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# Two highly divergent alcohol dehydrogenases of melon exhibit fruit ripening-specific expression and distinct biochemical characteristics

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**Abstract** Alcohol dehydrogenases (ADH) participate in the biosynthetic pathway of aroma volatiles in fruit by interconverting aldehydes to alcohols and providing substrates for the formation of esters. Two highly divergent *ADH* genes (15% identity at the amino acid level) of Cantaloupe Charentais melon (*Cucumis melo* var. *Cantalupensis*) have been isolated. *Cm-ADH1* belongs to the medium-chain zinc-binding type of ADHs and is highly similar to all *ADH* genes expressed in fruit isolated so far. *Cm-ADH2* belongs to the short-chain type of ADHs. The two encoded proteins are enzymatically active upon expression in yeast. *Cm-ADH1* has strong preference for NADPH as a co-factor, whereas *Cm-ADH2* preferentially uses NADH. Both *Cm-ADH* proteins are much more active as reductases with  $K_m$ s 10–20 times lower for the conversion of aldehydes to alcohols than for the dehydrogenation of alcohols to aldehydes. They both show strong preference for aliphatic aldehydes but *Cm-ADH1* is capable of reducing branched aldehydes such as 3-methylbutyraldehyde, whereas *Cm-ADH2* cannot. Both *Cm-ADH* genes are expressed specifically in fruit and up-regulated during ripening. Gene expression as well as total ADH activity are strongly inhibited in antisense *ACC* oxidase melons and in melon fruit treated with the ethylene antagonist 1-methyl-

cyclopropene (1-MCP), indicating a positive regulation by ethylene. These data suggest that each of the *Cm-ADH* protein plays a specific role in the regulation of aroma biosynthesis in melon fruit.

**Keywords** Alcohol dehydrogenase/aldehyde reductase · Aroma volatiles · Ethylene · Fruit ripening · Medium- and short-chain ADH · Melon

## Introduction

Alcohol dehydrogenases (ADH, EC 1.1.1.1) catalyze the reversible conversion of aldehydes to the corresponding alcohols. They have been involved in the stress response of plants, mainly in anaerobiosis where they are responsible for the production of ethanol. ADHs have also been implicated in the response to a wide range of other stresses, elicitors and abscisic acid (Matton et al. 1990; De Bruxelles et al. 1996; Peters and Frenkel 2004). However, *ADH* genes are also expressed in plant tissues in a developmentally-regulated manner, particularly during fruit ripening (Van der Straeten et al. 1991; Speirs et al. 1998, 2002; Echeverria et al. 2004). In tomato fruit, one of the two *ADH* genes, *Le-ADH2*, participates in the formation of flavor volatiles during fruit ripening. Over-expression of *Le-ADH2* has led to improved flavor of the fruit by increasing the level of alcohols, particularly Z-3-hexenol (Speirs et al. 1998). In grapes, three *ADH* genes are expressed during fruit development. *Vv-ADH1* and *Vv-ADH3* transcripts accumulate transiently in young developing berry, while *Vv-ADH2* transcripts strongly increase at the onset of ripening named véraison (Tesnière and Verriès 2000). Fruit-specific dehydrogenases so far characterized belong to the medium-size zinc-containing class (Chase

Daniel Manríquez and Islam El-Sharkawy contributed equally to the work. Accession numbers for *Cm-ADH1* (ABC02081), and *Cm-ADH2* (ABC02082).

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1999). Partial cDNA clones putatively encoding short-chain ADHs have been reported in tomato (Picton et al. 1993) and in pear (Fonseca et al. 2004). In melon, the step of conversion of aldehydes to alcohols is controlled by ethylene and is strongly inhibited by the ethylene antagonist 1-MCP and in fruit in which ethylene production has been suppressed by an antisense *ACC* oxidase gene (Flores et al. 2002). In the present study, two fruit-specific *Cm-ADH* genes belonging to both the medium- and short-chain types have been isolated. After expression in yeast and purification, we have found that the two encoded enzymes preferentially work as aldehyde reductases and have specific substrates preferences.

## Materials and methods

### Plant material and postharvest treatments

Wild-type (WT) and *ACC* oxidase antisense (AS) Charentais Cantaloupe melons (*Cucumis melo* var. *Cantalupensis*, Naud cv. *Védrantais*) were used (Ayub et al. 1996; Guis et al. 1997). They were grown on a trellis in a greenhouse under standard cultural practices for fertilization and pesticide treatments. Freshly opened female flowers were tagged on the day of hand-pollination to identify fruit of known age. Melons were harvested after 32, 35, 37, 39 and 42 days after pollination (DAP) and ethylene production measured immediately after harvest. Fruit were selected for homogenous ethylene production. Stages of ripening of WT fruit, and equivalent age for AS fruit, corresponded to mature green (32 DAP), onset of ripening (35 DAP), early climacteric (37 DAP), full climacteric (39 DAP) and late climacteric (42 DAP). Antisense fruits, harvested at 35 DAP, were exposed to 50  $\mu\text{l l}^{-1}$  ethylene for 3 days. The ethylene inhibitor 1-MCP was applied at 35 DAP to WT fruit on the vine at 1  $\mu\text{l l}^{-1}$  in 3-l jars for 3 days before harvesting with periodical flushing with air and re-injection of the inhibitor. Vegetative tissues (leaves, stems, seeds and roots) and flowers were collected from plants grown in a greenhouse. All plant material was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### RNA isolation

