

## Development of hypo-allergenic apples: silencing of the major allergen *Mal d 1* gene in ‘Elstar’ apple and the effect of grafting

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(Accepted 26 August 2009)

### SUMMARY

Many people who are allergic to birch pollen are also allergic to apple fruit, due to cross- allergenicity. Since apples are the most extensively consumed fruit in Europe, it is highly relevant to develop a hypo-allergenic apple. Apples with significantly reduced levels of the allergen, *Mal d 1*, may allow many apple allergics to eat them without an allergic reaction. We are currently collaborating to develop a hypo-allergenic apple within the European Integrated Research Project, ISAFRUIT ([www.isafruit.org](http://www.isafruit.org)). Hypo-allergenic apple plants (*Malus* × *domestica* Borkh., ‘Elstar’) with decreased levels of *Mal d 1* mRNA were produced by RNA interference (RNAi) technology. Ten genetically modified (GM) apple lines were selected. *In vitro* plantlets were first transferred to a greenhouse, then grafted onto wild-type M.9 rootstock to promote the development of fruit-producing trees. Levels of *Mal d 1* gene silencing were measured repeatedly by quantitative real-time PCR. Compared to leaf samples from wild-type ‘Elstar’, two GM lines showed modest levels of gene silencing (up to 250-fold), whereas the other eight GM lines were significantly silenced (up to 10,000-fold) in *Mal d 1* gene expression. These levels of silencing were unaffected by grafting, and have been stable over more than 3 years, and throughout all developmental stages.

According to The European Federation of Allergy and Airways Diseases Patients’ Association, approx. 2% of adults and 8% of children in the EU suffer from food-induced allergic responses (Helm and Burks, 2000). Approx. 70% of patients with birch pollen allergy are also allergic to apples (Dreborg and Foucard, 1983). This is especially true for people living in regions with many birch trees, such as northern and central Europe, as well as in northern America. The most common symptoms include itching and swelling of the lips, the tongue, and the throat after ingestion; but severe anaphylactic reactions have also been reported in rare cases (Amlot *et al.*, 1987). Patients allergic to apple therefore avoid fruit in their diet. From a nutritional point of view, this is unfortunate because fruits and vegetables contain many important micro-nutrients, and because a high intake of fruit is associated with lower risks of cancer and cardiovascular diseases. Therefore, it is of interest to develop new hypo-allergenic lines of fruits and vegetables that such patients can tolerate.

Birch pollen-associated fruit allergy arises as a consequence of the cross-reaction of pollen-specific IgE antibodies with highly homologous proteins found in fruit and vegetables. Apples contain the protein, *Mal d 1*,

which has high homology to the allergen Bet v 1 from birch pollen. The amino acid sequences of the two proteins are 64.5% identical (Vanek-Krebitz *et al.*, 1995). Other fruits such as pear, cherry, and peach as well as carrot, celery, and hazelnut can induce the same allergic reaction (Vieths *et al.*, 2002). Prick-to-prick skin tests and oral provocations on patients have been used to assess variations in allergenicity between apple cultivars. The apple cultivars ‘Golden Delicious’, ‘Granny Smith’, and ‘Cox’s Orange’ all belong to the high allergenic group. The cultivars ‘Elstar’ and ‘Gala’ had intermediate allergenicity, and cultivars such as ‘Belle de Boskoop’, ‘Jonagold’, and ‘Gloster’ provoked only low allergenic responses (Gao, 2005). This indicates that the degree of allergenicity against *Mal d 1* is cultivar-dependent. Storage of apples under certain conditions may increase the content of the allergen, but *Mal d 1* is labile and destroyed by cooking (Sancho *et al.*, 2006).

