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ORIGINAL PAPER

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Linkage map positions and allelic diversity of two *Mal d 3* (non-specific lipid transfer protein) genes in the cultivated apple (*Malus domestica*)

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Abstract Non-specific lipid transfer proteins (nsLTPs) of *Rosaceae* fruits, such as peach, apricot, cherry, plum and apple, represent major allergens for Mediterranean atopic populations. As a first step in elucidating the genetics of nsLTPs, we directed the research reported here towards identifying the number and location of nsLTP (Mal d 3) genes in the apple genome and determining their allelic diversity. PCR cloning was initially performed on two cultivars, Prima and Fiesta, parents of a core apple mapping progeny in Europe, based on two *Mal d 3* sequences (AF221502 and AJ277164) in the GenBank. This resulted in the identification of two distinct sequences (representing two genes) encoding the mature nsLTP proteins. One is identical to accession AF221502 and has been named *Mal d 3.01*, and the other is new and has been named *Mal d 3.02*. Subsequent genome walking in the upstream direction and DNA polymorphism analysis revealed that these two genes are intronless and that they could be mapped on two homoeologous segments of linkage groups 12 and 4, respectively. Further cloning and sequencing of the coding and upstream region of both *Mal d 3* genes in eight cultivars was performed to identify allelic variation. Assessment of the deduced nsLTP amino acid sequences gave a total of two variants at the protein level for *Mal d 3.01* and three for *Mal d 3.02*. The consequences of our results for allergen nomenclature and the breeding of low allergenic apple cultivars are discussed.

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Introduction

Fruits are generally considered to be an important component of a healthy diet for humans. However, in some individuals, several kinds of fruit may also cause severe allergic reactions. A food allergy results when the immune system reacts improperly to specific proteins and, once sensitized, the individual can become allergic to homologous proteins of other food (fruits) via cross-reactivity. There are two classes of food allergy. A class-I food allergy, in which the immune reaction takes place in the gastrointestinal tract, is especially prevalent in children. The allergens involved in this class are very stable. A class-II food allergy is initiated by proteins that come into contact with the immune system through inhalation; i.e. the inhalation of pollen from several tree species and grasses. Class-II food allergies are generally found in adults.

In apple, two major allergens have been identified: (1) a non-specific lipid transfer protein (nsLTP) called Mal d 3, which causes a class-I food allergy (Pastorello et al. 1999a); (2) Mal d 1, another major allergen, is a homologue of the sensitizing Bet v 1 allergen from birch pollen (Breiteneder and Ebner 2000) and causes a class-II food allergy. Many plant food allergens can be classified as members of the groups of pathogenesis-related (PR) proteins. Mal d 1 and Mal d 3 belong to the PR-10 and the PR-14 families, respectively (Breiteneder and Ebner 2000; Mills et al. 2003).

The nsLTPs were discovered about 30 years ago (for review, see Kader 1996). They form a multigene family encoding 9-kDa proteins (90–95 amino acids) that are distributed throughout the plant kingdom. In *Arabidopsis*, at least six individual nsLTP genes have been found on three chromosomes (Arondel et al. 2000). Kader (1996) suggested that nsLTPs are pathogenesis-related since they can participate in plant defense reactions through anti-fungal and anti-bacterial activities. It has recently been confirmed that nsLTPs lack any specificity for fatty acids, phospholipids or the cutin monomers (Douliez et al. 2001). With respect to food allergies, most of the nsLTP allergens identified to date belong to the nsLTP1 subfamily, whose members have been identified as major allergens in several *Rosaceae* fruits, including peach (Pastorello et al. 1999b; Ballmer-Weber 2002), apple (Pastorello et al. 1999a; Diaz-Perales et al. 2002), apricot (Pastorello et al. 2000a, 2000b), plum (Pastorello et al. 2001) and cherry (Scheurer et al. 2001). The prevalence of allergies to nsLTP-containing fruits is especially high in Mediterranean communities (Ballmer-Weber 2002). With respect to Mal d 3, two cDNA sequences obtained from apple fruit were published in the GenBank at the beginning of this study. The first, AF 221502, comprises the complete coding sequence for the Mal d 3 precursor (including a signal peptide), while the second, AJ277164 (Diaz-Perales et al. 2002), encodes for the mature Mal d 3 protein. These two sequences differ in only one nucleotide in the coding region for the mature protein. This sequence information was used as the start for genomic cloning.

The aim of this investigation was to contribute to the successful breeding of apple cultivars with reduced Mal d 3 allergenicity. At present, nothing is known about the genomic localization, the extension of the *Mal d 3* gene family and the degree of its allelic variation. To answer these questions, we carried out genomic cloning on ten apple cultivars, including Prima, Fiesta, Jonathan and Discovery, four parental cultivars of three mapping progenies. Two distinct *Mal d 3* genes were localized on molecular marker linkage maps. Allelic diversity studies in ten cultivars showed the existence of two and three protein variants of both genes, respectively. This is the first report on the linkage map position of apple allergen genes.

Materials and methods

Cultivars used for genomic cloning and DNA isolation

Apple (*Malus domestica*) cultivars Prima (PM) and Fiesta (FS) were used initially for genomic PCR cloning and sequencing because of the availability of a molecular marker linkage map from the progeny of PM × FS (Maliepaard et al. 1998). Subsequently, Jonathan (JO) and Discovery (DS) were added because maps from the progenies of JO × PM and FS × DS became accessible (van de Weg et al., unpublished). Ultimately six additional cultivars, Golden Delicious (GD), Ingrid Marie (IM), Priscilla (PS), Cox (CO), Red Delicious (RD) and Fuji (FJ), were included in our investigation of allelic variation. These specific six cultivars were chosen because they are either common apple cultivars and/or frequently used in our apple-breeding programme. Golden Delicious, Ingrid Marie and Priscilla are the founders of the newly released cultivar Santana [(GD × IM) × PS] by Plant Research International, Wageningen.

Genomic DNA was extracted from young leaves of the cultivars and the progeny plants using the CTAB-based large-scale nuclei-isolation method (Roche et al. 1997).

Genomic PCR cloning and sequencing

Four rounds of PCR cloning were performed to obtain sequences of the *Mal d 3* genes. The primer sequences, PCR conditions and product sizes are shown in Table 1. In the first round of cloning, *Mal d 3* genomic fragments of Prima and Fiesta were amplified by PCR using primers (Mald3-For and Mald3-Rev1) designed on the basis of two cDNA sequences, AF221502 and AJ277164 (Diaz-Perales et al. 2002). In the second round, genomic walking (see below) was carried out to reveal the upstream sequences of those obtained in the first round. The third round of PCR cloning was based on the sequences obtained by genome walking for the purpose of searching for polymorphisms in four parent cultivars of the three mapping progenies, while the fourth round was aimed at investigating allelic diversity of the identified *Mal d 3* genes in eight additional cultivars.

