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RESEARCH PAPER

Identification of a novel α-L-arabinofuranosidase gene associated with mealiness in apple

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Abstract

In order to investigate the genetic bases of the physiological syndrome mealiness that causes abnormal fruit softening and juice loss in apples, an integrative approach was devised, consisting of sensory, instrumental, biochemical, genetic, and genomic methods. High levels of activity of α-L-arabinofuranosidase (α-AFase), a hydrolase acting on the pectic component of the cell walls, were found in individuals exhibiting the mealiness phenotype in a segregating population. The expression levels of the previously uncharacterized apple *AF* gene *MdAF3* are higher in fruits from plants consistently showing mealiness symptons and high α-AFase activity. The transcription of *MdAF3* is differentially regulated in distinct genomic contexts and appears to be independent of ethylene. Thus, it is likely to be controlled by endogenous developmental mechanisms associated with fruit ripening. The use of integrative approaches has allowed the identification of a novel contributor to the mealiness phenotype in apple and it has been possible to overcome the problems posed by the unavailability of near-isogenic lines to dissect the genetic bases of a complex physiological trait in woody perennial species.

Key words: Apple segregating population, α-L-arabinofuranosidase, gene expression, mealiness.

Introduction

Mealiness is one of the most undesirable characteristics of the apple fruit for consumers. The sensory perception of this physiological syndrome is distinctive and consists of abnormal softness and lack of free juice; its causes encompass several physical and chemical changes in the fruit cells and cell walls. The onset of mealiness in apple fruits is controlled by complex interactions between development and environmental factors, and there is evidence of extensive genetic variation for the trait in apple germplasm (Harker and Hallet, 1992).

Instrumentally, apple mealiness is characterized by cell separation upon the application of a force on the fruit flesh; in contrast to the breakage of cells observed in non-mealy fruits (De Smedt *et al.*, 1998). Morphological analyses by transmission electron microscopy have shown that the middle lamella undergoes extensive dissolution in mealy fruits (Ben-Arie and Kislev, 1979). Similarly, differences in the chemical composition between the pectic components of the middle lamella from mealy and non-mealy fruits were also observed (Nara *et al.*, 2001). The release of the aldopentose monosaccharide arabinose from the terminal positions is markedly increased in mealy (Nara *et al.*, 2001) and soft (Pena and Carpita, 2004) apples in comparison with their non-mealy and firm counterparts; similar reports were found in others fleshy fruits (Gross, 1984; Gross and Sams, 1984; Ponce *et al.*, 2010; Yoshioka *et al.*, 2010).

The release of terminal arabinose is mediated by the activity of α-L-arabinofuranosidases (α-AFases) (EC 3.2.1.55) that hydrolyse non-reducing arabinofuranosyl residues from the pectin matrix. In higher plants, two

glycoside hydrolase (GH) families of α-AFases have been described; GH3 and GH51 (Tateishi, 2008). The activity of α-AFase has been demonstrated to be distinctly regulated among apple cultivars (Yoshioka *et al.*, 1995; Goulao *et al.*, 2007; Wei *et al.*, 2010) and appears to be associated with mealiness and fruit softening (Yoshioka *et al.*, 1995; Wei *et al.*, 2010). Although several isoforms may contribute to the detected α-AFase activity, a single transcript, *MdAF1*, has so far been isolated from apple fruits at harvest and post-harvest (Goulao *et al.*, 2008). *MdAF1* belongs to the GH51 family of α-AFases and its transcription profile does not appear to be associated with the enzyme activity profile (Goulao *et al.*, 2008; Wei *et al.*, 2010). In contrast, in Japanese pear (*Pyrus pyrifolia*), the transcription of a distinct GH3 α-AFase, labelled *PpARF2*, has been exclusively associated with ripe fruits (Tateishi *et al.*, 2005; Mwaniki *et al.*, 2007). However, *PpARF2* expression and enzymatic activity are not significantly correlated with flesh firmness in pear (Mwaniki *et al.*, 2007).

The complex phenotypes associated with the ripening and post-harvest processes in apples are thought to involve the activity of other enzymes and the function of genes associated with the cell wall and ethylene metabolism, such as polygalacturonase (PG), pectin-methylesterase (PME), pectin and pectate lyases (PLs), expansins, β-galactosidase (β-Gal), the *COLD BINDING FACTOR* (*CBF*) family of transcriptional regulators, and the *ETHYLENE-INSENSITIVE3*-like (EIN3) factor (Goulao *et al.*, 2007; Tateishi, 2008; Costa *et al.*, 2010; Tacken *et al.*, 2010).

In the present study, sensory, instrumental, biochemical, genetic, and genomic analyses were combined to characterize the complex phenotype of mealiness in apple. The employed integrative approach has shown α-AFase enzyme activity to be associated with mealiness in an apple segregating population, and two previously uncharacterized α-AFase genes, *MdAF2* and *MdAF3*, have been identified. Transcriptional profiling of the three known *MdAF* genes throughout *in planta* ripening and in harvested fruits suggests that *MdAF3* is the main contributor to the α-AFase activity associated with the mealiness phenotype in apple.

Materials and methods

Plant material and phenotyping

This study is based on the 'IM' segregate population, issued from the cross between X6681 and X6683, two genitors from the INRA breeding programme segregating for mealiness. It has been selected from preliminary and extensive work developed at GenHort-INRA (Angers-France) in 2005 where the fruits of almost 600 hybrids from 13 *Malus domestica* populations were tasted by at least two panellists. The IM population showed a clear-cut segregation for mealiness. Eleven hybrids, phenotyped during 4 years from 2005 to 2009, were selected for candidate gene analysis. According to the sensory analyses, the hybrids were classified as follows: five as mealy (M14, M40, M48, M55, and M74) and six as non-mealy (M16, M20, M21, M22, M38, and M49). The Golden Delicious, Greenleeves, Canada Rouge, Gala, and Jonagold apple varieties were sensorily evaluated as reference standards.

The fruits were sampled from 2006 and 2007 on the plant [100 and 140 days after flowering (DAF)], at harvest (H1), and during storage in a cold chamber at 1 °C for 2 months (S1) and for 4 months (S2). The sampled time points and the analyses performed (sensory, penetrometry, α-AFase activity, and transcriptional profiling) are summarized in Supplementary Table S1 available at *JXB* online. Harvested fruits (H1) and those transferred from cold storage at S1 and S2 were analysed 24 h after acclimation at room temperature.

Fruit sampling

For *in planta* analyses at 100 and 140 DAF, two fruits per tree were collected, peeled, and the fleshy tissue was immediately frozen in liquid nitrogen. At harvest (H1), at least 40 fruits per tree were collected. A two-step procedure has been set up to select the most homogeneous samples at optimum maturity. Initially, large numbers of fruits were harvested from the middle and outside parts of the canopy; different and complementary criteria were used to check the maturity of each sample: flesh colour and skin ground and colour coverage, and starch evaluation through instrumental and sensory approaches. Subsequently, 40–50 fruits were further selected and classified into three homogeneous lots according to size and colour, to be employed for analyses performed at H1, S1, and S2. The remaining fruits were discarded. Before their storage in a cold chamber (1 °C), fruits destined for analyses carried out at S1 and S2 were sampled and classified as described. Additionally, at H1, S1, and S2, two fruits were submitted to sensory analyses and 10 to destructive instrumental analysis.

The fruits were sampled in 2009, for ethylene quantification and transcriptional profiling, as described previously. A second haverst (H2) was done 15 d after the first one (H1) (see Supplementary Table S1 at *JXB* online). Ten fruits were transferred to a hermetically closed container and ethylene contents were analysed using an Ethylene Atmosphere Sampling Instrument (EASI-1) (Absoger, France).

