endemic. Apples with significantly decreased levels of Mal d 1 would allow most patients in these areas to eat apples without allergic reactions. **Objective:** The aim of this study was to inhibit the expression of Mal d 1 in apple plants by RNA interference.

Methods: *In vitro*-grown apple plantlets were transformed with a construct coding for an intron-spliced hairpin RNA containing a Mal d 1-specific inverted repeat sequence separated by a Mal d 1-specific intron sequence. The presence of the construct in transformants was checked by PCR. Expression of Mal d 1 in leaves was monitored by prick-to-prick skin testing in 3 patients allergic to apples and by immunoblotting with a Mal d 1-reactive mAb and with IgE antibodies against Mal d 1.

Results: After transformation, plantlets 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 gene-silencing construct was demonstrated. By skin prick test it was shown that a 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 M_r 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.

Conclusions: 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. (J Allergy Clin Immunol 2005;115:364-9.)

Key words: Gene silencing, Mal d 1, RNA interference, genetic modification, apple (*Malus domestica*), skin prick test, hypoallergenic, immunoblotting

Apple allergy is a common phenomenon in patients with birch pollen allergy. The explanation for this concordance is IgE cross-reactivity.¹⁻³ Approximately 90% of patients allergic to birch pollen have IgE antibodies against the birch pollen allergen Bet v 1.⁴ This allergen belongs to a group of so-called pathogenesis-related proteins, more specifically the PR10 proteins.^{5,6} Many plant foods, in particular fruits and tree nuts, contain homologous proteins that are recognized by the same Bet v 1-specific IgE antibodies. In apple, this allergen was designated Mal d 1.⁷ Cross-reactive IgE antibody responses against this allergen dominate the IgE response against apple in patients allergic to birch pollen.⁸ Approximately 70% of patients allergic to birch pollen have been reported to have adverse reactions to apple as a consequence of these cross-reactive IgE antibodies.⁹ Although birch pollen-related apple allergy is almost exclusively mild and restricted to the oral cavity, most patients allergic to apples avoid the fruit in their diet. Related fruits of the Rosaceae family, such as pear, cherry, and peach, as well as tree nuts such as hazelnut, can also induce adverse reactions on the basis of the same cross-reactive IgE antibodies.^{10,11} Therefore, avoidance often results in deprivation of the diet of a wide range of common plant foods that have important nutritional value.

Patients allergic to apples sometimes report that they tolerate one apple cultivar better than another. Differences in allergenicity have indeed been demonstrated.¹²⁻¹⁴ Recently, we screened a panel of apple cultivars by the prick-to-prick method and confirmed differences in

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Abbreviations used

BAP:	6-Benzylaminopurine
DBPCFC:	Double-blind placebo-controlled food challenge
RNAi:	RNA interference
SPT:	Skin prick test
UTR:	Untranslated region
WT:	Wild type

allergenicity by double-blind placebo-controlled food challenges (DBPCFCs; S. Bolhaar et al, unpublished data). Golden Delicious proved to be among the cultivars with high allergenicity, whereas Santana was identified as a low-allergenic cultivar, with a 30-fold reduced response in the DBPCFC. This suggests that conventional breeding strategies can result in the production of apple cultivars of even lower allergenicity that will allow normal consumption of their fruits by patients allergic to apples. Promising results in this respect have been obtained recently in fruits from the progeny plants of a crossing between 2 apple cultivars of different allergenicity (W.E. van de Weg et al, unpublished data). However, Mal d 1 represents an extended gene family of at least 18 members (loci) that are arranged on the apple genome in several gene clusters (Z. Gao et al, unpublished data). Of these genes, the individual level of expression in the fruit and the degree of allergenicity have not yet been elucidated. This makes breeding of a hypoallergenic apple plant that expresses only low-allergenic Mal d 1 variants a complex matter. In addition, the production of a market-valuable cultivar from a new genotype takes at least 15 years, including tests for taste and texture, production and storage, consumer acceptability, and economic viability (T. Browne, personal communication). Therefore, the production of an apple plant with a significant reduction of the overall expression of Mal d 1 from an existing economically successful cultivar seems an attractive time-saving (by a factor of 2) and simpler alternative because its effect is independent of the individual Mal d 1 genes and antibody specificities.