Based on sequence identity, the allergens *Mal d 1* and Bet v 1 have been classified as belonging to a group of pathogenesis-related proteins, the PR-10 class (Hoffmann-Sommergruber, 2000). *Mal d 1* genes form a gene family with at least 18 members, with *Mal d 1* loci present on three linkage groups (Gao *et al.*, 2005). The *Mal d 1* protein appears to be expressed constitutively or induced in response to bacterial or fungal attacks, or

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abiotic stress. It is also produced during fruit ripening (Atkinson *et al.*, 1996; Pühringer *et al.*, 2000). No clear function has yet been found for Mal d 1, although it has been suggested that PR-10 proteins may code for ribonucleases (Bantignies *et al.*, 2000). Recently, the Bet v 1 allergen was found to bind plant steroids (Marković-Housley *et al.*, 2003). PR-10 proteins have been identified in various plant families. All members are similar in size (17 – 18 kDa) with 158 – 159 amino acid residues containing conserved motifs and structures suggesting a common ancestor. Genes encoding Mal d 1 have been cloned from the leaves and fruits of several apple cultivars and, so far, more than 30 isoforms are known. Phylogenetic analysis indicates four distinct sub-families, based on the absence or presence of an intron, and on variations in intron length. A mixture of isoforms of the *Mal d 1* gene is present in each apple cultivar, some of which differ by only a single amino acid (Gao *et al.*, 2005). The genetic isoform of *Mal d 1* that was chosen for down-regulation here was *Mal d 1.02*. This is the most highly expressed gene sub-family in apple pulp and peel (Sancho *et al.*, 2006). In addition, Mal d 1.02 is also the most prevalent protein (Beuning *et al.*, 2004; Helsper *et al.*, 2002).

Three additional classes of allergens exist in apple. Mal d 2 is a thaumatin-like protein, classified as PR-5, with diverse functions including anti-fungal activity (Hoffmann-Sommergruber, 2000). Mal d 3 belongs to the PR-14 class, and is homologous to lipid transfer proteins (LTP). This allergen was identified as the major allergen in birch-‘free’ areas (e.g., among Mediterranean populations) and is present mostly in apple peel (Salcedo *et al.*, 2004; Marzban *et al.*, 2005). LTPs are resistant to heat treatment and proteolytic digestion. A third class of allergen found in apples includes Mal d 4. This allergen belongs to the group known as profilins, which regulate polymerisation of cytoskeletal actin and play a role in pollen-associated allergy. Mal d 4 is homologous to Bet v 2, found in birch pollen (Ma *et al.*, 2006).

In this paper, we describe the successful down-regulation of the most abundant allergen in apple (Mal d 1.02), quantified using real-time PCR technology. We investigated the stability of gene silencing, from plantlet formation through greenhouse transfer, until 2 years after grafting onto wild-type M.9 rootstock.

## MATERIALS AND METHODS

### *Silencing of Mal d 1*

Leaf segments of *in vitro*-grown plantlets of the apple cultivar ‘Elstar’ were transformed using *Agrobacterium tumefaciens* carrying a T-DNA construct (derived from the sequence of *Mal d 1.02*; Genbank Accession Number AF020242) that coded for an intron-spliced hairpin RNA containing a *Mal d 1*-specific inverted repeat sequence, separated by a *Mal d 1*-specific intron sequence (Gilissen *et al.*, 2005). In the nuclei of transformed cells, this construct initiated interference at the level of *Mal d 1* transcription (RNA breakdown), resulting in the inhibition of Mal d 1 protein production. Plantlets were regenerated from the resulting transformed calli. This work was carried out at Wageningen as a task in a previous EU-funded project (Gilissen *et al.*, 2005).

When a gene construct is inserted into a plant genome,

the activity of the gene depends on its site of insertion in the plant genome. With present technology, it is not possible to determine precisely where the gene construct is inserted, in advance, but it is possible to test whether the construct is present. Therefore, different independent insertion events [i.e., genetically modified (GM) lines of transformed plantlets] from different callus cultures were produced and analysed by PCR to verify the presence of the gene-silencing construct. Six *in vitro*-grown GM lines, positive by PCR analysis, were evaluated for their reduced allergenicity by prick-to-prick skin testing in patients allergic to apple, and were found not to provoke an allergic response (Gilissen *et al.*, 2005). Based on this success, ten GM lines (TB2, TB3, TB4, TG1, TG4, TI1, TJ1, TK2, TL1, and TM1) were selected and sent to Denmark for transfer to greenhouse conditions.