Total RNA from fruit samples was extracted using the methods described by Boss et al. (1996). For leaf, stem, seed, root, and flower material, total RNA was extracted using RNeasy Plant Mini Kit following the manufacturer's recommendations (Qiagen, Valencia, CA, USA). All RNA extracts were treated with DNase I (Promega, Madison,

WI, USA) and cleaned up by phenol–chloroform extraction.

### Isolation and in silico analysis of *Cm-ADH* sequences

*Cm-ADH1* and *Cm-ADH2* have been isolated by PCR from a cDNA library of ripe melon. The SK primer (in Bluescript: 5'-CGCTCTAGAACTAGTGGATCCC-3') was combined with the degenerated primers, *Cm-ADH1* (F): 5'-TCTASTTTTACGCGWRTACACTGTT-3', *Cm-ADH1* (R): 5'-AAGTCCAAYAGMTCCAAGTCCAAA-3', *Cm-ADH2* (F): 5'-CAGCCTTCAWSAGAAACCATG-3', and *Cm-ADH2* (R): 5'-AAGAGACTGTGCTCCATCAAC-3' designed from a conserved region among plants alcohol dehydrogenase. The isolated fragments were cloned using Qiagen PCR Cloning<sub>plus</sub> Kit (Qiagen, Valencia, CA, USA), sequenced and compared with database sequences using the BLAST program (Altschul et al. 1997). Extension of the partial cDNA clones was carried out using the 3'- and 5'-RACE kit (Invitrogen, Paisely, UK). First strand cDNA synthesis was carried out using 10  $\mu\text{g}$  of total DNase-treated RNA in a 50  $\mu\text{l}$  aliquot followed by PCR with specific *Cm-ADH* primers using 1  $\mu\text{l}$  of cDNA. A high fidelity PCR system (BMB Indianapolis, IN, USA) was used with the following PCR parameters: 3 min template denaturation at  $95^{\circ}\text{C}$  for one cycle, followed by 5 cycles at  $95^{\circ}\text{C}$  (30 s),  $58^{\circ}\text{C}$  (1 min), and  $72^{\circ}\text{C}$  (1 min 30 s), then 25 cycles at  $95^{\circ}\text{C}$  (30 s),  $58^{\circ}\text{C}$  (1 min), and  $72^{\circ}\text{C}$  (2 min) with a final 10 min extension step at  $72^{\circ}\text{C}$  to isolate the full length *Cm-ADH* sequences.

Alignments of the predicted protein sequences were performed with ClustalX (Thompson et al. 1997) and GENEDOC (Nicholas and Nicholas 1997). Phylogenetic analysis of ADH sequences was performed using the neighbor-joining method (Saitou and Nei 1987) of PHYLIP package (Felsenstein 1992). Bootstrapping was performed by resampling from the data 1000 times.

### Real time quantitative RT-PCR

DNase-treated RNA (4  $\mu\text{g}$ ) was reverse transcribed in a total volume of 40- $\mu\text{l}$  using Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed using 100 ng of cDNA in a 20- $\mu\text{l}$  reaction volume using SYBR GREEN PCR Master Mix (PE-Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence-detection system. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers (Table 1). For all the genes studied here, optimal primer concentration was 300 nM. RT-PCR conditions were as follow:  $50^{\circ}\text{C}$  for 2 min, followed by  $95^{\circ}\text{C}$  for 10 min,

then 40 cycles of 95°C for 15 s and 60°C for 1 min. All RT-PCR experiments were run in triplicate with different cDNAs synthesized from three biological replicates. Samples were run in triplicate on each 96-well plate and were repeated at least two plates for each experiment. For each sample, a Ct (threshold sample) value was calculated from the amplification curves by selecting the optimal  $\Delta R_n$  (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the  $\beta$ -actin as an internal standard. A cDNA clone was isolated with homology to a  $\beta$ -actin sequence (AY859055). It was checked by Northern analysis (data not shown) that the  $\beta$ -actin mRNA level was similar in all treatments. To demonstrate that the efficiencies of the different genes primers were approximately equal, the absolute value of the slope of log input amount versus  $\Delta C_t$  was calculated for both the *Cm-ADH* and  $\beta$ -actin genes and was determined to be  $<0.1$ . To determine relative fold differences for each sample in each experiment, the Ct value for both *Cm-ADH* genes was normalized to the Ct value for  $\beta$ -actin and was calculated relative to a calibrator (seeds for *Cm-ADH1*, wild-type melon fruit treated 3 days with 1-MCP for *Cm-ADH2*) using the formula  $2^{-\Delta\Delta C_t}$ .