In all of the cloning rounds with the exception of genome walking, PCR was performed in two steps using *Pfu* and *Taq* polymerase. In the first round, the PCR analysis was carried out using *Pfu* polymerase (Stratagene, La Jolla, Calif.) in a total reaction volume of 50 µl containing 40 ng genomic DNA (gDNA) of PM or FS, 5 µl of 10× *Pfu* buffer, 20 pmol forward and reverse primers, 0.2 µl 10 m *M* dNTPs and 2.5 U *Pfu* polymerase enzyme. This PCR round was performed using the following amplification programme: an initial 2-min denaturation at 95°C; 25–30 cycles of 2 min at 95°C, 30 s at the optimized annealing temperature (see below), 3 min at 74°C (elongation); a final 10 min at 74°C. If this

Table 1 Primer pairs used for four successive cloning rounds of two different *Mal d 3* genes

Round	Primer sequence (5′– 3′)	Position[b]	PCR[c]	Super-*Taq*	Product[d]	Reference
First	Mald3-For: ATAACATGTGGCCAAGTGA Mald3-Rev1: ACTTCACGGTGGCGCAGTTG	73–91	60/30	64/2	274	AF221502
Second[a]	Mald3.01GSP1: AGGAAGCCCTGCTGCATTGTTA Mald3.01GSP2: AGCCAATGCATGGCGCAAGGCTGCTGG Mald3.02GSP1: TGACTCCACACTTTCCAGGAAGCGATT Mald3.02GSP2: GTAGTCAAAGCATGGTACAAGGTTGGA					First-round sequences
Third	Mald3.01UP-For: CATAGTTGTTGAATCATTGACCA Mald3.01SSR-Rev: GTTTCTATTCCGTCTTCCTTC	35–57 399–419	58/30	60/2	385–390	AY572500
Fourth	Mald3.01-For: TATGTTTCTCACCATACTAGCCGA Mald3-Rev2: TTACTTCACGGTGGCGCAGTTG	1,515–1,538 2,394–2,415	60/30	62/2	901	AY572500
	Mald3.02-For: GCTGCTGCTGTTCCTCCAGAT Mald3-Rev2: TTACTTCACGGTGGCGCAGTTG	7–27 970–991	60/30	62/2	985–986	AY572517

[a] The second round was genomic walking; PCR procedures followed the manufacturer's instructions (see text)
[b] Refers to reference sequence
[c] Temperature (°C)/number of cycles (*Pfu*)
[d] Nucleotides (number)

PCR resulted in the expected fragment, an additional first-round PCR was performed by adding 50 µl reaction mixture containing 5 µl 10× super-*Taq* buffer, 20 pmol of forward and reverse primers, 0.2 µl 10 m *M* dNTPs and 0.25 U super-*Taq* polymerase to obtain A-tailing of the PCR product, which facilitates subsequent cloning. Amplification was performed for 2 min at 94°C, followed by two cycles of 94°C for 1 min, 30 s at the optimized temperature, 2 min at 72°C and completed by 10 min at 72°C. The PCR product was separated on a 1.2% agarose gel, and the target fragment was excised and purified using the Qiaquick Gel Extraction kit (Westburg, Germany) following the protocol supplied by the manufacturer. PCR products were then ligated into pGEM-T easy vector (Promega, Madison, Wis.) and used to transform XL1 Blue competent cells (Stratagene) according to the protocol recommended by the supplier. For each fragment, 8–16 white colonies were subcultured for plasmid DNA isolation using the Qiaprep Turbo BioRot kit (Westburg) and DNA sequencing (ABI 3700 Sequencer; Applied Biosystems, Foster City, Calif.).

Genome walking (the second round) was applied to two cultivars, Prima and Fiesta, using the Universal Genome Walker kit (Clontech, Palo Alto, Calif.) to explore polymorphisms in the upstream region. For each cultivar, four libraries were constructed using *Dra*I, *EcoR*V, *Pvu*II and *Stu*I restriction enzymes to digest 2.5 µg of the gDNA. Adaptors were ligated to the digested DNA fragments. Nested PCR was performed using two gene-specific reverse primers, GSP1 and GSP2, which were based on new sequences from the first-round cloning (Table 1), together with two adaptor primers (AP1 and AP2). The products obtained from two to three of the four libraries were excised from the gel and subsequently purified, ligated, transformed and sequenced as already described.

Single nucleotide polymorphisms (SNPs) were analysed using the SEQMAN programme (DNAstar, Madison, Wis.). The phylogenetic tree and sequence identity plot were created using the CLUSTAL W method of the MEGALIGN programme (DNAstar). Multiple DNA and amino acid sequence alignments were performed using the GENEDOC programme.

Allele-specific (AS) primers

To discriminate *Mal d 3* allelic sequences, we designed primer pairs using the software programme PRIMER DESIGNER ver. 2.0 (Scientific and Educational Software, Cary, N.C.). The 3′ end in one or both of the forward and reverse primers was exactly located at polymorphic nucleotides found anywhere in both the coding and the upstream sequences. To reduce the chance of primers annealing to the untargeted template, we employed the addition of a designed mismatch at the second or the third position at the 3′ end of the primer. This kind of marker has been called a single nucleotide amplified polymorphisms (SNAP) (Drenkard et al. 2000). In most cases, the designed mismatches were just complementary to the matching nucleotide, which were supposed to have a stronger effect. If this change did not meet the primer setting criteria, two other nucleotides were examined. In case of ambiguity with respect to the allele specificity of primer pairs at a single annealing temperature, a touch-down PCR was performed. Two steps of nested PCR (Bonants et al. 1997) were also applied to detect an allele that lacked a unique SNP.

Test for authenticity of the polymorphisms revealed by cloning

The validity of some polymorphisms in the sequences was checked using a two-step SNAP marker test. The candidate AS primer pair was tested first with gradient PCR on the cloned plasmid DNA of an allelic sequence and the results were compared with a negative plasmid DNA control with another allelic sequence. The primer pair was then applied to gDNA of the representative cultivar from which the sequence was obtained and to two negative cultivars. In a final step, the primer pairs

were tested on all ten cultivars examined in this investigation and for which specificity could be validated due to obtained sequence information.

Optimization of annealing temperature of PCR

This step was followed for each new primer pair. PCR was performed in 20-µl reaction volumes, each containing 20 ng gDNA or 0.1 ng of cloned plasmid DNA, 2 µl 10× super-*Taq* buffer (HT Biotechnology, UK), 0.2 m *M* of each dNTP, 5 pmol of forward and reverse primer and 0.25 U super-*Taq* polymerase. PCR was carried out using a MultiCycler PTC-200 machine (MJ Research.Waltham, Mass.) and the following PCR conditions: one cycle at 94°C for 2.5 min (initial denaturation) followed by 35 cycles of 94°C for 30 s, a 50.5–66.6°C gradient for 30 s and 72°C for 1.5 min (amplification). The PCR products were analysed by agarose gel electrophoresis to determine the optimum annealing temperature.