### *Growth and grafting*

Eleven plantlets (2 – 4 cm in height) of each GM line (110 plantlets in all) were transferred to soil conditions and kept under artificial light with a 16 h photoperiod ( $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 18°C with 60% relative humidity in growth chambers during Winter 2006. The trees (60 – 80 cm in height) were then transferred to a GM-approved greenhouse facility in February 2006. In parallel, wild-type M.9 rootstock trees were planted in 1.5 l containers, moved to the same greenhouse, and kept at 20°C day and night. As an internal control, five non-GM M.9 grafted ‘Elstar’ trees were included. At the end of March 2006, ten trees of each GM line (100) were planted, together with a full-leaved M.9 rootstock tree, in a 3.5 l container. The remaining tree from each GM line was not grafted in order to investigate if grafting had any effect on the gene silencing process. Trees were grafted 15 – 20 cm above the root neck using a ‘Floor’s X’ cutting (Garner, 1967). Ten weeks after grafting, each scion was disengaged from its own root by a cut below the graft union. At the same time, half of the apical sprout of each scion was removed, to reduce evapotranspiration. Reciprocal grafts were also produced on one tree of each GM line (10), with a GM rootstocks and an M.9 scion, for later analysis of the possible movement of silencing signals. Ten weeks later, the top of the M.9 rootstock was cut-off above the graft union (Figure 1).

Trees were fertilised from March until September by drip irrigation one-to-three times per tree  $\text{d}^{-1}$  (150 ml in total  $\text{d}^{-1}$ ) depending on the solar radiation and temperature in the greenhouse, with 560 mg  $\text{l}^{-1}$  PIONER NPK 19-2-15 + Mg (Brøste, Lyngby, Denmark) acidified with 250 mg  $\text{l}^{-1}$  62% (v/v)  $\text{HNO}_3$  (BRENTAG Nordic A/S, Hellerup, Denmark). From September until leaf fall, trees were drip-irrigated with 150 ml per tree  $\text{d}^{-1}$  250 mg  $\text{l}^{-1}$  62% (v/v)  $\text{HNO}_3$  (BRENTAG Nordic A/S) alone. To improve the initiation of flower buds, 1-year-old branches were bent down with small concrete bricks in September 2007 (Tukey, 1964).

### *RNA isolation and cDNA synthesis*

Two leaf samples were collected from each tree in May and September, immediately transferred to liquid nitrogen, then stored at –80°C. Total RNA from 100 mg leaf tissue was isolated after disruption in a Polytron homogeniser (Ultra Turrax T25, IKA® Werke GmbH &