Sensory and instrumental evaluation

The sensory panel included four permanent experts working in two separate groups for each sample at each stage (two fruits per sample per date); two sensory scores were given for each of the six texture attributes: crunchiness, firmness, juiciness, meltiness, granularity, and mealiness (Kouassi *et al.*, 2009). For this last trait, the cultivars Greensleeve and Jonagold were considered as the references of extremely mealy and absence of mealiness, respectively. Each trait was scored on a 9-point scale-based grid, ranging from 1 (low) to 5 (high); it also includes intermediate scores (1.5, 2.5, 3.5, and 4.5). An individual fruit was considered as mealy if its sensory score was >2.5 at least at S2. In addition, instrumental firmness was assessed by an automate penetrometer (TA.XT.-PLUS, Stable Micro System) on 10 fruits per individual plant. Since all the parameters of the penetrometry curves are well correlated (F. Laurens, personal communication), Ff, the force in Newtons (N) for a 7 mm displacement, which correlates with the Magness–Taylor firmness (Camps *et al.*, 2005), was chosen for use.

Protein extraction and enzymatic activity

Cell wall proteins were extracted according to Brummell *et al.* (2004), with minor modifications. Briefly, 5 g of frozen flesh tissue was ground into a fine powder with a pestle and mortar, and homogenized in 4–5 vols of ice-cold 12% polyethylene glycol (PEG) 3350 containing 0.2% (w/v) sodium bisulphite. Insoluble material was collected on a Miracloth, washed with 2.5 vols of ice-cold 0.2% (w/v) sodium bisulphite, and extracted in 1.25 M NaCl/4 mM EDTA, pH 6.5. Subsequently, the extract was incubated in a cold chamber at 4 °C for 1 h under agitation. The insoluble residue was removed by filtration through one layer of Miracloth, and the supernatant was clarified by centrifugation at 16 000 *g* for 10 min and stored at –80 °C.

The activity of α-AFase was evaluated by adding 0.1 ml of the enzyme extract to 1 ml of 0.1 M sodium acetate, pH 5.0, and 0.2 ml of 10 mg ml^{-1} 4-nitrophenyl α-L-arabinofuranoside (Sigma, USA). Reactions were incubated at 30 °C, and at 30 min intervals 0.25 ml aliquots were added to equal volumes of 1 M ammonium hydroxide. The α-AFase activity was correlated to the sample spectrophotometric absorbance at 400 nm. It was linear for at least 6 h. One enzyme unit was defined in moles of nitrophenol h^{-1} mg^{-1}.

Gene cloning and sequence analyses

The novel apple AF homologues were identified by BLAST searches (Altschult *et al.*, 1997) of open access *Malus* expressed sequence tag (EST) databases at the Genome Database for *Rosaceae* (GDR) (http://www.bioinfo.wsu.edu/gdr/), the Institute of Genome Research, TIGR (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=apple), GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), and the Genome Database from 'Fondazione Edmund Mach Istituto Agrario San Michele All'Adige', Italy, IASMA (http://genomics.research.iasma.it/index.html) using apple *MdAF1* (GenBank accession no. AY309436; Goulao *et al.*, 2008) and pear *PpARF*2 (GenBank accession no. AB195230; Tateishi *et al.*, 2005) nucleotide sequences as queries. The retrieved apple sequence was used to design internal primers to clone the complete mRNA, using a Creator SMART cDNA library construction kit (Clontech, USA).

Phylogenetic analyses of α-AFase complete amino acid sequences were carried out using the Neighbor–Joining distance matrix, using the default parameters of ClustalX software (Thompson *et al.*, 1997).

RNA extraction, cDNA synthesis, and PCR quantitative (qPCR)

Total RNA was extracted from 3 g of fruit flesh tissue, as described by Gasic *et al.* (2004), with minor modifications. Initially, 1.5 μg of total RNA was treated with 2 U of DNase I (Promega, USA), according to the manufacturer's instructions. cDNA synthesis was then performed using an ImProm™ II Reverse Transcription System (Promega, USA). The qPCRs were performed in triplicate, using 3 μl of the product of reverse transcription (1/50 dilution) in a final volume of 15 μl containing 1× IQ SYBR Green Supermix (Bio-Rad), and 0.3 μM of each primer. Amplifications were performed using an Opticon 4 RealTime PCR detector (Bio-Rad, USA) as follows: 95 °C, 3 min; 40× (95 °C, 15 s; 60°C, 1 min). Amplification and dissociation curves were monitored and analysed employing Opticon Monitor (Bio-Rad, USA). The PCR efficiency curve was performed for each primer pair at each sampling date (i.e. 100 DAF, 140 DAF, H1, H2, S1, and S2) using a cDNA pool. The PCR efficiency was determined by serial dilutions of 0.1, 0.04, 0.02, 0.01, 0.004, and 0.002, according to the equation: PCR efficiency=$[10^{(-1/\text{slope})}-1]\times100$. Primers were designed with Beacon Designer 7 (PREMIER Biosoft International) (Supplementary Table S2 at *JXB* online). The constitutively expressed genes *PROTEIN DISULPHIDE ISOMERASE* (*MdPDI1*-CN494516), *POLYUBIQUITIN* (*MdUBI2*-CN491263) (Wiersma *et al.*, 2007), *ACTIN* (*MdACT*- CN938023) (Li and Yuan, 2008), and *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GAPDH*- CN494000) (Defilippi *et al.*, 2005) were evaluated for stability to normalize the qPCR using geNorm software (http://allserv.ugent.be/;jvdesomp/genorm/index.html) (Vandesompele *et al.* 2002). Under the tested conditions, the most stably expressed were *GAPDH* and *MdPDI1*, and *GAPDH*, as a frequently used reference gene, was employed. Relative quantification was determined by two distinct methodologies: comparing the transcriptional expression between tagged genes and the constitutive reference gene *GAPDH* ($2^{-\Delta\Delta Ct}$) by a calculation formula derived from the $2^{-\Delta\Delta Ct}$ method [$\Delta Ct=(Ct_{tag}-Ct_{ref})$, where Ct is the threshold cycle, tag is the tag gene, and ref is the reference gene (Livak and Schmittgen, 2001), and by evaluating the differential expression between the mealy and non-mealy hybrids (Pfaffl *et al.*, 2001).

Data analysis

Data were processed and statistically analysed by analysis of variance (ANOVA), and Pearson's correlation employing SAS software (SAS Institute, USA). The ANOVA general linear model (GLM) procedure provides matrices of additive genetic, residual, and phenotypic variances/co-variances. The narrow-sense heritability (h^2) was estimated, $h^2=\sigma_a^2/(\sigma_a^2+\sigma_e^2)$, where σ_a^2 is the additive genetic variance between individual genotypes and σ_e^2 is the residual variance.

Expression patterns were identified by hierarchical clustering analyses of *MdAF3* transcription in individuals of the population during sampling dates in 2006 and 2007. Normalized expression data were arcsine transformed and analysed using Pearson's correlation parameters from the MultiExperiment Viewer v.4.6 (Saeed *et al.*, 2006), correlating *MdAF3* transcription levels in individuals and sampling dates. Statistical significance of the expression analyses was determined employing the multiclass model from the Significance Analysis of Microarrays (SAM) feature of the software (Saeed *et al.*, 2006).

Results

Mealiness sensory and instrumental analyses

Fruits from hybrids previously selected from the IM breeding population and the parents were submitted to sensory analysis for mealiness and to instrumental analysis for firmness. Mealiness perception in fruits from the selected plants was consistent during 4 years of evaluation and increased after fruits were harvested from the plants (Table 1). A slight seasonal influence was observed in mealiness perception, since mealiness was perceived earlier in 2006 for the fruits from the majority of the hybrids than in following years (Table 1). The parent X6681 and the hybrids M16, M20, M21, and M49 exhibited non-significant mealiness (Table 1). However, in 2006, a slight perception of mealiness was observed for fruits from the male parent X6683, which was not repeated in the following years (Table 1). The mealy phenotype was consistent in fruits from the hybrids M14, M40, M48, M55, and M74, although the yearly score was variable (Table 1). A similar year effect was not noticeable on the non-mealy phenotype. The association between mealiness and fruit softening was confirmed by the negative correlation between the mealiness sensory scores and fruit firmness, which was of –0.67 (<0.0001).