Mapping genes on molecular marker linkage maps

Molecular marker linkage maps from the crosses PM × FS and JO × PM were used to map *Mal d 3*. The PM × FS map (Maliepaard et al. 1998) has been extended within the European DARE project (FAIR5-CT97-3898) (Lespinasse et al. 2000) by Plant Research International and the Swiss Federal Institute of Technology (van de Weg and Liebhard, unpublished). The JO × PM map was generated at Plant Research International (van de Weg et al., unpublished). These two maps cover about 2,140 cM each and consist of 700 markers and 620 markers, respectively. Of the three parental cultivars, two are genetically related: Jonathan is a grandparent of Fiesta [Cox's Orange Pippin × Idared (= JO × Wagner Apfel)]. This relationship is useful because when one allele is present in homozygous condition in Fiesta, and can not be mapped in PM × FS, it may be mapped in JO × PM.

The grouping and mapping of AS markers were performed with JOINMAP 3.0 (Van Ooijen and Voorrips 2001) using the Kosambi mapping function. The LOD and recombination threshold was 4 and 0.45, respectively. Final drawings of the marker maps were generated with MAPCHART (Voorrips 2001).

Nomenclature of the *Mal d 3* sequences

Current allergen nomenclature (King et al. 1995) has been followed and extended to name *Mal d 3* gene sequences using suffixes consisting of six Arabic numerals. The first two numerals refer to different genes of the same allergen, such as *Mal d 3.01* and *Mal d 3.02*. Alleles are differentiated at two levels: firstly, at the level of the deduced amino acid sequence of the mature nsLTPs (variant), which wis designated by the middle two numerals; secondly, at the level of the silent mutation in the coding sequence—indicated by a dot and the final two numerals. For example, *Mal d 3.0101.01* refers to a silent mutation of the first allele (variant) from the *Mal d 3.01* gene. In addition, a final lowercase letter was used to indicate DNA polymorphisms in the flanking region of an allele.

Results

Genomic cloning and sequencing of *Mal d 3* genes from PM and FS

In the first round of PCR cloning using the gDNA of Prima and Fiesta as templates (Table 1), we obtained two distinct sequences, both of which were present in Prima and Fiesta. Both have a length of 274 nt, which is 2 nt shorter than the published cDNA due to the reverse primer lacking the final 2 nt of the gene, and they share 93% and 84% identity at the DNA and amino acid level, respectively. One of the sequences is identical to AF221502 in the GenBank. We next developed two sequence-specific markers and tested these in the three mapping progenies, PM × FS, JO × PM and DS × FS; the two markers were present in all of the samples (data not shown), indicating that these two sequences are not allelic variations at the same locus but are located at different loci, which we denoted as *Mal d 3.01* and *Mal d 3.02*, respectively.

Genome walking (the second round) was carried out on parental cultivars Prima and Fiesta to identify DNA polymorphism in the upstream region of the two *Mal d 3* genes. When the sequence identified in the first-round PCR cloning is included in the calculation, the total length of the sequences was 2,415–2,418 nt and 990–991 nt for *Mal d 3.01* (accession: AY572500-AY572502) and *Mal d 3.02* (accession: AY572503-AY572505), respectively, for both cultivars. These are presented as two consensus sequences in Figs. 1 and 2. Both consensus sequences contain a single exon of 348 nt, of which the first 72 nt code for a putative signal peptide that directs the molecule to the proper location and cleaves off from the mature protein (Kader 1996). These two *Mal d 3* consensus sequences share 89% identity in the last 600-nt region including the coding sequences. Upstream of these 600-nt regions, the similarity is very low. *Mal d 3.01* is highly conserved, not only in the coding region for the mature protein but also up to at least 2 kb upstream. Polymorphisms were only found in a poly-T region about 1,730 nt upstream of the *Mal d 3* reading frame (Fig. 1). The number of T repeats varied from 14 to 16 in Prima and from 15 to 17 in Fiesta with steps of 1 nt. Each of these poly-T sequences was observed in at least two clones. To see whether any of these poly-T alleles segregated in the PM × FS progeny, two primers (forward: 5-TTAGCTAGAG-ACCCATCGAGAG-3; reverse: 5-GTTTCTATTCCG-TCTTCCTTC-3) flanking this region were designed and