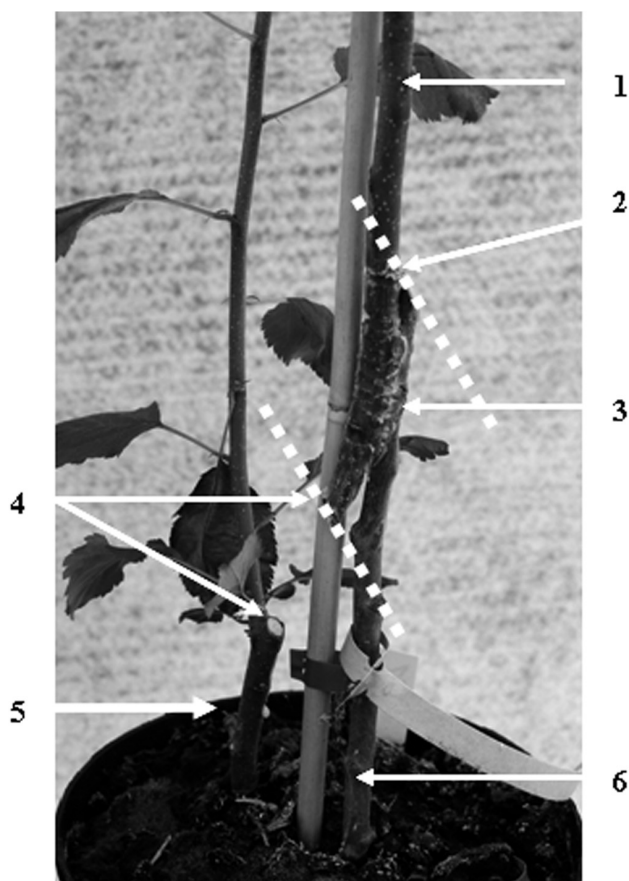


FIG. 1

Illustration of grafting in 'Elstar' apple (scion) with M.9 rootstock. 'Elstar' apple GM line TJ1 scion (1). The top of the M.9 rootstock was cut-off immediately above the graft union (2). The graft union (3). The 'Elstar' GM scion was cut free from its own root 1–2 cm below the graft union (4). The rooted 'Elstar' GM TJ1 line, with a new lateral shoot, could be used again for a new grafting on the same rootstock, if the first grafting failed (5). The M.9 rootstock (6). Pot diameter 18 cm.

Co., Staufen, Germany) using the Qiagen Plant Mini-kit (Qiagen, Hilden, Germany). The concentration and purity of each RNA was measured by reading its absorbance at 260 nm and 280 nm. cDNA was synthesised using 250 ng total RNA, in a final volume of 10 µl, and the Random Hexamer Primer and Superscript™ II RNase H-Reverse Transcriptase kits, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

#### Analysis of *Mal d 1* gene silencing

Quantitative real-time PCR assays of the expression levels of the *Mal d 1* and 18S rRNA genes were performed in triplicate on cDNA samples using a 7900HT FAST Real-Time PCR System (Applied Biosystems, Stockholm, Sweden). To measure *Mal d 1* gene expression, specific primers and a TaqMan® MGB probe were designed using Primer Express software, Version 2.0 (Applied Biosystems). The primer pair and probe sequences were based on Genbank Accession Number AF020242, which was also the sequence used for building the intron-spliced, RNAi hairpin construct (Gilissen *et al.*, 2005). The amplicon was designed to span the intron, in order to avoid genomic DNA amplification during PCR. Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) was used as an internal

normalisation standard. Each PCR reaction was performed in a total volume of 10 µl containing 3 ng cDNA according to the Applied Biosystems protocols. Primer and probe concentrations were 900 nM and 300 nM, respectively. The amplification conditions were: 95°C for 20 s, then 45 cycles of 95°C for 1 s and 60°C for 20 s. The results were calculated using the comparative Ct method ( $\Delta\Delta C_t$  method) as described previously (ABI PRISM 7700 Sequence Detection System User Bulletin #2; Applied Biosystems, 2001) with the *Mal d 1* gene as the target, 18S rRNA as the endogenous control, and non-GM 'Elstar' (non-GM-EL) leaves as the calibrator.

#### Statistics

The levels of expression of the *Mal d 1* gene in GM leaves relative to non-GM-EL leaves were determined for all GM lines, both grafted and non-grafted. These values were not normally distributed, due to the bimodal nature of the data set. We used the Kruskal-Wallis test (SAS Version 9.01; SAS Institute, Cary, NC, USA) to determine the differences between GM-lines and to test for the effect of grafting on *Mal d 1* gene expression. A *P*-value of 0.05 was considered significant.

#### RESULTS AND DISCUSSION

In this study, we successfully grafted transformed 'Elstar' plants onto M.9 rootstocks and developed a method to measure the efficiency of silencing of the gene for the major allergen, *Mal d 1*, using real-time PCR.

At present, the most efficient treatment for apple allergy is consumer avoidance. Many different strategies have been adopted in order to help allergy sufferers eat apples without eliciting an allergic reaction. One approach has been testing and selecting conventionally-grown apple cultivars with low allergenicity, evaluated by prick-to-prick skin tests (SPT) and double-blind, placebo-controlled food challenge (DBPCFC) tests (Bolhaar *et al.*, 2005a). In this way, the hypo-allergenic cultivar 'Santana' was discovered and recently introduced onto the market in The Netherlands for individuals suffering from mild apple allergy (L. J. W. J. Gilissen, personal communication). Another strategy is allergen-specific immunotherapy. A mutant *Mal d 1* gene with five amino acids exchanged (Bolhaar *et al.*, 2005b) has been obtained by site-directed mutagenesis. This *Mal d 1mut* gene was derived from *Mal d 1.0108* and shown by SPT and DBPCFC to result in a reduced IgE-binding response.