Recent advances in plant biotechnology have enabled the (almost) complete silencing of specific genes, including genes that encode for allergenic proteins, eg, in rice¹⁵ and soy.¹⁶ One of the approaches is RNA interference (RNAi).¹⁷ This method for posttranscriptional gene silencing seems to be highly efficient, especially when a gene construct is used that consists of an inverted repeat of a fragment of the targeted gene sequence separated by an intron. Such a construct results in the formation of a so-called intron-spliced hairpin RNA. This method operates by 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.¹⁸ In this way, the endogenous RNA is prevented from passing from the nucleus to the ribosomes where it normally directs protein production.

In apple, representatives of the Mal d 1 gene family contain a single intron or are intronless (National Center for Biotechnology Information [NCBI] database; Z. Gao et al, unpublished data).¹⁹ One such intron-containing Mal d 1 gene (from the cultivar Gala) has been isolated to build the construct for posttranscriptional gene silencing of Mal d 1. The method is highly sequence specific. Nevertheless, it still enables silencing of entire gene families on the basis of sequence homology.^{20,21} Therefore, this RNAi strategy has been chosen to reduce overall Mal d 1 expression.

Normally it takes approximately 5 years to grow an apple fruit-producing tree from seed or *in vitro* culture. This would imply an extended lag in time between the transformation and the testing on successful reduction of Mal d 1 expression in the apple fruit. However, because Mal d 1 genes are expressed in leaves as well as in fruits (L. Beuning et al and G. Marzban et al, unpublished data), evaluation of effective Mal d 1 gene silencing should already be possible at an earlier stage, ie, in the leaves of young apple shoots growing *in vitro*. Using this strategy, the present study has provided support for the feasibility of producing hypoallergenic apples by silencing the genes for Mal d 1.

METHODS

Plant cultures

For transformation, the apple cultivar Elstar (E94) was used. This cultivar was grown under *in vitro* conditions on medium containing MS medium (4.6 g/L),²² sucrose (30 g/L), 6-benzylaminopurine (BAP) (0.7 mg/L), ethylenediamine di-2-hydroxyphenyl acetate ferric (FeDDHA) (96 mg/L), and Daishin agar (9 g/L). For transformation, the *in vitro* leaflets were subdivided into 4 explants. After the transformation, the explants were cultured on the following media, in order of use.

Shoot induction medium consisted of MS medium (4.6 g/L), sorbitol (30 g/L), thidiazuron (8.8 mg/L), 1-naphtalene acetic acid (0.1 mg/L), and phytagel (3 g/L); to this medium, kanamycin (150 mg/L) and cefotaxim (250 mg/L) were added to select for outgrowth of only the transformed plant cells and to kill *Agrobacterium tumefaciens* cells. Shoot elongation medium consisted of MS (4.6 g/L), galactose (10 g/L), sucrose (20 g/L), BAP (1 mg/L), GA3 (0.1 mg/L), cefotaxim (250 mg/L), and Daishin agar (9 g/L). Shoot propagation medium consisted of MS medium (4.6 g/L), sucrose (30 g/L), BAP (0.7 mg/L), FeDDHA (96 mg/L), and Daishin agar (9 g/L).

Before autoclaving, all media were adjusted to pH 5.7.

Construct for gene silencing

Primers were designed, according to a published sequence of Mal d 1 that was designated as Mal d 1b by Son et al¹⁴ (AF020542), to include the single Mal d 1 intron in the construct. The methods involved the following successive steps (see also Table 1 and Fig 1; restriction sites in the primers used for the cloning procedure are given in bold).

The 2 fragments for synthesis of the hairpin construct were obtained by PCR from genomic DNA isolated from the cultivar Gala. For fragment 1, the 5'-end primer 1 (All1HpaI) and the 3'-end primer 2 (All2SmaI) were used, resulting in a 516-bp fragment that encompassed the 5'-untranslated region, exon 1, intron, and a small part of exon 2. For fragment 2, the 5'-end primer 1 (All1HpaI) and the 3'-end primer 3 (All3SmaI) were used, resulting in a 276-bp fragment that encompassed the 5'-untranslated region and exon 1. Primers All1HpaI and All2SmaI were designed on the basis of the sequence of the Mal d 1 gene obtained from GenBank accession AF020542.⁶

TABLE I. Designed oligos for building the construct (based on the sequence from GenBank accession AF020542.⁶; primers 1 to 3) and for testing the presence of the construct in putative transformants (primers 4 and 5)