Instrumental measurements of flesh firmness, using the Ff parameter, were correlated to sensory evaluations of firmness [$\rho_{Pearson}$=mealy 0.49 (<0.0001)], and slightly negatively correlated to mealiness [$\rho_{Pearson}$=mealy –0.37 (<0.0008)]. These observations are confirmed by the firmer phenotype of fruits from the parent X6681 and from non-mealy hybrids in comparison with the softer fruits from the mealy hybrids and from parent X6683 (Table 2). However, exceptions to those correlations are clearly present, as observed in the dispersion graph of sensory mealiness scores versus instrumental Ff force analysis (Fig. 1). For example, fruits from plant M74

Table 1. Sensory evaluation of mealiness for individual hybrids from the IM breeding population and commercial cultivars. The mealy hybrids are in bold.

	2006			2007			2008			2009		
IM population	**H1**	**S1**	**S2**	**H1**	**S1**	**S2**	**H1**	**S1**	**S2**	**H1**	**S1**	**S2**
M14	2.9	2.5	2.8	1.3	1.0	3.0	1.0	2.8	NA	1.0	1.0	2.3
M40	4.0	3.8	4.8	2.0	1.5	4.3	NA	1.8	NA	1.5	4.0	2.8
M48	1.0	3.3	4.0	1.0	2.0	2.8	1.3	1.0	3.3	1.0	2.5	1.9
M55	3.0	3.0	4.5	2.0	2.5	NA	2.3	2.3	NA	1.0	2.0	1.5
M74	1.8	1.0	4.0	1.0	1.0	3.5	1.0	2.0	NA	1.5	2.0	3.0
M16	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.3	1.0	1.0	1.0
M20	1.0	1.0	1.0	1.0	1.0	1.5	1.0	1.0	NA	1.0	1.0	1.0
M21	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
M22	1.0	1.0	1.3	NA	NA	NA	1.5	1.0	NA	NA	NA	NA
M38	1.0	1.0	1.0	NA	NA	NA	1.0	1.0	2.3	NA	NA	NA
M49	1.0	1.0	1.0	1.0	1.0	1.3	NA	1.3	NA	1.0	1.0	1.0
X6681	1.2	1.1	1.0	1.0	1.0	1.0	1.0	1.0	NA	1.0	1.0	1.0
X6683	1.1	1.5	1.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Varieties												
Greensleeves	1	3	3.25	NA	NA	NA	3.25	3.75		2.5	4.75	4.25
Gala	1	1	1	1	1	1.25	1	1	1	1	1	1
G. Delicious	NA	NA	NA	1	1	1	NA	1	NA	1	2	1.5
C. Rouge	NA	1.25	2	1.75	3	NA	2	1.75	NA	1	1.5	2.25
Jonagold	1	1.75	1.25	1	1.5	2	1	1	1.5	NA	NA	NA

Scores range from 1 (low mealiness perception) to 5 (high mealiness perception) for evaluations carried out during four consecutive years, from 2006 to 2009. Light grey shading represents mealy hybrids and dark grey shading, non-mealy hybrids. The population parental plants and commercial cultivars are shown without shading. H1, the first harvest; S1, after storage in a cold chamber at 1 °C for 2 months; and S2, after storage in a cold chamber at 1 °C for 4 months.
G. Delicious, Golden Delicious; C. Rouge, Canada Rouge; NA, not analysed.

exhibit significant values for mealiness, especially at S2, and displayed very high firmness in comparison with the remaining fruits from the lot, even after 4 months of cold storage at 1 °C. In contrast, fruits from plant M49, classified as non-mealy (highest mealiness score=1.3), exhibit low firmness scores in comparison with fruits from the other hybrids.

The heritability of the mealiness and firmness phenotypes, evaluated sensorially and instrumentally, was calculated employing data from 4 years of evaluations, from 2006 to 2009 at H1, S1, and S2. For sensory mealiness, the values were h^2=50.7, h^2=57.4, and h^2=72.2, and for firmness (instrumental), they were h^2=41.7, h^2=29.2, and h^2=45.9 at the three dates investigated, respectively.

α-AFase enzymatic activity

The activity of α-AFase was higher in individual mealy hybrids in comparison with non-mealy hybrids at the three sampling dates, for two years (Fig. 2). In non-mealy hybrids α-AFase activity ranged from 157 to 872 mol of nitrophenol h^{-1} mg^{-1}, with higher rates found after 4 months storage (S2). In mealy hybrids, α-AFase activity was more variable; ranging from 280 to 3914 mol of nitrophenol h mg^{-1}. In general, fruits from hybrids classified as mealy exhibited higher levels of α-AFase activity, such as M55 and M48, which displayed the highest enzyme activity. In contrast, in fruits from M74, classified as mealy but firm, the levels of α-AFase activity were low (Fig. 2).

In the time-course investigations at harvest (H1) and after storage (S1 and S2), α-AFase activity remained stable for the parent X6681 and non-mealy hybrids. In contrast, the enzyme activity showed a significant increase for the mealy hybrids and X6683 parent, especially at the later dates. In general, the α-AFase activity was higher in 2006 than in 2007 (Fig. 2), coinciding with higher occurrence of mealiness by phenotypic evaluation in 2006 (Table 1). For fruits sampled from the trees (100 and 140 DAF), no differences in α-AFase activity were detected between mealy and non-mealy hybrids and the parents (data not shown).

The most significant positive correlations between α-AFase activity and sensory mealiness were observed after a storage period of 2 months at 1 °C (S1) (Table 3). Similar significant but negative correlations were observed for firmness and mealiness (Table 3).

Sequence analyses

Three contigs showing significant sequence conservation with *MdAF1* (Goulao *et al.*, 2008) were identified by querying apple GDR databases (*Malus* assembly 4): Contig18999, Contig9012, and Contig666, showing 99, 98, and 92% nucleotide identity to *MdAF1*, respectively. Two other sequences showing significant conservation with *MdAF1* were identified by querying apple IASMA gene set databases using a protein query to search the translated nucleotide databases: MDP0000055078 and MDP0000256049. The extensive sequence conservation observed between Contig18999, Contig9012, and *MdAF1* suggests that these sequences may correspond to allelic variations. The sequence of Contig666 corresponds to a previously unidentified *AF* gene labelled here as *MdAF2*. From a similar search, employing pear *PpARF2* (Tateishi *et al.*, 2005) as query, Contig21129 was identified, exhibiting 96% nucleotide sequence identity to the pear gene. The Contig21129 sequence was used to design an internal primer to clone the complete cDNA sequence, and a full-length clone encoding a novel apple α-AFase (named *MdAF3*; accession no. GU937612) was obtained. The cloned cDNA fragment (2609 p) consisted of an open reading frame coding sequence of 2322 nucleotides and the corresponding untranslated region at the 5' and 3' ends (73 bp and 214 bp, respectively). Five sequences showing significant conservation with *MdAF3* were identified by querying apple IASMA translated nucleotide databases with a protein query: MDP0000140483, MDP0000121374, MDP0000174676, MDP0000121374, and MDP0000174676.

Phylogenetic analyses of the full-length deduced amino acid sequence of α-AFase accessions available from GenBank resulted in two distinct clusters (Fig. 3); the first one consisted of *Md*AF1 and other GH51 family sequences, the second

Table 2. Fruit firmness for individual hybrids and parents from the IM breeding population evaluated by penetrometry

The numbers represent the average penetrometry force (Ff) in Newtons (N) for 7 mm displacement. Data correspond to evaluations performed during two consecutive years, 2006 and 2007 at harvesting (H1) and after 2 (S1) and 4 months (S2) of cold-storage at 1 °C. Lettering (from a to e) represents variance analysis (ANOVA) groups. The mealy hybrids are in bold.