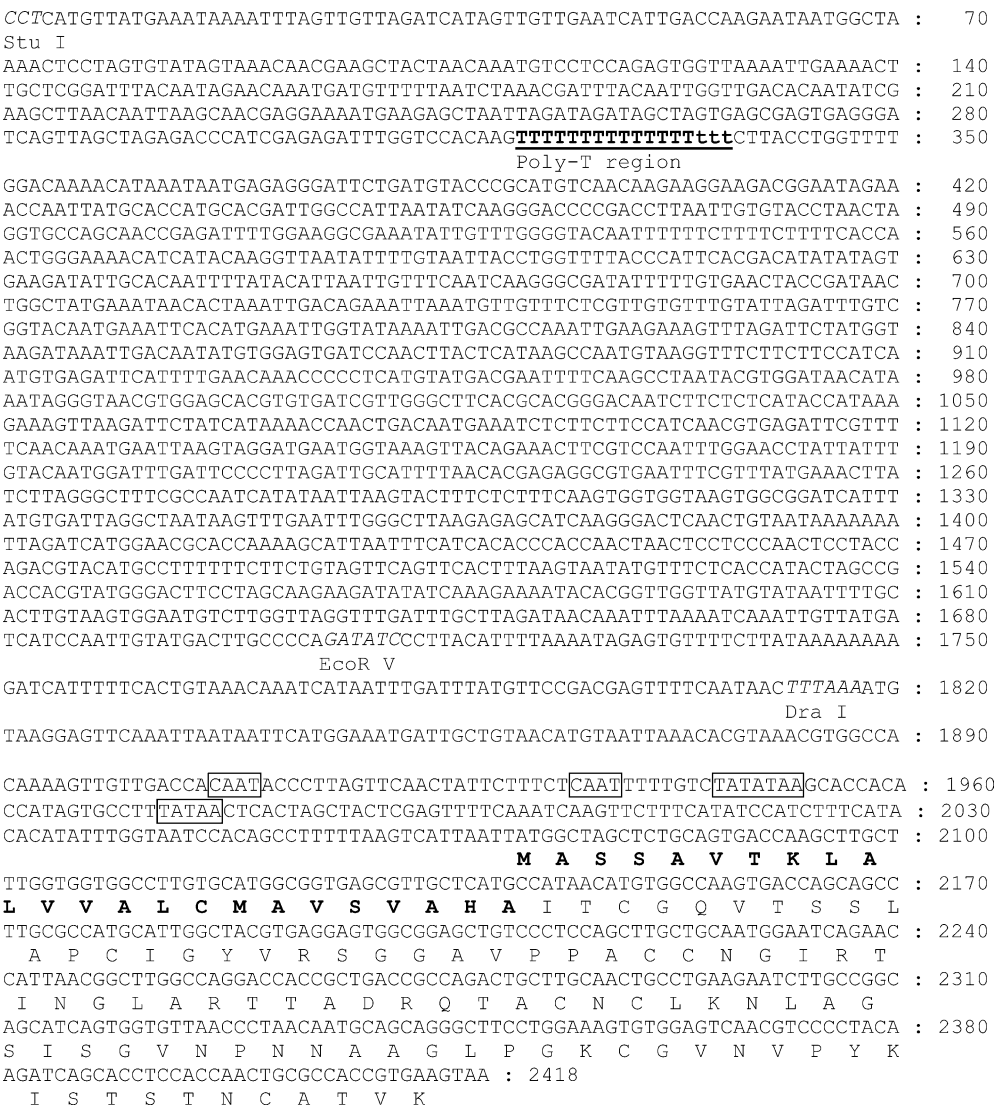

Fig. 1 Consensus sequence of gDNA and deduced amino acids of *Mal d 3.01* derived from apple cultivars Prima (PR) and Fiesta (FS). Nucleotides shown in *italics* indicate the three restriction sites used for genome walking. Putative CAAT and TATA *boxes* for the promoter near the start code are indicated. Nucleotides in *bold and underlined* indicate poly-T regions where T repeats varied by 14,15, 16 for PR (accession nos.: AY572500, AY572501 and AY572502) and by 15,16,17 for FS (accession nos. AY572503, AY572504 and AY572505). Amino acids in *bold* comprise the signal peptide

used to amplify the polymorphic fragments which were visualized by polyacrylamide gel electrophoresis with [^{33} P]-labeling. Prima and Fiesta were each shown to have three major bands that varied in length from 136 nt to 138 nt and from 137 nt to 139 nt, respectively; this corresponds to the T-repeat numbers and were uniformly present in all descendants. Therefore, it was impossible to map *Mal d 3.01* with the simple sequence repeat (SSR) marker.

To further examine the poly-T region, a third round of PCR cloning (Table 1) was conducted on Prima and Fiesta to confirm the presence of poly-T alleles, as well as on Jonathon and Discovery to find additional polymorphisms. The amplified fragments were about 390 nt. Following sequencing from both sides, all six poly-T alleles reappeared in Prima and Fiesta accordingly, while Discovery possessed four major poly-T repeats of 15, 17, 18 and 19 nt (accession nos.: CL449239, CL449240, CL449241 and CL449242). Surprisingly, Jonathan had eight identical nucleotide sequences with 5T and 1A followed by 10T (accession CL449238), three sequences with 15T (CL449236) and four sequences of 16T (Cl449237). All other sequences flanking this poly-T region were identical. Based on these data, we concluded that the multiple number of poly-T sequences within a diploid apple cultivar indicates that *Mal d 3.01* is not a single-copy gene.

Conversely, *Mal d 3.02* proved to be more variable than *Mal d 3.01*. When we aligned the two sequences from Prima and one sequence from Fiesta of an even shorter length of 991 nt, we were able to identify 14 SNPs. Most of these were located in the upstream region; only one was located in the part encoding for the signal peptide (Fig. 2).

Development of AS markers to map *Mal d 3.01* and *Mal d 3.02*

Polymorphisms in the upstream of *Mal d 3* coding sequences were used to create markers to map *Mal d 3.01* and *Mal d 3.02*. For *Mal d 3.01*, a forward primer

Fig. 2 Consensus sequence of gDNA and deduced amino acids of *Mal d 3.02* derived from cvs. Prima and Fiesta. Nucleotides in *italics* indicate the restriction sites used for the genome walking. *Bold and underlined letters* denote the SNPs within and between alleles identified from PR and FS: Y = C or T; W = A or T; R = G or A; M = A or C; S = C or G. Putative CAAT and TATA *boxes* for the promoter near the start code are indicated. In the poly-T region, one allele from PR is one T shorter. Amino acids in *bold* indicate the signal peptide. Accession nos.: AY572517, AY572518 and AY572519

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CTGTGAGCTGCTGCTGTTCCTCCAGATCGGGCCAYGGGAGAGCAAGCACTGCCGACTGTCTAGCCACART :  70
Pvu II
GGCGGGGCCGTAGAAGAAAGAAGTTAGAAGCCCWGGGACTGGATGTTGGTGCCGACATCRATCYGAGTCT : 140
GGGTTTTCGGTGCAAAGAGATAGAGGAAGGGAGAGAGAGGATGCGAAGTGCAGGAACAGACGAGAGAAGA : 210
YGAAGGTCTTAGCAATCCYGTGGTTTTGCRGGGGTCTCGCTAAGAACTMCTAGCGAGGCCTCTAATCCAC : 280
                                                    Stu I
GCRAGTCCCCTTTAGTCCCATTTAACTTAGTCCCTATACAAACCAAACATGGGACTACAGTCTARTTCAA : 350
TCCARTCCCAGTTAACGAGGTCAAACAAMCGCCCCCTTAAAATATAAGGAGTACTTCAAGGTTATAATTC : 420
ATCGCCATGATTGAGGTAACAAGTAATTAAACACGTAAAGCGTTGCCACAAATTTTTTTtGACCACTATAC : 490
                                                  Poly-T region
CCTTCGTTCACACGTTTTCTCTCTTAATTTTTCTCTATATAAGCACCACCATAGTGCCTTTACAACTCAC : 560
TAGCTACACGAGTCTTCAAATCAAGTTCTTTCACATCCATTACTCATACACACTTTGGTAATCCACATCC : 630
TAATTAATTCGTTATGGCTAGCTCTGCAGTGATCAASCTTGCTTTGGTGGTGGCCTTGTGCATGGCGGTG : 700
            M  A  S  S  A  V  I  K/NL  A  L  V  V  A  L  C  M  A  V
AGCGTTGCTCATGCCATAACATGTGGCCAGGTGAGCTCCAACCTTGTACCATGCTTTGACTACGTGAGGA : 770
S  V  A  H  A  I  T  C  G  Q  V  S  S  N  L  V  P  C  F  D  Y  V  R  S
GTGGCGGACCTGTCCCTCCAGCTTGCTGCAATGGAATCAGAACCATTAACGGCTTGGCCAAGACCACCCC : 840
  G  G  P  V  P  P  A  C  C  N  G  I  R  T  I  N  G  L  A  K  T  T  P
TGACCGCCAGGCTGCTTGCAACTGCCTGAAGAGTCTTGCCGGCAGCGTCAGTGGTGTTAACCCTGGCAAT : 910
 D  R  Q  A  A  C  N  C  L  K  S  L  A  G  S  V  S  G  V  N  P  G  N
GCCGAATCGCTTCCTGGAAAGTGTGGAGTCAACGTCCCCTACAAGATCAGCACCTCCACCAACTGCGCCA : 980
A  E  S  L  P  G  K  C  G  V  N  V  P  Y  K  I  S  T  S  T  N  C  A  T
CCGTGAAGTAA : 991
  V  K
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(Mald 3.01Jo-f1; Table 3) was designed based on a single SNP (A/T) in the poly-T region of Jonathan with an introduced mismatch base G in the third position. Together with a common reverse primer (Mald 3.01SSR-r; Table 3), we first performed gradient PCR on DNA templates from Jonathan and Prima. An expected clear band of 116 nt occurred over a range of annealing temperatures (T_m, 51.4–60.6°C; Fig. 3a) for Jonathan but not for Prima. Secondly, a touch-down PCR programme consisting of annealing temperatures of 58°C for five cycles and 56°C for 35 cycles was applied to Jonathan, Prima and 12 of their descendants. This PCR test proved that Mal d 3.0101a-JO is allele-specific (with respect to the other parental cultivar Prima) and segregating (Fig. 3b). Thirdly, 144 descendants were tested and shown to have a 1:1 segregation ratio (72 : 72).