Primer 1 (All1HpaI) (5'-onward oligo introducing the <i>HpaI</i> site): 5'-CCAACAGTTAACTCTCAACCTCACTAAAACCATC- ATCC-3'
Primer 2 (All2SmaI) (5'-onward oligo for the 3'-end introducing the <i>SmaI</i> site within exon 2): 5'-CAAAGCCCCGGCGTAT- GAGTAGTTTGCTTCGTCACAC-3'
Primer 3 (All3SmaI) (5'-onward oligo for the 3'-end introducing the <i>SmaI</i> site at the end of exon 1): 5'-CTGACCTTACCCG- GGGTGATCTTTTG-3'
Primer 4 (Apple1): 5'-GCAAACACTACTCATACGCCCGGGTGAT- CTTTTG-3'
Primer 5 (Tnos-50): 5'-ATGATAATCATCGCAAGACCG-3'

Primer All3SmaI was designed on the basis of the sequence of fragment 1.

For amplification, the DNA was denatured at 96°C for 2 minutes and subjected to 40 cycles of PCR with Pwo polymerase (Roche Diagnostics, Almere, The Netherlands). Each PCR cycle consisted of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, and primer extension at 72°C for 45 seconds. The amplified fragments were purified from agarose gel, digested, cloned in the pRAP 37 expression cassette (D. Florack, unpublished data), and verified by sequencing. The pRAP 37 expression cassette contains a cauliflower mosaic virus 35S promoter with a double enhancer sequence and a viral leader sequence followed by unique restriction sites for cloning and a nopaline synthase terminator sequence. The complete expression cassette containing the promoter-gene fusions was cloned into the binary expression vector pBINPLUS²³ by using the *AscI* and the *PacI* restriction sites and was named pBiMald1.

Plant transformation

For transformation, the protocol according to Puite and Schaart was used.²⁴ In 3 independent transformation experiments, leaf explants from *in vitro* culture of the cultivar Elstar were placed for 30 minutes in liquid MS medium (4.6 g/L) with sucrose (20 g/L) at pH 5.2 (MS20) containing a log-phase suspension culture of *A tumefaciens* strain AGL0 carrying the pBiMald1 vector. They were then transferred to fresh solid MS20 medium for 4 days (cocultivation). Next, the explants were placed on shoot induction medium containing kanamycin (150 mg/L) and cefotaxim (250 mg/L). After callus formation, calli were isolated and subcultured individually. Newly appearing shoots were cut off from the callus and subcultured on shoot elongation medium. Elongated shoots were transferred to shoot propagation medium. In shoot elongation medium and shoot propagation medium, the kanamycin was absent. Finally, putatively transformed shoots were adapted for miniprep isolation and tested by PCR for the presence of the construct. Genomic DNA was isolated by grinding leaf material under liquid nitrogen and transferring the powder in urea extraction buffer; extracted with phenol-chloroform-isoamyl alcohol (25:24:1 by volume); and precipitated in isopropanol according to Greene et al.²⁵ To test for the presence of the construct in the isolated DNA, PCR was performed by using the 5'-end primer 4 (Apple1) and the 3'-end primer 5 (Tnos-50), resulting in a 330-bp fragment (see Table I for the primer sequences). For amplification, the DNA was denatured at 94°C for 1 minute and subjected to 35 cycles of PCR with SuperTaq polymerase (Sphaero Q, Leiden, The Netherlands). Each PCR cycle consisted of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and primer extension at 72°C for 1 minute. After the last cycle, the

samples were incubated at 72°C for 7 minutes for a final extension step. PCR products were loaded on gel. Shoots showing the presence of the construct were used for further analysis in skin prick tests (SPTs) and immunoblotting. Three *in vitro* plants that lacked the construct, as well as the untransformed wild-type (WT) plant, were used as controls for normal Mal d 1 expression. Because Elstar was not available at the time of the experiments, apple fruit from the cultivar Fiesta was used as a control in SPT. This cultivar was shown by SPT to have allergenicity in Bet v 1-sensitized individuals similar to that of Elstar (Bolhaar et al, unpublished data).