2006						2007					
H1		S1		S2		H1		S1		S2	
IM	Ff (N)	IM	Ff (N)	IM	Ff (N)	IM	Ff (N)	IM	Ff (N)	IM	Ff (N)
M74	**19.9 a**	M21	16.3a	X6681	20.4 a	**M74**	**19.5** a	**M74**	**13.2 a**	**M74**	**9.8 a**
M48	**14.4 b**	M22	11.4 b	**M74**	**13.8 b**	**M40**	**15.3** b	M20	12.9 a	X6681	9.6 a
M22	13.6 c,b	X6681	11.4 b	M16	10.1 c	**M48**	**14.4** b	X6681	11.9 b,a	**M48**	**8.2 b**
X6681	13.5 c,b	M20	10.6 b	M21	9.9 c	M20	13.8 b,d	**M48**	**11.2 b,c**	X6683	8.2 b
M16	12.8 c,d	M16	10.4 b	M22	9.5 c	X6681	13.5 c,d	X6683	10.8 b,c	M14	8.1 b
M20	12.5 c,d	**M55**	**8.1 c**	M38	9.3 c	**M14**	**13.2** c,d	M16	10.3 c	M16	8.1 b
X6683	12.4 c,d	M49	7.7 c	M20	9.1 c	X6683	12.4 d	**M14**	**10.2 c**	**M55**	**7.9 b**
M21	11.5 d	**M14**	**7.1 c**	**M48**	**7.6 d**	M21	10.9 e	M49	10.1 c	M21	7.6 b
M49	11.5 d	M40	NA	M49	7.4 d	M55	NA	M21	9.8 c	M49	7.6 b
M55	**9.7 e**	M48	NA	**M55**	**7.3 d**	M16	NA	**M40**	**9.7 c**	**M40**	**7.1 b**
M40	**9.3 e**	M74	NA	**M14**	**6.5 d**	M22	NA	**M55**	**8.5 d**	M20	NA
M14	**9 e**	M38	NA	**M40**	**4.2 e**	M38	NA	M22	NA	M22	NA
M38	NA	X6683	NA	X6683	NA	M49	NA	M38	NA	M38	NA

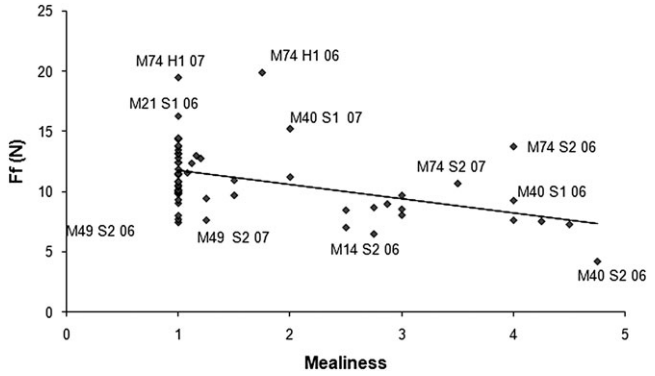

Fig. 1. Dispersion graph of the correlation between flesh firmness (Ff) and mealiness in fruits for the years 2006 and 2007. The *y*-axis represents Ff in Newtons (N) and the *x*-axis represents mealiness. H1, the first harvest; S1, during storage in a cold chamber at 1 °C for 2 months; and S2, during storage in a cold chamber at 1 °C for 4 months. The years are represented by 06 for 2006 and 07 for 2007.

cluster contained *Md*AF3 and sequences from the GH3 family, whereas *Aspergillus niger* sequences, classified as α-AFases from families GH54, GH62, and GH43 by the Carbohydrate-Active enZYmes Database (CAZy; http://www.cazy.org/Glycoside-Hydrolases.html), constituted solo branches. The novel apple α-AFase sequence, *Md*AF3, clustered in close proximity to MDP0000740483, *Pp*ARF2 (96% identity), MDP0000121374 and MDP0000174676. The most similar genomic sequence, MDP0000140483, probably corresponds to the *MdAF3* locus. In contrast, the other two sequences, MDP0000121374 and MDP0000174676, did not exhibit sufficient sequence similarity to previously characterized transcript sequences to be attributed to the same genome locus. The absence of similar transcripts from EST databases suggests that they are rarely transcribed or not transcribed in apple. Both genomic sequences belonging to the GH51 group, MDP0000055078 and MDP0000256049, probably correspond to *MdAF1* and *MdAF2* loci, respectively. The family GH51 consists solely of enzymes displaying α-AFase activity, whereas GH3 consists of proteins showing β-xylosidase and β-glucosidase activity, in addition to the α-AFase activity (Gilbert, 2010).

Functional domain analyses have demonstrated the presence of a conserved α-L-arabinofuranosidase motif, characteristic of GH51 family proteins, at the C-terminus of *Md*AF1 and *Md*AF2. In contrast, *Md*AF3 exhibits N- and C-terminal domains more closely related to those of proteins from the GH3 family (Supplementary Figure S1 at *JXB* online).

MdAF gene expression analysis

The expression profiles of *MdAF1*, *MdAF2*, and *MdAF3* were investigated by qPCR, in mealy (hybrids M14, M55, and M40) and non-mealy (hybrids M16, M20, and M21) and the parents (i.e. X6681 and X6683) at five sampling points, corresponding to *in planta* fruits at 100 DAF and 140 DAF, and at H1, S1, and S2 (Fig. 4). The expression of *MdAF1* was low for *in planta* fruits and exhibited a marked increase, peaking at harvest and decreasing during storage for mealy and non-mealy hybrids and the parents (Fig. 4A). Although the induction profile was similar, *MdAF1* transcript levels were relatively higher for X6681 (Fig. 4A). A constitutive expression pattern was observed for *MdAF2* at all investigated times and in all invetigated fruit samples (Fig. 4B). In contrast, *MdAF3* expression was markedly induced at harvest in fruits from the mealy group in comparison with those from the non-mealy group (Fig.

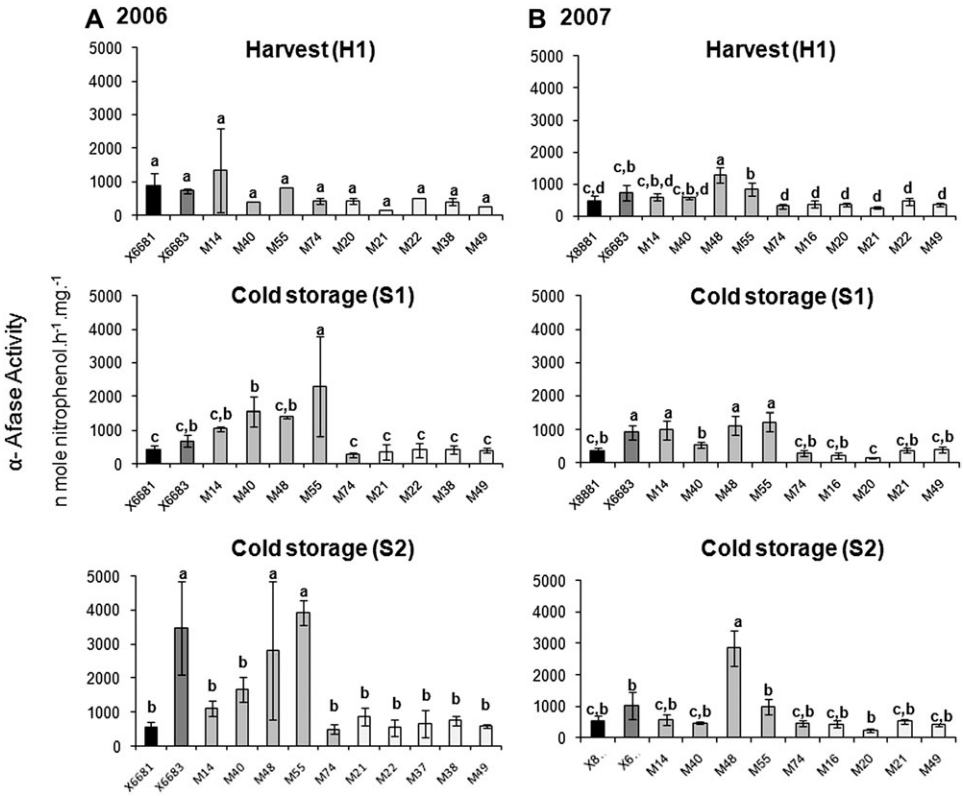

Fig. 2. Analysis of α-AFase activity in fruits from individual plants in the years 2006 (A) and 2007 (B). The fruits were analysed on the plants at 100 and 140 days after flowering (DAF), at the first harvest (H1), and after 2 (S2) and 4 months (S2) of cold storage at 1 °C. The individuals are represented by disticnt column colours: black, X6681 parent; dark grey, X6683 parent; light grey, mealy hybrids; white, non-mealy hybrids. Bars represent standard deviation for three and two independent technical and biological replicates, respectively. Lower case lettering (from a to d) represents analysis of variance (ANOVA) groups.