With respect to the *Mal d 3.02* locus, Prima possesses two sequences that are different from that of Fiesta. Similarly, an AS marker Mal d 3.0201c-PM (Table 3) segregated in a 71:68 ratio in PM × FS and in a 69:75 ratio in the JO × PM progeny. The new *Mal d 3* marker data allowed the mapping of both genes in the first or second round with Chi-square contributions of 0.53, 0.33 and 0.38 in the PM4-PM × FS, PM4-JO × PM and JO12-JO × PM maps, respectively (Fig. 4).

Allelic diversity

To obtain information on the allelic diversity for both *Mal d 3* genes, We carried out additional cloning (the fourth round) on eight cultivars using gene-specific forward primers (Mald3.01-For, Mald3.02-For) with one common reverse primer (Mald3-Rev2) (Table 1). Their PCR products were expected to be around 901 nt and 986 nt in length, respectively (Table 1).

Comparison of all sequences of the same length (901–986 nt) from the ten cultivars and subsequent verification tests revealed only three different sequences in the group of *Mal d 3.01* and seven in that of *Mal d 3.02*. These sequences were further classified into variants based on the deduced mature nsLTP proteins: two variants for Mal d 3.01 and three variants for Mal d 3.02 (Table 2). Figures 5 and 6 show the alignment of the complete coding DNA sequences and deduced amino acid sequences of the identified *Mal d 3.01* and *Mal d 3.02* alleles together with reference sequences.

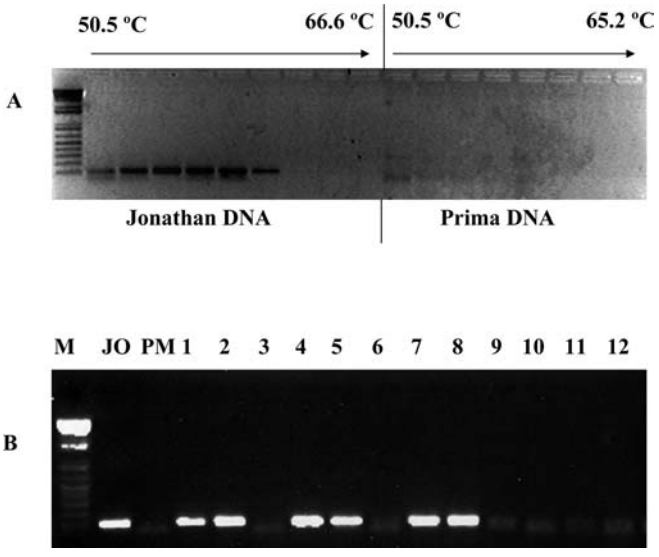

Fig. 3 Test of the AS marker Mal d 3.0101a-JO in the progeny of JO × PM. **a** Test on specificity by gradient PCR. Only Jonathan shows the expected 116-nt band over a wide range of T_m (*lanes 2–6*, 51.4–60.6°C). **b** Test for segregation on 12 descendants of the cross JO × PM (*lanes 1–12*). *Lanes*: *M* marker (1 kb plus DNA ladder), *JO* Jonathon, *PM* Prima

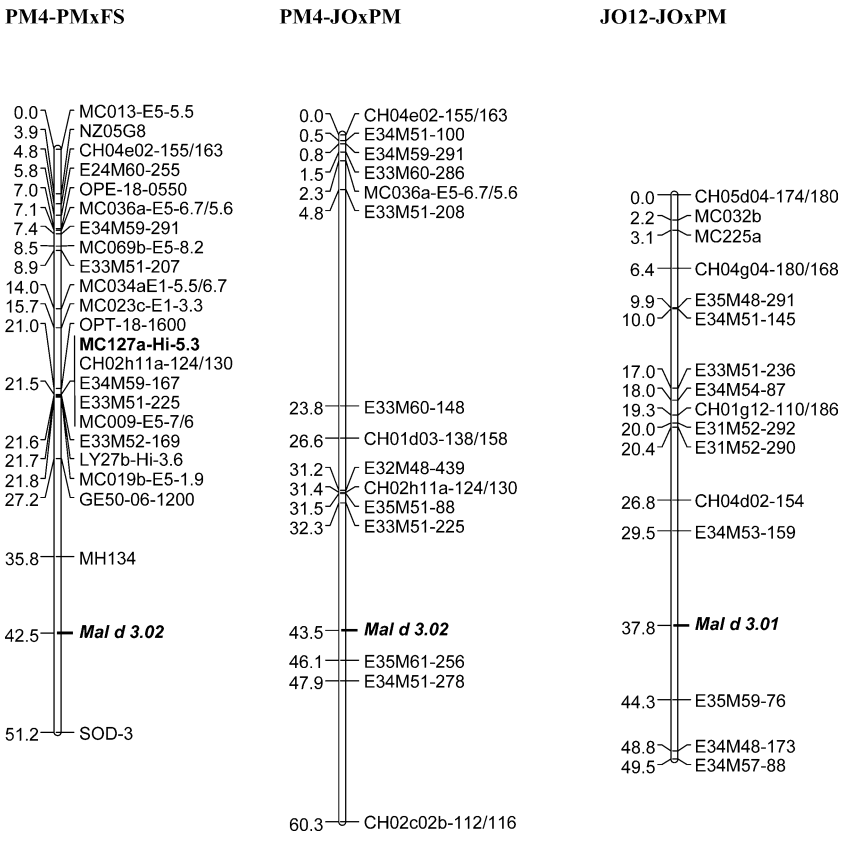

Fig. 4 Map position of *Mal d 3.01* and *Mal d 3.02* on the two homoeologous linkage groups 12 and 4 (*JO12* and *PM4*, respectively) based on segregation patterns of the Mal d 3.0101a-JO and Mal d 3.0201c-PM markers

Table 2 Systematic classification of allelic variations of *Mal d 3.01* and *Mal d 3.02* among ten apple cultivars (*PM* Prima, *FS* Fiesta, *GD* Golden Delicious, *PS* Priscilla, *IM* Ingrid Marie, *JO* Jonathan, *CO* Cox, *RD* Red Delicious, *FJ* Fuji, *DS* Discovery)

Allergen[a]	Iso- allergen[a]	Variant[a]	Additional polymorphism[a,b]	PM	FS	GD	IM	PS	JO	CO	RD	FJ	DS
Mal d 3	01	01	a	+ + [c]	+ +	+ +	+	+	+ +	+ +	+	+ +	+ +
			b				+				+		
		02	a					+					
	02	01	a		+ +			+	+ +	+	+ +	+	
			b	+		+ +							+
			c	+				+					
			d									+	
			e										+
		02	a				+			+			
		03	a				+						