Skin prick test

Three adult patients with a history of birch pollinosis and apple allergy were recruited via the outpatient clinic of the University Medical Center Utrecht. Apple allergy was confirmed in these patients by a positive DBPCFC with apple, a positive skin test (wheal surface of ≥ 7 mm²), and/or a positive RAST (≥ 0.7 IU/mL) for birch pollen and apple. All patients had seasonal rhinitis during the period of February to April, the tree pollen season in The Netherlands. SPTs were performed on the flexor surface of the patient's forearm by using a standardized prick needle (ALK Lancet; ALK, Hørsholm, Denmark) according to Dreborg²⁶ and Dreborg and Foucard.²⁷ The transformed and control *in vitro* plantlets were applied by using the prick-to-prick method. Histamine dihydrochloride (10 mg/mL) was used as a positive control, and the glycerol diluent, normally applied for SPT extracts, was used as a negative control (ALK-Abelló, Nieuwegein, The Netherlands). SPT reactivity was measured after 15 minutes by copying the wheal reaction with transparent adhesive tape onto a record sheet for later comparison. The skin wheal areas were evaluated by computer scanning according to Poulsen et al.²⁸ SPT results were expressed relative to the histamine control. SPTs were regarded as positive when this ratio was ≥ 0.25 , ie, when the wheal induced by the plantlet was at least 25% of that of the positive control. The study was reviewed and approved by the local medical ethics committee. Informed consent was obtained from all subjects before enrollment in the study.

SDS-PAGE/immunoblotting

Equal amounts of leaves from plantlets were homogenized in 6% wt/vol PBS. Subsequently, sample mix was added, and the samples were heated at 100°C for 10 minutes. Equal amounts of protein were loaded, and proteins were separated by SDS-PAGE (NuPAGE® 4% to 12% Bis-Tris gel; protein, 10 µg/cm) according to the protocol of the manufacturer (Invitrogen, Carlsbad, Calif). Western blotting was performed by semidry transfer to nitrocellulose on a Novablot electrophoretic transfer apparatus according to the manufacturer's instructions (Invitrogen). For detection of Mal d 1, either mAb 5H8 directed to Bet v 1 and cross-reactive to Mal d 1 or a pool serum of patients allergic to apples and birch pollen was used. After blocking with PBS/10 mmol/L EDTA/0.3% BSA for a minimum of 10 minutes, blots were incubated overnight with 150 µL of serum pool or 10 µL of mAb 5H8²⁹ in 3 mL of PBS/0.3% BSA/0.1% Tween 20. After washing with PBS/0.1% Tween 20, radiolabeled sheep antibodies against human IgE or goat antibodies against mouse IgG (Sanquin, Amsterdam, The Netherlands) were used for detection of bound IgE or mAb 5H8, respectively. Blots were exposed to x-ray film (Eastman Kodak Company, Rochester, NY).

RESULTS

Plant transformation

From 3 independent transformation experiments with the construct pBiMald1 using 2770 Elstar *in vitro* leaf explants, approximately 1000 calli were formed after growth on shoot induction medium. Almost 340 of these

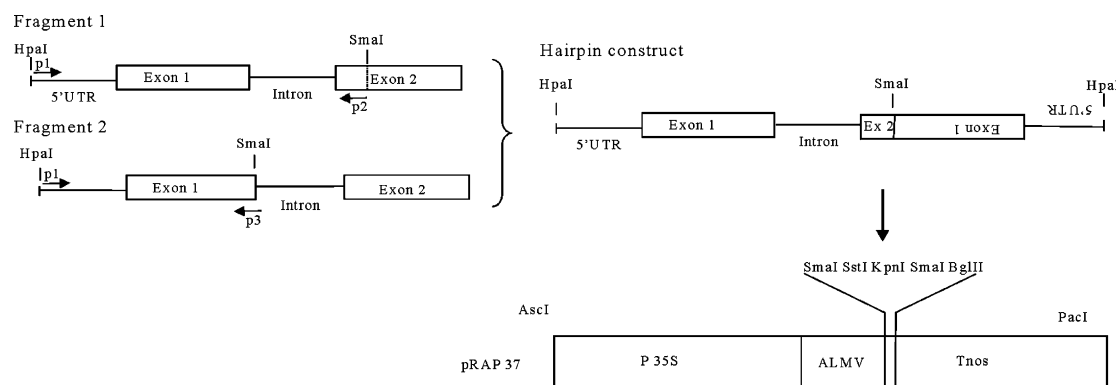


FIG 1. The construct for gene silencing was built up by linkage of fragment 1 (obtained by PCR using primer 1 [All1HpaI; p1] and primer 2 [All2SmaI; p2] and fragment 2 (from primer 1 [All1HpaI; p1] and primer 3 [All3SmaI; p3]) at the *SmaI* site and insertion into the expression cassette pRAP 37. *UTR*, Untranslated region.