Table 3. Pearson correlation between α-AFase activity and sensory mealiness, firmness, penetrometry, or gene expression analysis at harvest (H1), after storage in a cold chamber at 1°C for 2 months (S1) and for 4 months (S2)

Bold numbers represents Prob > |r| under H0: Rho=0 at least > 0.09.

α-AFase	Sensory				Penetrometry		Gene expression	
	Mealiness		Firmness		Ff (N)		*MdAF3*	
	2006	2007	2006	2007	2006	2007	2006	2007
H1	0.35	0.26	**–0.41**	–0.43	–0.28	0.18	**0.62**	**0.96**
S1	**0.63**	**0.73**	**–0.65**	**–0.73**	–0.50	–0.50	**0.9**	**0.61**
S2	0.50	0.17	–0.44	–0.43	–0.42	–0.03	–0.05	**0.63**

4C). *MdAF3* expression levels in mealy fruits displayed a 36- and 26-fold up-regulation at H1 and S1, respectively, in comparison with the transcription in non-mealy fruits (Fig. 4C). In fruits from both parents, *MdAF3* transcription was also induced at harvest although more markedly so for X6683 (Fig. 4C). For fruits on the tree, the transcription of *MdAF3* was low for mealy and non-mealy pools and for both parents (Fig. 4C). At harvest, *MdAF3* exhibited a marked induction in fruits from the parent trees and in the mealy group of hybrids (Fig. 4C).

In order to gain further insight into the influence of the genomic context in *AF* gene family expression in apple, an additional relative quantification analysis for *MdAF1* and *MdAF3* was performed for fruit samples from individual hybrids. Similar to the results from the pooled analyses, *MdAF1* showed no contrasting differential expression among the samples (hybrids and parents) within the collection dates (100 DAF, 140 DAF, H1, S1, and S2) (data not shown). In contrast, the *MdAF3* gene exhibited differential expression patterns between mealy and non-mealy individuals at the H1, S1, and S2 investigated collection dates in both harv vesting seasons (Fig. 5). Non-mealy hybrids consistently displayed lower levels of *MdAF3* transcription, whereas, in the hybrids classified as mealy,

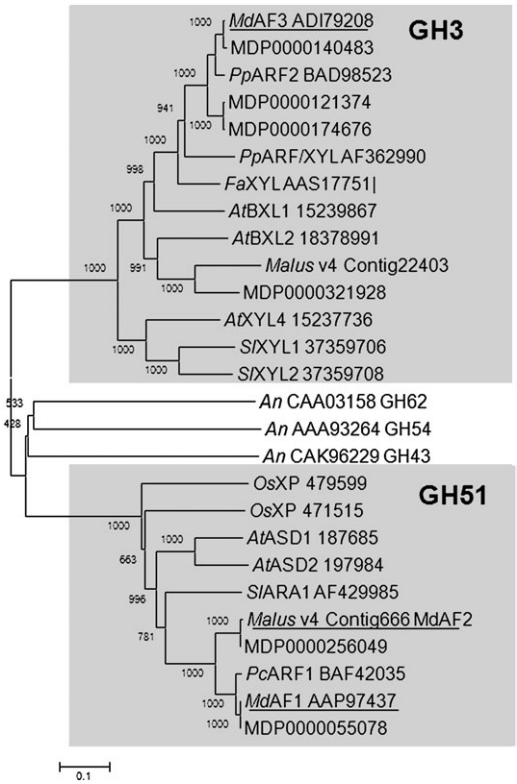

Fig. 3. Phylogenetic analysis of the deduced amino acid sequences of AFase sequences. Apple sequences correspond to *Md*AF1 and *Md*AF3 (GenBank protein sequences), *Md*AF2 and Contig22403 (GDR databases), and MDP0000140483, MDP0000121374, MDP0000174676, MDP0000121374, MDP0000174676, MDP0000055078, and MDP0000256049 (IASMA databases). Additionally sequences from other organisms (GenBank protein accessions) were included and are represented by the initials of their binomial nomenclature. A Neighbor–Joining (NJ) tree was generated using the default parameters of ClustalX software. Bootstrap values are represented close to the branch knots. Accession numbers from the NCBI database are shown following the protein labels. *Pp*, *Pyrus pyrifolia*; *Pc*, *Pyrus communis*; *Fa*, *Fragaria*×*ananassa*, *At*, *Arabidopsis thaliana*; *Sl*, *Solanum lycopersicum*; *Os*, *Oryza sativa. Aspergillus niger* (*An*) GH62, GH43, and GH54 sequences, classified by the CAZYy database, were use as the outgroup.

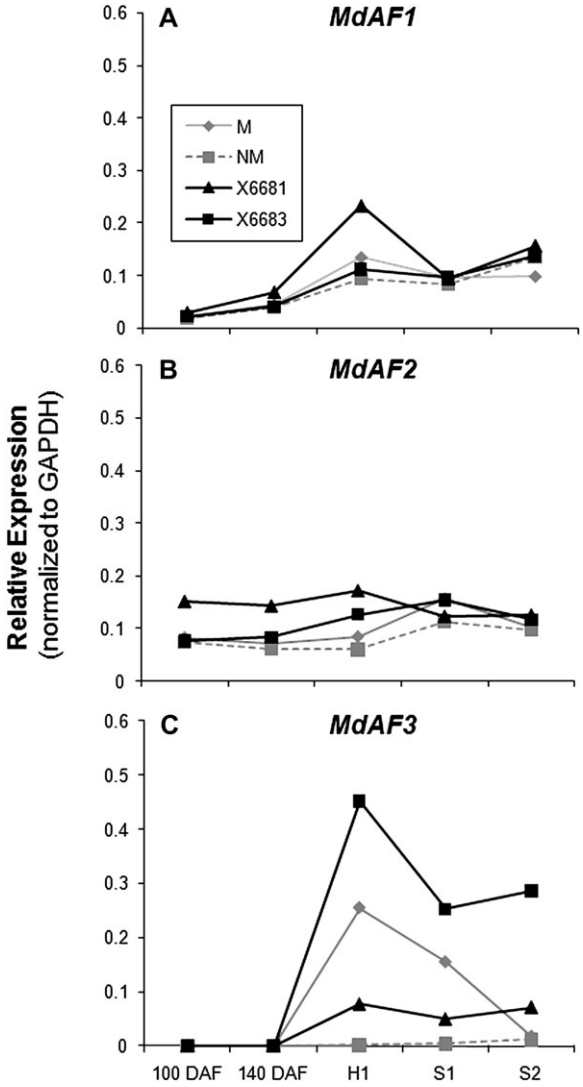

Fig. 4. Transcriptional profile of *MdAF* genes in pooled samples of mealy (M) (M14, M55, and M40) and non-mealy (NM) hybrids (M16, M20, and M21), and the population parents X6681 and X6683. (A) *MdAF1*, (B) *MdAF2* (C) *MdAF3.* The values represent relative transcription in comparison with *GAPDH* expression. The fruits were analysed on the plants at 100 and 140 days after flowering (DAF); at the first harvest (H1) and after 2 (S1) and 4 months (S2) of cold storage at 1 °C.

MdAF3 transcription was predominantly higher, as shown by clustering analyses (Fig. 5), thus confirming the association between mealy fruit phenotype and higher levels of *MdAF3* expression. The expression level of *MdAF3* was differentially regulated at the sampling dates; however, the influence of the growing season (2006 versus 2007) was more marked (Fig. 5). In fruits from parent X6683 and non-mealy soft M49, the levels of *MdAF3* transcription were higher than those found in non-mealy hybrids for both investigated seasons (Fig. 5).