We have used an alternative strategy, post-transcriptional silencing of the gene coding for the allergen, *Mal d 1*, using RNAi technology, which has been shown to be efficient in other contexts (Nakamura and Matsuda, 1996; Herman, 2003). The *Mal d 1.02* (*Mal d 1b*) isoform of the gene was an obvious choice to silence at the time when the ten GM lines were generated (Gilissen *et al.*, 2005). Several studies have shown this isoform to be the most abundant, both at the transcriptional level as well as at the protein level. In addition, the *Mal d 1.02* gene is highly expressed in ripe fruit (Puehringer *et al.*, 2003; Helsper *et al.*, 2002; Marzban *et al.*, 2005).

To overcome the time-lag between *in vitro* transformation and having the final product (apples) for

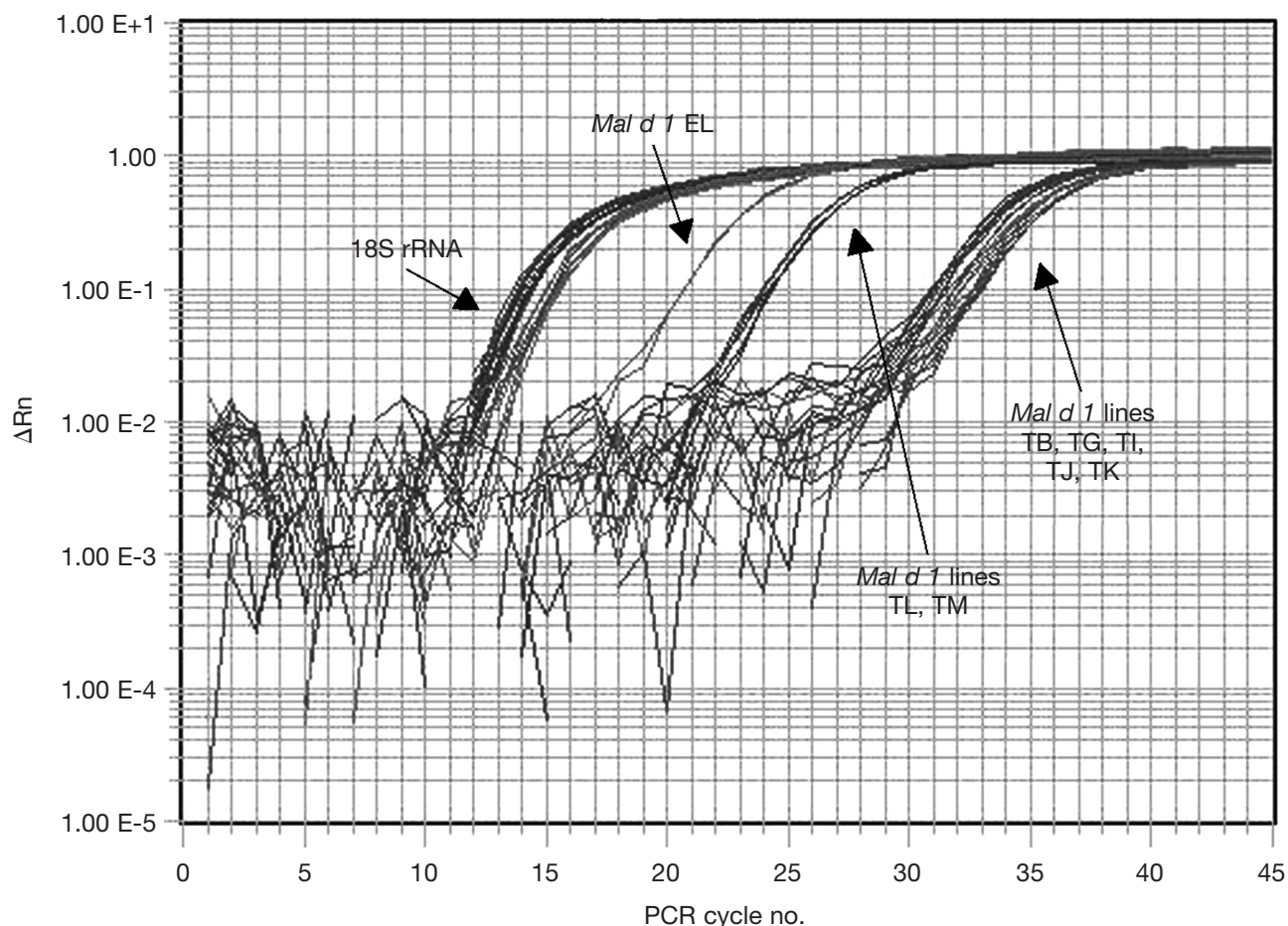


FIG. 2

Real-time quantitative PCR amplification plot showing the amount of 18S rRNA and *Mal d 1* mRNA in leaves of selected GM 'Elstar' apple lines. *Mal d 1* EL was the non-GM-EL control. 18S rRNA was the endogenous control. *Mal d 1* lines TM1 and TL1 were moderately silenced GM-lines. *Mal d 1* lines TB, TG, TI, TJ, and TK were efficiently silenced GM-lines.

testing, we grafted nine trees of each GM plant line onto wild-type M.9 rootstock, in order to promote the development of fruit-producing trees. The unique feature of this approach to grafting was that both those plants to be joined were brought together while both retained a root system to supply water and a top for active photosynthesis during coalescence. One tree of each GM line was reciprocally grafted with an M.9 scion on the GM rootstock. Of the 100 grafted plants thus produced, only three failed.