[a] NCBI GenBank accession numbers:
Mal d 3.0101a: AY572500, AY572501, AY572502, AY572503, AY572504, AY572505, AY572506, AY572507, AY572508, AY572509, AY572510, AY572511, AY572512, AY572513;
Mal d 3.0101b: AY572514, AY572515;Mal d 3.0102a: AY572516;
Mal d 3.0201a: AY572517, AY572520, AY572521, AY572522, AY572523, AY572524;
Mal d 3.0201b: AY572518, AY572525, AY572526;
Mal d 3.0201c: AY572519, AY572527;
Mal d 3.0201d: AY572528;Mal d 3.0201e: AY572529;
Mal d 3.0202a: AY572530, AY572531;
Mal d 3.0203a: AY572532

[b] Polymorphisms in the region of about 600 nt upstream of the coding DNA sequences for the mature Mal d 3 proteins

[c] +, Indicates heterozygous presence of polymorphic allele; + +, indicates homozygous presence of polymorphic allele

Only two variants were found in *Mal d 3.01*. One of these is *Mal d 3.0101*, which was present in all ten cultivars tested and is fully identical to AF221502 from Golden Delicious. Compared to AJ277164, *Mal d 3.0101* has one nucleotide difference at position 105, where we found the consensus G instead of A (Fig. 5). However, this change does not lead to amino acid change. The second *Mal d 3.01* allele, *Mal d 3.0102*, is present only in Priscilla. This allele has two SNPs at positions +128 and +178 which cause two amino acid changes—from S to N and from G to S, respectively—and an additional 15 SNPs in the upstream sequences. Through the breeding pedigree we were able to trace the special allele (*Mal d 3.0102*) from cv. Priscilla back to its original source, *Malus floribunda* 821, and forward to a new variety, *Santana*. The finding of just one prevalent allele for *Mal d 3.01* coincided with little variation in the upstream region of the gene. The *Mal*

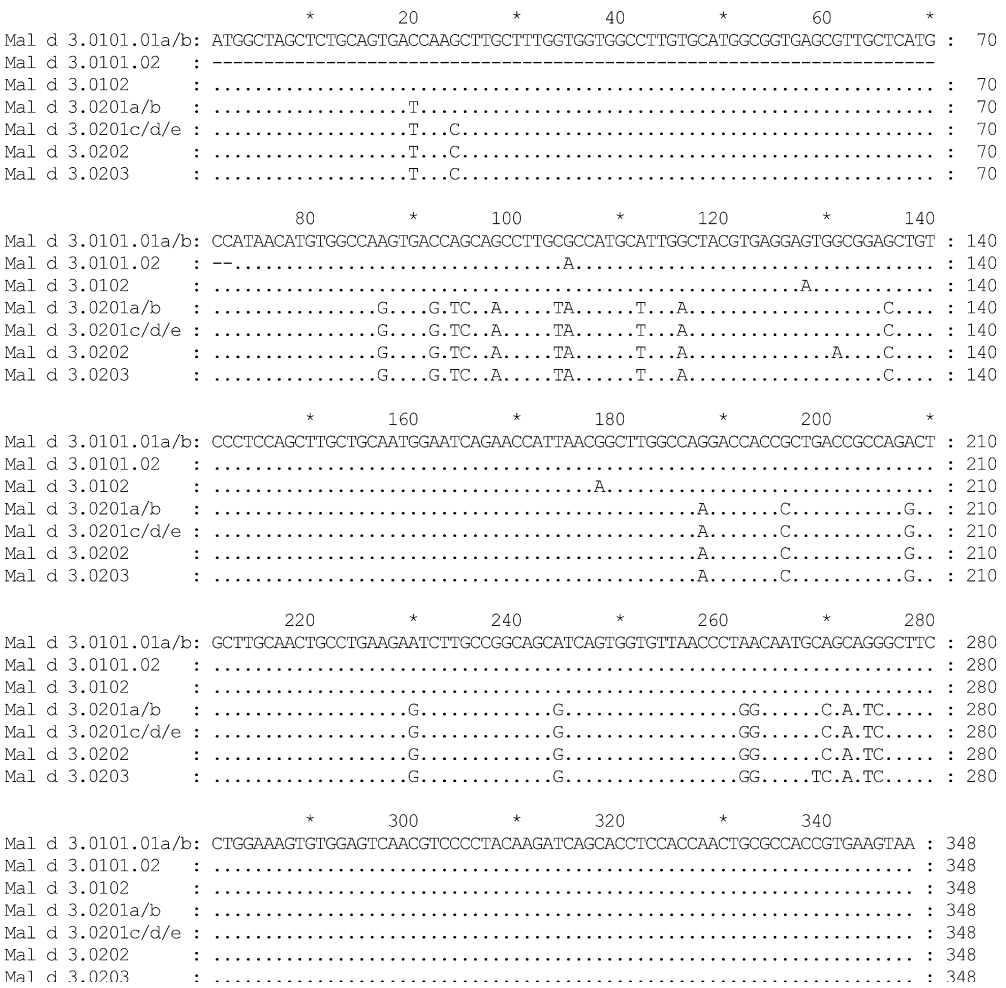

Fig. 5 Alignment of coding sequences of *Mal d 3.01* and *Mal d 3.02* among the ten cultivars together with two Mal d 3 reference sequences. Reference Mal d 3 sequence AF221502 is identical to Mal d 3.0101.01. AJ277164 has been proposed to be named Mal d 3.0101.02

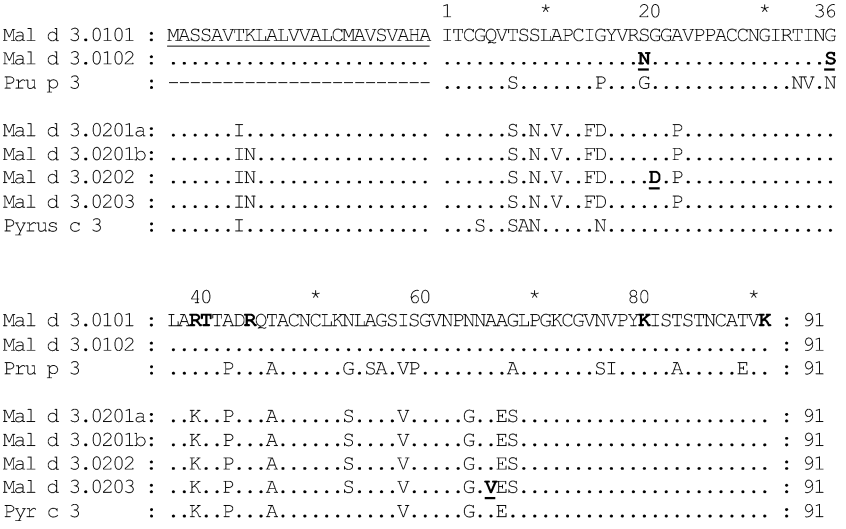

Fig. 6 Alignment of deduced amino acid sequences of the variants of Mal d 3.01 and Mal d 3.02 observed in the ten apple cultivars together with reference sequences from apple, pear and peach. The *first 24 underlined* amino acids are the signal peptide, amino acids in *bold* are the same as the predicted antibody recognition sites of Pru p 3 (AJ277163). Amino acids (*in bold and underlined*) indicate amino acid substitutions. *Pyr c 3* Accession AF221503

d 3.02 sequences were more diverse than those of *Mal d 3.01*. Four different *Mal d 3.02* amino acid sequences of precursor nsLTP were obtained from ten cultivars, but these resulted in three variants of the mature protein after removal of the signal peptides. A single amino acid substitution, 20D/G or 66V/A, was found in two variants. More polymorphisms occurred in the upstream region.