Clustering analyses of *MdAF3* expression in fruits from the population parents and hybrids, in comparison with its transcription in non-mealy individuals M49, M20, and M21, confirmed the previously observed association between high levels of *MdAF3* transcripts and the mealy phenotype for three growing seasons (Fig. 6). Moreover, an environmental component was observed in the regulation of *MdAF3* expression, for example lower transcription levels in 2007 in comparison with 2006 and 2009 (Fig. 6A, C). Developmental factors also influence *MdAF3* transcription, since higher levels of transcript were consistently found in fruits at harvest (H1 and H2), whereas the gene was significantly down-regulated in stored fruits (S1 and S2) (Fig. 6). Hierarchical clustering analyses indicate that although environmental and developmental factors influence *MdAF3* transcription, it is consistently up-regulated in mealy fruits in comparison with non-mealy fruits (Fig. 6). In 2006, *MdAF3* expression was up-regulated in all mealy

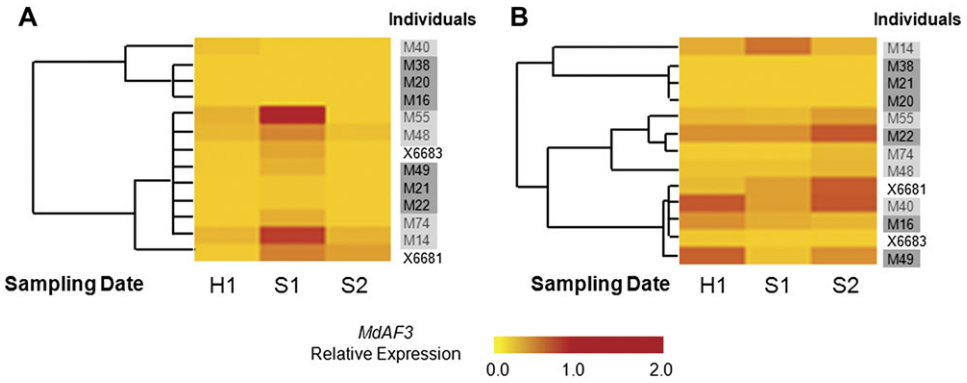

Fig. 5. Transcriptional profile of *MdAF3* expression in the parents and individual mealy (light grey squares) and non-mealy (dark grey squares) plants from the segregating populations at harvest (H1) and during storage in a cold chamber at 1 °C for 2 months (S1) and for 4 months (S2) for the years 2006 (A) and 2007 (B). Relative expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method to represent transcript induction in comparison with GAPDH expression and arcsine transformed for hierarchical clustering analysis based on Pearson's correlation, employing default parameters of the Multiexperiment Viewer v4.6.1 software (Saeed *et al.*, 2006). The parental individuals are not highlighted.

hybrids in comparison with non-mealy M49, at all sampling dates (Fig. 6A). Although *MdAF3* was generally down-regulated in 2007, the transcription levels found in mealy fruits were higher than those in non-mealy fruits (Fig. 6A). Similarly, *MdAF3* transcription in mealy hybrids (i.e. M14, M55, and M48) and the parental X6683 was highly induced in comparison with the transcription levels observed for non-mealy hybrid M20 at harvest in 2007 (Fig. 6B). In 2009, *MdAF3* transcription was also up-regulated in mealy fruits, in comparison with the levels found in non-mealy apples from M21 (Fig. 6C).

The differences in *MdAF3* transcription observed at harvest and after cold storage prompted the investigation of the role of ethylene in regulation of its expression. Fruits from three mealy (M14, M40, and M74) and two non-mealy hybrids (M49 and M21) harvested in 2009 were sampled for qPCR and ethylene dosage analyses (M21 was excluded from the last analysis). As observed in the previously investigated seasons, *MdAF3* transcription is associated with mealiness, since the transcription levels in mealy fruits (from hybrids M14, M40, and M74) were higher than those found in non-mealy fruits from hybrids M21 and M49 (Fig. 6C). The profile of *MdAF3* expression was variable in non-mealy hybrids M21 and M49; in fruits from M21, *MdAF3* expression was constitutively low and, in M49, the expression was increased upon cold storage (Fig. 7).

Ethylene production was low for all tested hybrids upon harvesting (ranging from 7 µl h^{-1} kg^{-1} to 84 µl h^{-1} kg^{-1}) (Fig. 7). The hormone levels gradually increased from harvest to 4 months of cold storage for fruits from hybrids M49 and M74. In fruits from hybrid M14, ethylene contents reached a plateau after 2 months of cold storage (S1), whereas for fruits from M40, the highest level of ethylene (3263 µl h^{-1} kg^{-1}) was reached at the second harvest. The patterns of *MdAF3* transcription in fruits from the investigated hybrids were distinct from the ethylene accumulation kinetics. Similarly, increased ethylene release from the fruits did not induce *MdAF3* transcription (Fig. 7).

Discussion

The mealy phenotype of apples has been associated with losses in the cell wall pectic sugar arabinose, caused by enzyme-mediated hydrolysis (Yoshioka *et al.*, 1995; Nara *et al.*, 2001; Wei *et al.*, 2010). Genetic studies using a refined and precise sensory analysis methodology (F. Laurens, personal communication) have revealed contrasting mealiness phenotypes in an apple breeding population and the presence of high heritabilities (0.80–0.89) for the trait. By combining sensory, instrumental, genetic, and genomic analyses, a novel *AF* family gene has been identified and demonstrated to contribute to mealiness in apple fruits.

α-AFase activity associated with fruit texture changes, mealiness, and firmness

Mealiness is a complex attribute that cannot be predicted by a single instrumental variable (Mehinagic *et al.*, 2004; Camps *et al.*, 2005). In the current study, the methodological difficulties in phenotyping were overcome by longer evaluation periods (from 2005 to 2009) and integrative approaches. Among a number of cell wall hydrolases investigated, α-AFase enzymatic activity exhibited a consistent positive correlation with fruit firmness, which increased with extended storage periods. During the ripening process, the activity of arabinosidases has been associated with softening in several fruits (Pena and Carpita, 2004; Goulao *et al.*, 2008; Wei *et al.*, 2010). In the present work, the integrative approach revealed high levels of α-AFase activity in fruits classified as soft and mealy, by penetrometry and sensory analyses, respectively. Moreover, the use of a segregating population has allowed the detection of the influence of the genetic context on the associations observed between α-AFase activity and fruit firmness or mealiness. The levels of enzyme activity and *MdAF3* transcriptional levels in fruits from the parent X6683, characterized as non-mealy and soft, were similar to those found in mealy