#### Analysis of the level of *Mal d 1* silencing

Since *Mal d 1* is expressed in leaves as well as in fruit, it is possible to follow and evaluate the efficiency of silencing at all developmental stages. We developed a quantitative real-time PCR assay specifically to measure the silencing efficiency of *Mal d 1* at the mRNA level. Based on the sequence used to build the intron-spliced hairpin RNAi construct, we designed a primer pair and a probe to amplify *Mal d 1.02* mRNA, specifically. The expected amplicon (90 bp) was "Blasted" (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to test if any other isoforms of *Mal d 1* could be amplified using the same set of primers. Only *Mal d 1.02* sequences were found. Thus, the designed primer pair and probe were *Mal d 1.02*-specific. The expected size of the PCR product was also verified by electrophoresis in 2% (w/v)

agarose gels, followed by staining with  $0.5 \mu\text{g ml}^{-1}$  ethidium bromide and visualised under UV light. Only one band, at 90 bp, was visualised for all amplification reactions investigated (data not shown).

Other research groups have used SYBR-Green for real-time quantitative PCR analysis of the expression of *Mal d 1.01*, *1.02*, and *1.03* (Puehringer *et al.*, 2003; Botton *et al.*, 2008). We used a TaqMan<sup>®</sup> MGB probe. Real-time PCR measures the amount of the amplicon as the reaction proceeds. The amount of the PCR product is directly related to the fluorescence of a reporter dye. Two methods are available: TaqMan<sup>®</sup> probe or SYBR Green-based technology. SYBR Green-based detection is cheap and easy to perform, and requires only two primers and the dye, but the dye binds to all double-stranded DNA making it non-specific. In addition to the two primers, Taqman<sup>®</sup> probe technology also requires a third primer labelled with a dye and a quencher. This method is more expensive, but much more specific. Detection of the fluorescent signal occurs only if all three oligonucleotides bind. The amplicon can be designed to be very short, thereby saving time in the PCR. We used the Taqman<sup>®</sup> probe technology because of its specificity.