The signal peptides are identical for both variants of *Mal d 3.01*, while those of *Mal d 3.02* differ in one or two amino acids from *Mal d 3.01*. Eight typical conserved cysteine residues (Kader 1996) have been

Table 3 Description of AS markers for *Mal d 3* genes

Marker name	Primer pairs	Primer sequence [a] (5′-3′)	Product (number of nucleotides)	Annealing temperature (°C)[b]	Cycles[c]
Mal d 3.0101a-JO[d]	Mald 3.01Jo-f1	GATTTGGTCCACAAGTTT GT**A**	116	58/56	5/35
	Mald 3.01SSR-r	GTTTCTATTCCGTCTTCCTTC			
Mal d 3.0201c-PM[d]	Mald 3.02-Pm-f1	GTTGGTGCCGACATC**G**ATC**C**	571	63	35
	Mald 3.02-Pm-r1	AGGCCACCACCAAAGCAA T**G**			
Mal d 3.0101a	M3.0101a-f	ATGTTCCGACGAGTTTTC T**A**	259	58/57	5/35
	M3.0101ab-r	GATTACCAAAT ATGTGTATGAA T**G**			
Mal d 3.0101b	Mald 3.01-f1	ATGTTCCGACGAGTTTTC T**G**	565	60	35
	Mald 3.01-r1	AGGAAGCCCTGCTGCATTGTTA			
Mal d 3.0102a	Mald 3.01-f2	CCTACATTTTAA CATAGAGTGT**CG**	503	53	35
	Mald 3.01-r2	GAGGGACAGCTCCGC GA**T**			
Mal d 3.0201a	Mald 3.02-f1	ACGAAGGTCTTAGCAATC A**T**	665	53	40
	Mald 3.02-r1	ACTCTTCAGGCAGTTGCAAGC			
Mal d 3.0201b	Mald 3.02-f2	TGCCGACTGTCTAGCCA GA**A**	825	65/63	5/30
	Mald 3.02-r1	See above			
Mal d 3.0201c	Mald 3.01-f3	CAGTTAACGAGGTCAAACA T**C**	516	60	35
	Mald 3.02-r1	See above			
Mal d 3.0201d[e]	Mald 3.02-f4	GAAGAAAGAAGTTAGAAGCC G**A**	316	53/52	5/30
	Mald 3.02-r2	TTTCCAGGAAGCGATT A**G**G			
	Mald 3.02-r3	CTTATATTTTAAGGGGGC A**T**			
Mal d 3.0201e	Mald 3.02-f5	TAACGAGGTCAAACAAAC A**T**	512	60/58	5/30
	Mald 3.02-r1	See above			
Mal d 3.0202a	Mald 3.02-f6	GAAGAAAGAAGTTAGAAGCC GT**A**	712	63/61	5/35
	Mald 3.02-r4	AGCTGGAGGGACAGGTC GG**T**			
Mal d 3.0203a	Mald 3.02-f7	ATTCATCGCCATGATTG T**T**	458	55	35
	Mald 3.02-r1	See above			

[a] In bold, Allele-specific SNPs; underlined, designed mismatch nucleotide

[b,c]PCR annealing temperatures (T_m) and number of cycles, respectively. In the case of two values, a touch-down PCR was performed in two steps: the first number then refers to the T_m and the number of cycles, respectively, of the first step

[d]Markers used for mapping

[e]This marker is obtained by nested PCR: 1 μl of the PCR product of primer pair Mal d 3.02-f4 and-r2 was used as the template in a second-round PCR with primer pair Mal d 3.02-f4 and -r3. The same touch-down procedure was followed for both PCR rounds

found in all variants of Mal d 3.01 and Mal d 3.02. The Mal d 3.02 allelic constitution of the cultivars reflects their pedigree relations: Ingrid Marie inherited the Mal d 3.0202a allele from Cox, while Mal d 3.0201a in Fuji and Priscilla came from Red Delicious.

Development of AS markers

Wherever the SNPs were located, they were used to develop SNAP markers for tagging the different *Mal d 3* genomic sequences. In all, 18 SNPs from *Mal d 3.01* sequences and 21 SNPs of *Mal d 3.02* were found. On the basis of these SNPs, we developed ten allele-specific SNAP markers and tested these on the cultivars using the *Mal d 3* sequence information and other cultivars of interest in order to approve their authenticity. Their primer sequences, PCR conditions and product sizes are given in Table 3.

Discussion

Mal d 3 is one of the four allergens in apple identified to date. We report here, for the first time, linkage mapping of apple allergen genes. By means of genomic PCR cloning and subsequent genome walking, we characterized the genomic sequences of two Mal d 3 (nsLTP) isoallergen genes, which we denoted *Mal d 3.01* and *Mal d3.02*, and mapped these two genes on linkage groups 12 and 4, respectively. Comparison of the deduced amino acid sequences from ten apple cultivars showed that *Mal d 3.01* is highly conserved, since only two variants were observed, while *Mal d 3.02* is more variable, with three variants.

The amphidiploid nature of the apple genome

It has been proposed that cultivated apple is an amphidiploid ($x = 17$) evolved from a hybrid of two closely related ancestors (Chevreau and Laurens, 1987). The large number of duplicated restriction fragment length polymorphism (RFLP) markers that have been found in the PM × FS linkage map (Maliepaard et al. 1998) support this hypothesis. In our updated map, the lower part of linkage groups 4 and 12 share two common RFLP markers, MC127 and MC105 (van de Weg, unpublished). Alignment of the updated linkage groups 4 and 12 derived from Prima, Fiesta and Jonathan showed that *Mal d 3.01* and *Mal d 3.02* are located just between these two RFLP markers. The fact that these two genes are located on homoeologous chromosomes is clear evidence that *Mal d 3.01* and *Mal d 3.02* have originated from different ancestral genomes. Because the genome constitution of apple and pear is highly similar (Yamamoto et al. 2002, 2004; Hemmat et al. 2003), we expected to locate: (1) the known pear nsLTP gene (Pyr c 3, accession AF221503), which is 93% identical to Mal d 3.02 (Fig. 6), on linkage group 4; and (2) another yet unidentified pear nsLTP gene on linkage group 12.