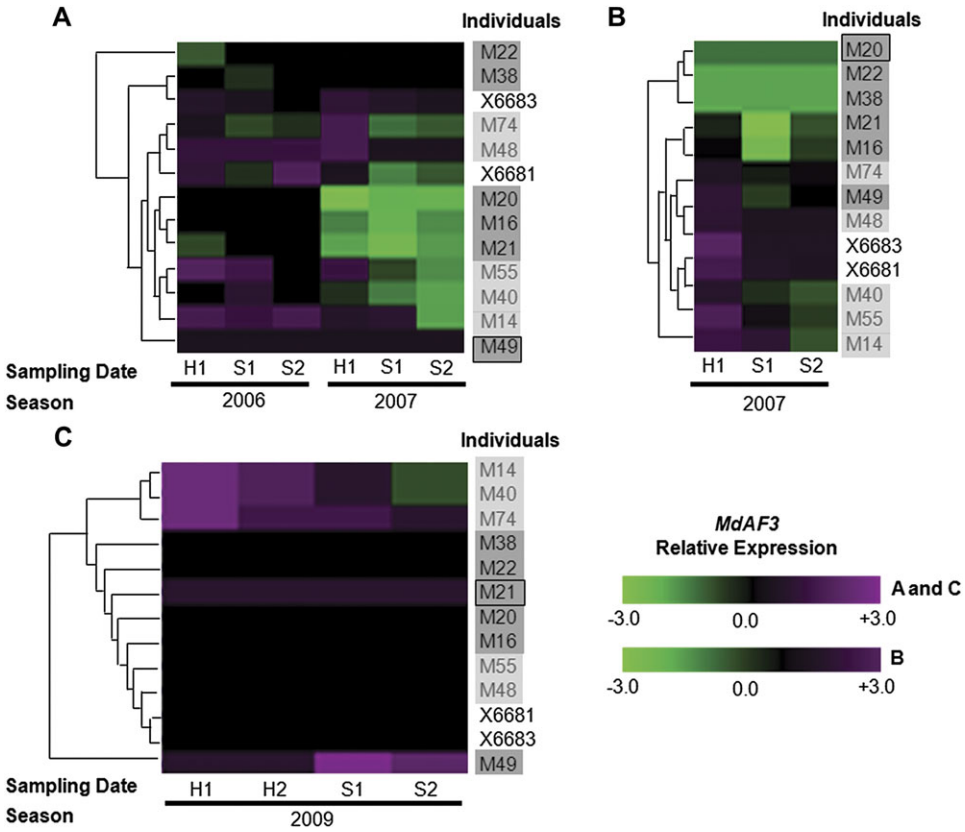

Fig. 6. Transcriptional profile of *MdAF3* expression in the parents and individual plants from the segregating populations at the first harvest (H1), 15 d after the first harvest at the second harvest (H2), and during storage in a cold chamber at 1 °C for 2 months (S1) and for 4 months (S2). Relative expression levels were calculated based on Pfaffl *et al.* (2002) to represent transcript induction in comparison with controls: M49 (A), M20 (B), and M21 (C), and arcsine transformed for hierarchical clustering analysis based on Pearson's correlation, employing default parameters of the Multiexperiment Viewer v4.6.1 software (Saeed *et al.*, 2006). Mealy hybrids are highlighted by light grey shadowing, whereas non-mealy hybrids are shaded in dark grey. The parental individuals are not highlighted.

hybrids, suggesting that the high levels of α-AFase activity are preferentially associated with softness for that particular genomic context. In contrast, in apples from the hybrid M49, classified as non-mealy and soft, α-AFase activity and *MdAF3* transcriptional levels were relatively low, whereas in fruits from M74, considered mealy but firm, the enzyme activity and *MdAF3* transcriptional levels were low. Thus, although associated with mealiness, α-AFase activity as well as *MdAF3* transcriptional levels are not solely responsible for the complex mealy phenotype and its effect is modulated by the genomic context. In tomato, the *colourless-non-ripening* (*Cnr*) mutant fails to soften during maturation and shows a mealy texture (Thompson *et al.*, 1999). The mealiness in *Cnr* fruits is hypothesized to be associated with a severe reduction in cell–cell adhesion in the pericarp (Thompson *et al.*, 1999; Orfila *et al.*, 2001). The firm phenotype of *Cnr* tomatoes and apples from the M74 hybrid suggests that the physiological basis for firmness and mealiness are, at least, partially independent.

Peach is a well-characterized system for the development of a mealiness-like physiological condition, labelled woolliness. Even though peach is considered a melting fruit and apple is a crisp fruit, previous studies with both species have associated α-AFase activity with mealiness under post-harvest conditions (Yoshioka *et al.*, 1995, 2010). Apples from the cultivar Starking Delicious, stored at 25 °C immediately after harvesting, exhibited gradual flesh softening and mealiness 10 d after harvest (Yoshioka *et al.*, 1995). The authors report that α-AFase activity was not detectable at harvest, appearing 5 d after harvest, and rapidly increased in apples 10 d and 20 d after harvest. In peach, α-AFase activity and the loss of arabinosyl residues associated with mealiness have been elegantly demonstrated by comparisons of the melting texture of 'Akatsuki' and mealiness, induced in stony-hardy 'Odoroki' treated with propylene. For both species, mealiness development is observed at room temperature (Yoshioka *et al.*, 1995, 2010). Interestingly, the present data demonstrate that α-AFase activity in mealy fruits and the parent X6683 fruits is induced after harvesting, regardless of cold storage. In contrast, in fruits from non-mealy hybrids and from the parent X6681, α-AFase activity was only slightly increased, if at all, under cold storage, as observed for fruits from 'Fuji' and 'Golden Delicious' varieties (Wei *et al.*, 2010).

It has been hypothesized that α-AFase activity contains an endo-type of glycosidase, which releases ethanol-soluble

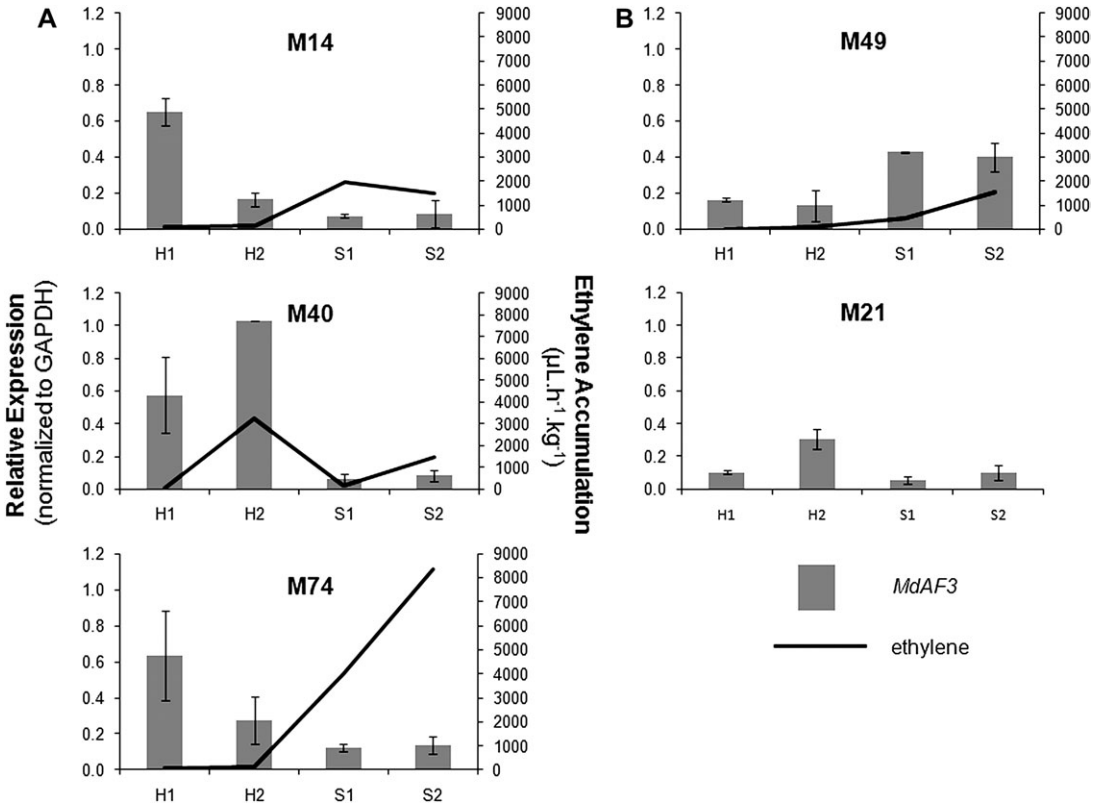

Fig. 7. Relative quantification of *MdAF3* transcription and ethylene accumulation in fruits from individual plants showing mealiness (A) and non-mealiness (B). The values represent relative transcription in comparison with *GAPDH* expression. The fruits were analysed at the first harvest (H1), 15 d after the harvest (H2), and after 2 (S1) and 4 months (S2) of cold storage at 1 °C. Bars represent the standard deviation for three and two independent technical and biological replicates, respectively.

oligosaccharides containing arabinosyl, galactosyl, and glycosyl residues from arabinogalactan and polyuronide (Yoshioka *et al.*, 1995). In comparison with the exo-type of cell wall-degrading enzymes, those containing the endoglycosidase activity seem to be more effective to solubilize cell wall polysaccharides. Thus, α-AFase activity during the softening of apples and peaches may indicate a role for the enzyme in the loss of arabinosyl residues.