calli produced shootlike structures. These structures were initially vitrified in appearance; vitrification disappeared in only some of the structures after subculturing. Their growth was very slow. Slow growth and vitrification are normal phenomena in the shoot regeneration process from apple calli. Fifty-seven shootlike structures developed further and were transferred to shoot elongation medium. Of these, 17 produced very small plantlets with a normal phenotype that were subsequently transferred to shoot propagation medium for further outgrowth. The best-growing plantlets were subcultured to be used for analysis of the presence of the construct for gene silencing, for application in SPT, and for further immunoblotting. These plantlets were A11, A12, B1, B2, B4, D2, D3, F1, G1, and the nontransformed WT. PCR analysis revealed that D2, D3, and F1 did not contain the construct. These lines were further used as positive controls for gene silencing, in addition to the WT. The phenotype of the transformed plantlets was indistinguishable from that of the WT.

Assessment of hypoallergenicity of transformants by SPT

Six plantlets with an established presence of the gene-silencing construct and the controls (WT, D2, D3, and F1) were evaluated for their allergenicity by prick-to-prick SPT in 3 patients allergic to birch pollen and apples. In addition to the *in vitro* plantlet controls, prick-to-prick testing with an apple fruit from the cultivar Fiesta was performed as a further positive control. Fig 2 shows the consistently reduced allergenicity of the transformants A11, A12, and B1 in all 3 patients. In several cases the skin reaction was scored negative (<0.25). This was observed 2 out of 3 times for B1 and 1 out of 3 times for A11 and A12. The transformants (except B4) induced a significantly reduced SPT response ($P < .05$) compared with the control plantlets (Table II). WT and F1 showed a degree of biological activity close to that observed for the Fiesta apple.

Immunoblot analysis: significant reduction of Mal d 1 expression

To further confirm that the expression of Mal d 1 was indeed significantly reduced, extracts of all plantlets were

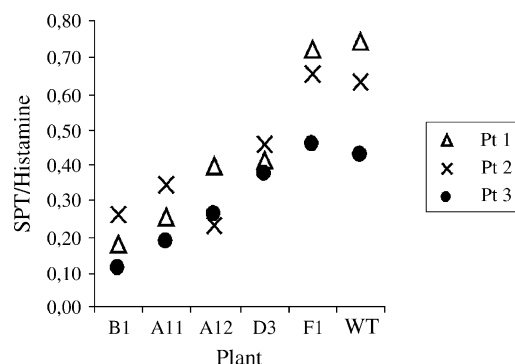


FIG 2. SPT reactivity of 3 transformants and 3 control plantlets in 3 patients. Reactivity was expressed relative to the histamine control. *Pt*, Patient.

evaluated by immunoblot analysis after separation by SDS-PAGE. All transformants showed little (B4) or no reactivity on immunoblot with mAb 5H8. In contrast, this mAb detected a clear band at the expected M_r of approximately 17 kDa for WT, D2, D3, and F1 (Fig 3A); this is in line with the observed absence of the construct in these plants. A serum pool with specific IgE against Mal d 1 showed weak but significant reactivity with the same band. This was not observed in the transformants that carry the construct (Fig 3B), thus confirming the hypoallergenicity for Mal d 1 of these plantlets.

DISCUSSION

In this study we have successfully demonstrated the feasibility of reducing Mal d 1 expression by RNAi. The efficiency of transformation of apple plants has been reported to be low.²⁴ Indeed, in our study only a few dozen plants were obtained from almost 3000 original explants. Of these plants, only a limited number ($n = 5$) showed the desired effect of strongly reduced expression of Mal d 1. The effect of gene silencing was monitored by both *in vivo* SPT and immunoblotting with leaves of apple plantlets growing *in vitro*. The fact that Mal d 1 has been shown to

TABLE II. Mean SPT/histamine reactivity and the SEM of 6 transformants and 5 controls (D2, D3, F1, WT, and Fiesta fruit)