Multigene family of nsLTPs

Plant nsLTPs (PR-14) are encoded by a small gene family which is present throughout the plant kingdom (Mills et al. 2003). nsLTPs are generally secreted (Sterk et al. 1991) and externally associated with the cell wall (Clark and Bohnert 1999). While their precise biological function remains uncertain, it has been postulated that they are involved in the formation of the protective hydrophobic cutin and/or suberin layer and play a role in the defence of plants against microbial pathogens (Mills et al. 2003). One subfamily, nsLTP1, is characterized by a molecular weight of 9 kDa, a strictly conserved eight-cysteine motif and resistance to pepsin hydrolysis and thermal denaturation. Most nsLTP allergens identified to date belong to the nsLTP1 subfamily (Pastorello et al. 2000a, b; Chardin et al. 2003), including both of the apple nsLTPs described here.

Each nsLTP member may have a different expression profile. In peach, Botton et al. (2002) identified two LTP genes in the reproductive organs that had different expression levels, while Jung et al. (2003) identified three pathogen-inducible LTPs from pepper that were differentially activated by pathogens, abiotic and environmental stress. It is quite clear that in apple *Mal d 3.01* is being expressed in the fruit since both cDNA (Diaz-Perales et al. 2002) and its protein (Pastorello et al. 1999a) have been identified. However, the expression of *Mal d 3.02* in the fruit has not yet been shown. Typical TATA and CAAT boxes in the promoter are present in the proximity of the start codon at different positions for both *Mal d 3* genes (Figs. 1,2). There is also a large sequence variation between the upstream regions of *Mal d 3.01* and *Mal d 3.02* that points towards a potential individual expression profile. Furthermore, one or two amino acid differences in the signal peptide for *Mal d 3.01* and *Mal d 3.02* (Fig. 6) may lead to different trafficking to the target compartment of these two genes (quality of transport, in terms of time, and possibly also efficacy in cleaving off the signal peptide while retaining the function of the mature protein).

Potential cross-reactivity with peach nsLTP (Pru p 3)

The peach fruit is one of the most frequent causes of food allergy in Mediterranean populations and is considered to be the primary sensitizing agent in this type of food allergy (Asero et al. 2000). Garcia-Casado et al. (2003) used the peach allergen, Pru p 3, as a model to find IgE epitopes. They identified three amino acid sequence regions (11–25, 31–45 and 71–80) as major IgE

epitopes and predicted five amino acid residues of R39, T40, R44, K80 and K91 as antibody recognition sites using IgE immuno-detection and synthetic peptides. Mal d 3.01 appears to be more similar to Pru p 3 than does Mal d 3.02 with respect to the amino acid sequence of the total protein as well as to the supposed epitope regions and recognition sites (Fig. 6). Compared with Pru p 3, the Mal d 3.01 and Mal d 3.02 protein sequences share 80% and 77% identity, respectively. Considering the recognition sites, Mal d 3.01 and Pru p 3 are fully identical, while Mal d 3.02 differs at a single position (R/K39). This may imply that Mal d 3.01 is more relevant to apple LTP allergenicity. Structure modelling and immunological testing of the recombinant proteins of all identified apple Mal d 3 isoallergens and variants will provide further information about their individual allergenicity.

Allelic diversity and association with phenotype

Neither of the *Mal d 3* genes was observed to be very diverse in the ten cultivars investigated; in particular, *Mal d 3.01*, which we assume to be the more important of the two considering its expression in fruit and potential allergenicity, showed very little diversity. Therefore, it is necessary to obtained more detailed information on allelic diversity for more cultivars and selections. Consequently, additional AS markers can be developed and used in screening *Mal d 3* genotypes. Once the *Mal d 3* allelic constitution and the Mal d 3-related allergenicity for a wider range of apple cultivars have been assessed, it will be possible to associate individual genes and alleles to low Mal d 3 allergenicity. On that basis, AS markers can be applied in breeding for low nsLTP allergenic cultivars.

Nomenclature of allergen genes

The occurrence of different loci and multi-allelism has consequences for the nomenclature of these allergen genes. We followed the current allergen nomenclature (King et al. 1995) when naming the genomic sequences of *Mal d 3* as much as possible. Firstly, the current allergen nomenclature provides some guidelines for discriminating isoallergens and variants on the basis of amino acid and DNA sequence similarity. The distinction between isoallergens and variants is arbitrary since there is no single threshold of amino acid identity that can be applied in all situations. In the case of this investigation, two similar *Mal d 3* genes share 85% identity at the amino acid level and have been mapped onto two linkage groups, so we denoted them as two isoallergen genes: *Mal d 3.01* and *Mal d 3.02*. The Allergen Nomenclature Sub-Committee has accepted these names. Secondly, current nomenclature protocols recognize that each isoallergen may have multiple forms (designated as variants by two numerals) of cDNA sequences with nucleotide mutations which are either silent or can lead to single or multiple amino acid substitutions. The silent mutations will be designated by new variant numerals. This means that different variant names may represent the same protein, which may cause confusion about just how many different proteins are actually present when more allelic sequences from different sources become available. To avoid this confusion, we distinguished variants solely on the basis of amino acid sequence and indicated silent mutations with two additional numerals preceded by a dot. For example, accessions. AF221502 and AJ277164, which have a silent mutation at position 105 (Fig. 5), will be named Mal d 3.0101.01 and Mal d3.0101.02, respectively. This is a recommendation for updating the current allergen nomenclature. Thirdly, any polymorphism in an intron (if present) or flanking region is not covered in the current nomenclature. However, recognition of their existence by different names is necessary in some cases. Therefore, we extended the allele names by adding a lowercase letter. For example, Mal d 3.0101a and Mal d 3.0101b refer to gDNA sequences for which the polymorphisms are located beyond the coding region of variant Mal d 3.0101.

Mapping genes of interest, genetic analysis and breeding aspects

With the advancement of sequencing facilities and the availability of the complete genome sequence data for several model plant species, it can be expected that many gene sequences will be determined in the very near future for other plants too. With respect to genetics and breeding, knowledge of the location of these genes on the chromosome will be of great help in the analysis of allelic diversity related to phenotypes. As illustrated in this paper, two almost identical *Mal d3* cDNA sequences in the GenBank were explored in order to generate genomic sequences and to assess linkage map positions and allelic diversity of two *Mal d 3* genes. In addition to Mal d 3, three more apple allergens have been identified by gene cloning and sequencing: Mal d 1 (Vanek- Krebitz et al. 1995), Mal d 2 (Krebitz et al. 2003) and Mal d 4 (Van Ree et al. 1995). Similarly, the number of loci and their location on the linkage groups will also be determined in the same way.

For a long time fruit genetics and breeding aimed at parameters such as high yield, resistance and quality. At the present time there is a growing awareness of the paradox that healthy fruits can cause allergic reactions in some sensitive individuals. Based on knowledge acquired from genetics and genomic research, future fruit breeding may be directed towards a new quality trait: the reduction of allergenicity.

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