MdAF *sequence analyses*

Two novel *MdAF* sequences, *MdAF2* and *MdAF3*, were identified in apple by comparative genomic approaches. The protein domain and phylogenetic analysis has demonstrated that the apple AF proteins *Md*AF3 and *Md*AF2 belong to the GH3 and GH51 families, like other α-AFases from higher plants (Tateishi *et al.*, 2005; Di Santo *et al.*, 2009). *In silico* functional predictions indicate that *Md*AF3 is able to catalyse the hydrolysis of a wider range of glycosyl residues from the pectin component of apple cell walls. Moreover, *Md*AF3 shows extensive sequence conservation with *Pp*ARF2 (96% deduced amino acid identity), demonstrated to hydrolyse preferentially *p*-nitrophenyl α-arabinofuranoside but also to react bifunctionally with β-D-xylopyranoside (Tateishi *et al.*, 2005). The pear enzyme has been demonstrated to release exclusively arabinose from native cell wall polysaccharides, indicating that *in vivo* it preferentially exhibits α-AFase activity (Tateishi *et al.*, 2005). A similar protein from strawberry, *Fa*XYL (83% deduced amino acid sequence identity), has been demonstrated to have solely β-xylosidase activity (Bustamante *et al.*, 2006). The preferential hydrolysis activity of GH proteins appears to be determined by their folding structure rather than by sequence homology (Henrissat and Bairoch, 1996). Thus, although *Md*AF3 is expected to exhibit substrate specificity similar to that of *Pp*ARF2, further functional analyses are required.

In tomato, the principal model species for fleshy fruit investigation, only two closely related xylosidase sequences, *Sl*XYL1 and *Sl*XYL2, displaying moderate conservation with *MdAF3* were found. The tomato sequences are relatively distinct from GH3 enzymes from the other fruit species, indicating that further functional investigations of *Md*AF3 in heterologous systems will require complementary studies in apple.

Expression profiling of α-AFase-coding genes

The investigation of the transcriptional profiling of *MdAF1* and *MdAF2* during ripening and post-harvest storage of apples has demonstrated that these genes are unlikely

candidates for the mealy phenotypes observed in this breeding population.

The expression of *MdAF1* has been previously demonstrated to be up-regulated at harvesting in the apple cultivar Mondial Gala (Goulao *et al.*, 2008). Accordingly, the expression of *MdAF1* in the mealiness segregating population is associated with developmental ripening processes but appears to be independent of specific texture changes. In contrast, the level of *MdAF2* transcripts was constant throughout fruit development and ripening, thus appearing not to be correlated to the observed texture changes. The expression of the GH3 family member *MdAF3* was induced in fruits at harvest and under post-harvest conditions, coinciding with the occurrence of texture changes related to softening and mealiness and with the observed high α-AFase activity in apples. Time-course analyses showed that the peaks of α-AFase enzyme activity occurred under post-harvest conditions. In contrast, the induction of *MdAF3* transcription occurred earlier, during *in planta* ripening stages. Thus, the accumulation of *MdAF3* mRNA throughout the fruit ripening stages in the plant is likely to be the major contributor to the higher levels of α-AFase activity observed in mealy apples. The lag phase observed between the transcription peaks and the enzyme activity suggests that AF3 function in apple is regulated by translational or post-translational mechanisms. Although still largely unknown, translational and post-translational mechanisms have been demonstrated to be involved in plant carbohydrate metabolism (Houtz *et al.*, 2008; Kötting *et al.*, 2010).

Ripening-associated changes in the same fruit species are associated with induction in the expression of *MdAF3* orthologues, such as *PpARF2* in pear (*Pyrus pyrifolia*) and *PpARF/XYL* in peach (*Prunus persica*) (Hayama *et al.*, 2006; Mwaniki *et al.*, 2007). In pear, the induction of *PpARF2* expression was associated with increased α-AFase activity, yet not associated with the fruit texture change (Mwaniki *et al.*, 2007). The levels of *MdAF3* and *PpARF2* transcripts exhibit a distinct correlation with fruit texture change, probably due to distinct features of apple and pear flesh texture. In contrast, in peach, the up-regulation of *PpARF/XYL* expression was associated with fruit softening (Hayama *et al.*, 2006). The expression of *PpARF/XYL* reached its maximum in fully ripe peach fruit with normal texture, coinciding with the peak of ethylene production (Di Santo *et al.*, 2009). *PpARF/XYL* and its highly similar sequence *PpAZ152* (Ruperti *et al.*, 2002) were ubiquously expressed (e.g. fruits, fruitlet, fruitlet abscission zone, flowers organs, leaves, and seedling roots) (Di Santo *et al.*, 2009). In apple, *MdAF3* appears to be preferentially expressed in fruits, as suggested by *in silico* transcription analysis of the GDR data set (Supplemental Figure S2 at *JXB* online). The remaining known GH3 plant proteins, from tomato and strawberry, are likely to be functionally distinct from the novel apple AF3 identified in the present study. The strawberry enzyme *Fa*XYL has been demonstrated to have exclusively β-xylosidase activity (Martínez *et al.*, 2004), whereas, although the substrate specificity of the tomato Sl*XYL1* remains unknown, its sequence conservation with *Md*AF3 is low, indicating divergent functions.

The metabolism and signal transduction of the hormone ethylene have been extensively associated with fleshy fruit texture changes during ripening, mainly due to cell wall component hydrolysis (Goulao and Oliveira, 2007; Bapat *et al.*, 2010). However, the function of several genes involved in cell wall loosening is also triggered by ethylene-independent pathways (Goulao and Oliveira, 2007; Vicente *et al.*, 2007). The induction of *MdAF3* transcription is independent of ethylene and occurs during the early ripening phase, where cellular events are hypothesized to be less dependent on ethylene (Johnston *et al.*, 2009). The expression profile of *PpARF/XYL* has been evaluated in peach fruits with suppressed ethylene production during ripening and exogenously treated with several concentrations of the growth regulator. In contrast to the results shown in the current work, higher concentrations of ethylene appear to regulate *PpARF/XYL* expression (Hayama *et al.*, 2006). However, other factors may be regulating *PpARF/XYL* expression, since its transcripts were observed at different developmental stages and in different organs even when ethylene biosynthesis was barely detectable (Di Santo *et al.*, 2009). The responsiveness of *MdAF3* transcription to the hormone remains to be tested under controlled conditions, such as in the presence of the ethylene receptor inhibitor 1-methylcyclopropene (1-MCP).

Hierarchical clustering analyses of *MdAF3* expression demonstrate its association with the mealiness phenotype in apple fruits and suggest that it is differentially regulated by environmental and developmental factors in distinct genetic backgrounds. Investigations employing high-throughput integrative methodologies have uncovered similar layered regulatory systems for ripening-associated genes in several species, including tomato, strawberry, grape, and *Arabidopsis* (Böttcher *et al.*, 2010; Fujisawa *et al.*, 2011; Karlova *et al.*, 2011; Seymour *et al.*, 2011).

Concluding remarks

The present study has demonstrated the association of a novel α-AFase with the mealy phenotype of apple fruits. Transcriptional induction of *MdAF3* is differentially regulated in distinct genomic contexts and is independent of ethylene accumulation, suggesting the action of endogenous developmental regulators. The lag between transcription and enzymatic hydrolysis peaks indicates that α-AFase activity in apple fruits is submitted to translational, post-translational, or metabolic regulation. The use of a segregating population has allowed the investigation of the genetic bases of a complex trait without the need to generate near-isogenic lines, unavailable for woody fruit species. Moreover, the integrative approach devised here permitted the identification of a previously uncharacterized contributor to the complex cellular changes involved in ripening of fleshy fruits.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Amino acid sequence alignment showing the GH3 functional domain in plants.

Figure S2. *In silico* transcription analysis of Contig21129 (very high nucleotide sequence similarity to *MdAF3* at 99%) from the open-access *Malus* EST databases at the Genome Database for *Rosaceae* (GDR) (http://www.bioinfo.wsu.edu/gdr/) data set.

Table S1. Summary of the integrative approach used to investigate the genetic bases of mealiness in apple. *In planta* fruits were sampled at 100 and 140 days after flowering (DAF). The first harvest corresponds to H1 and a second, 15 d later, to H2. Post-harvesting sampling points correspond to 2 and 4 months under cold storage at 1 °C, represented by S1 and S2. The analyses were carried out for 4 years, as shown.

Table S2. Primer sequences for qPCR analysis.

Acknowledgements

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