Leaf samples from the GM lines TB2, TB3, TB4, TG1, TG4, TI1, TJ1, TK2, TL1, and TM1 of 'Elstar' apple were collected in May and September each year to assess the degree of *Mal d 1* gene silencing, and the consequent

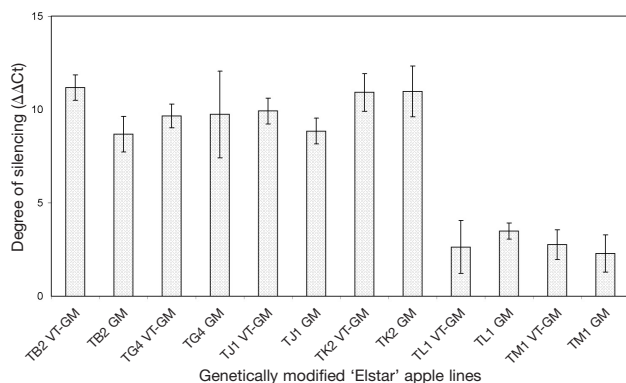


FIG. 3

Relative levels of *Mal d 1* gene expression in GM apple lines TB2, TG4, TJ1, TK2, TL1, and TM1 compared to non-GM-EL. VT-GM refers to a M.9 rootstock with the GM line as the scion. GM refers to a non-grafted GM line. No statistically significant influence of grafting was observed. Expression levels of *Mal d 1* in the GM apple lines TL1 and TM1 were statistically significantly different from all other lines ( $P < 0.0001$ ).

reduction in *Mal d 1* mRNA levels. Figure 2 illustrates a plot from a typical PCR amplification. The later the curve rises, the less *Mal d 1* mRNA was present in the sample. The  $\Delta R_n$  value on the Y-axis is an indicator of the relative magnitude of the signal generated by PCR.

The gene expression plot in Figure 3 shows the quantitative results for *Mal d 1* mRNA levels in the ten different GM lines. The Y-axis ("ΔΔCt") denotes the difference in the total number of PCR cycles required to produce a certain amount of *Mal d 1* mRNA PCR product (the data are normalised using 18S rRNA transcripts) between the non-GM-EL and GM lines. This difference illustrates the difference in the initial amount of *Mal d 1* mRNA. Ideally the amount of PCR product doubles for each cycle ( $2^n$ ). These representative data show that the GM lines TL1 and TM1 were only modestly silenced (up to 250-fold) for *Mal d 1* mRNA expression compared to the non-GM-EL *Mal d 1* mRNA, whereas the other eight GM lines (TB2, TB3, TB4, TG1, TG4, TJ1, TJ1, and TK2) were silenced up to 10,000-fold in *Mal d 1*

mRNA expression compared to non-GM-EL *Mal d 1* mRNA. In addition, the data showed no difference in *Mal d 1* mRNA level between leaves from GM 'Elstar' scions and from non-grafted GM plants (see VT-GM vs. GM; Figure 3) indicating that the grafting process had no influence on *Mal d 1* gene expression.

### Morphology

The morphology of some of the GM 'Elstar' trees was changed, probably due to *in vitro* culture (somaclonal variation) and/or the processes of genetic modification. For example, the highly-silenced GM line, TJ1, had darker-green leaves with more serrated edges, and the plants grew more slowly than all other GM lines.

### CONCLUSIONS

We have succeeded in producing, and growing, ten independently transformed constructs of *Mal d 1*-silenced apple callus cells into whole transgenic apple trees with the aim of producing hypo-allergenic apple fruit by Autumn 2009. The plants will continue to be evaluated once or twice a year by measuring the degree of *Mal d 1* silencing in randomly sampled leaves. In 2009, the *Mal d 1* allergen-free apple fruit will be evaluated by real-time PCR and tested by a panel of apple-allergic patients by prick-to-prick skin tests and, later, by oral provocation.

We thank Elin Rosenstrøm and Karen Hedegaard for assistance with the plants in the greenhouse

The ISAFRUIT Project is funded by the European Commission under Thematic Priority 5 – Food Quality and Safety of the 6th Framework Programme of RTD (Contract No. FP6-FOOD-CT-2006-016279).

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