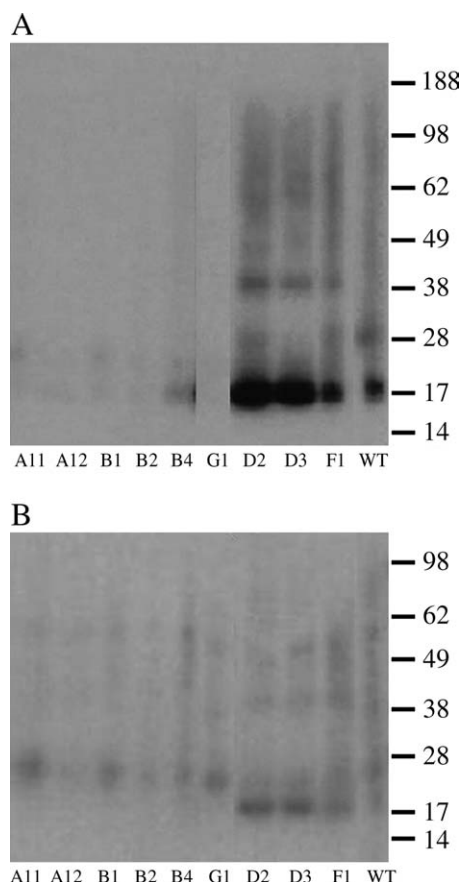
Plant	Mean SPT/histamine	SEM
B1	0.18	0.05
A11	0.26	0.05
G1	0.28	0.10
A12	0.29	0.05
B2	0.31	0.11
B4	0.38	0.11
D3	0.41	0.03
D2	0.46	0.15
WT	0.60	0.09
F1	0.61	0.08
Fiesta	0.86	0.07

be expressed in leaves as well as in fruits (L. Beuning et al and G. Marzban et al, unpublished data) allowed us to screen for effective gene silencing at a very early stage of plant development.

The prick-to-prick method has been proven to be reliable and reproducible for testing the differences in allergenicity of a wide range of apple cultivars on a ranking scale (S. Bolhaar et al, unpublished data). The advantage of SPT is its directness; no preparation of the material is required. This is especially relevant in the case of testing for allergenicity to Mal d 1, which is known as a protein sensitive to processing. A disadvantage of the SPT is its limitation to quantify the actual *in vivo* allergenicity because it does not allow titration. The other method used, immunoblotting, needs homogenization of the plant material and heating, which might affect the integrity of Mal d 1. According to the immunoblotting results, a reduction of at least 10-fold in the concentration of Mal d 1 in the transformants is suggested. Therefore, both methods can be characterized as being at best semi-quantitative. Nevertheless, the results obtained from both methods clearly confirmed each other. Recently, several new plantlets have been developed from the calli. These plantlets, together with the most promising plants from this study, are now being grown to sizes that will allow further confirmation and quantification of the observations reported here. In these studies, additional control apple cultivars and more subjects will be included.

At the *in vitro* plantlet stage, no phenotypic abnormalities were observed among the plantlets as compared with the WT. This suggests that there is a good chance that healthy fruit-bearing plants can be grown from the transformed *in vitro* material. Because the Mal d 1 gene family codes for proteins that are related to plant defense, ie, pathogenesis-related proteins,^{19,30} the effects of the current level of Mal d 1 silencing on the strength of defense against microbial pathogens and other stress-causing stimuli still need further study. Of course, new studies will have to be extended to the phase of fruit-bearing trees.

The gene-silencing construct was designed on the basis of sequence information of Mal d 1 from Gala apples but

**FIG 3.** Cross-reactivity of the monoclonal antibody 5H8 (directed to Bet v 1) (**A**) and of human IgE (**B**) to Mal d 1 in 6 *in vitro*-grown transformants and 4 control plants. Mal d 1 was separated by SDS-PAGE. For detection, radiolabeled goat antibody against mouse IgG or sheep antibody against human IgE was used, respectively.

was capable of silencing Mal d 1 in Elstar apples. Silencing was successful, suggesting that the sequence homology of the construct was stringent enough. This notion is also supported by the fact that 18 loci for Mal d 1 genes have recently been identified on the apple genome (Gao et al, unpublished data). Although no detailed information is presently available about the expression of all the individual Mal d 1 genes, the degree of homology with the introduced gene-silencing construct was apparently sufficient to overall silence the endogenous Mal d 1 mRNAs. The potential of RNAi to silence complete gene families has already been demonstrated in wheat, in which a conserved α -gliadin sequence, cloned into an RNAi construct, was able to silence the entire α -gliadin gene family in a hexaploid wheat variety.²⁰

Despite the lack of accurate quantification, our observations have clearly indicated that further research into RNAi as a means of producing hypoallergenic apple cultivars is certainly promising.

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