#### Expression of *Cm-ADH*

The two *Cm-ADH* cDNAs were cloned in the pYES2.1 TOPO-TA vector for regulated protein expression in yeast following the instructions provided by the manufacturer (Invitrogen, Paisely, UK). Auto-ligated construct was used as negative control. All the constructs were transformed into the *Saccharomyces cerevisiae* cell line INVSc1. The strain harboring the correct constructions were grown in selective medium (SC-U) with 2% galactose as inducer of the recombinant protein expression, at 30°C and 250 rpm, according to manufacturer's recommendations, until the  $OD_{600}$  of the culture reached  $\sim 4$  U.

**Table 1** Real time quantitative PCR primers

Name	Oligonucleotide sequence
Cm-actin-344 (F)	5'-GTGATGGTGTGAGTCACACTGTTC-3'
Cm-actin-426 (R)	5'-ACGACCAGCAAGGTCCAAAC-3'
Cm-ADH1-518 (F)	5'-GTGTTCTTAGCTGCGGCATTT-3'
Cm-ADH1-588 (R)	5'-TTGACCCTTTTtaggCTTTGCA-3'
Cm-ADH2-145 (F)	5'-GCGGAATCGTTAAAGGGTGTACT-3'
Cm-ADH2-216 (R)	5'-AGCCGCCTCTCTCTTCTTC-3'

#### Purification of recombinant ADH and electrophoresis methods

Cells were collected by centrifugation ( $1800 \times g$ , 10 min at room temperature) from 150 ml of yeast cultures induced with galactose and resuspended in buffer A (50 mM sodium phosphate pH 7.5, 10% v/v glycerol, 0.3 M NaCl) containing 2 mM  $\beta$ -mercaptoethanol. The cells were mechanically ground in liquid nitrogen for 2 min and stored at  $-80^\circ\text{C}$  until needed. To extract the ADH enzyme, the powder was thawed and centrifuged at  $45,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The crude extract obtained was concentrated by addition of  $(\text{NH}_4)_2\text{SO}_4$  to 80% saturation. The suspension centrifuged at  $45,000 \times g$  for 20 min at  $4^\circ\text{C}$  and the pellet suspended in 1 ml of tampon A and desalted in Sephadex G-25 columns equilibrated with buffer A (Amersham Biosciences, Chalfont Buckinghamshire, UK). The recombinant protein was purified by a metal affinity resin designed to purify polyhistidine-tagged proteins (BD Talon metal affinity resin, BD Biosciences), according to the manufacturer's protocol. Briefly, the enzyme was fixed to the resin in the presence of buffer A, and after removing the unbound proteins with several washes with the same buffer the recombinant protein was eluted with buffer A containing 150 mM imidazole. The extract was desalted in Sephadex G-25 equilibrated with buffer A. A second purification was performed with the metal affinity resin. The highly purified protein was quantified according to Bradford (1976) using bovine serum albumin as standard. Protein purity was verified by separation on SDS-PAGE (12% acrylamide gel) and staining with silver nitrate (Amersham Biosciences).

#### ADH enzyme activity and kinetic parameters with recombinant proteins

Reductase and dehydrogenase activities of ADH were evaluated by spectrophotometry according to Molina et al. (1987). Briefly, reductase activity was assayed in 1 ml total volume containing 100  $\mu\text{l}$  of purified protein (1–2  $\mu\text{g}$ ), 5 mM aldehyde, 0.25 mM NADH or NADPH and adjusted to final volume with sodium phosphate 50 mM buffer (pH 5.8). Dehydrogenase activity was measured in the presence of 5 mM ethanol, 0.25 mM NAD or NADP and adjusted to final volume with glycine-NaOH buffer (pH 9.4). Different concentrations of NADH/NAD or NADPH/NADP (from 0.015 mM to 1 mM) or acetaldehyde/ethanol (from 0.15 mM to 10 mM) were used for kinetic parameters determinations.

## ADH enzyme activity assay of melon fruit crude protein

One gram of mesocarp tissue was ground mechanically in the presence of 1 ml of extraction buffer (250 mM Tris/HCl, pH 7.5, 0.1% Triton X-100 and 2 mM  $\beta$ -mercaptoethanol) in liquid nitrogen during 2 min and the protein crude extract was stored at  $-80^{\circ}\text{C}$  until needed. The protein extract was thawed in ice and centrifuged at  $45,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The supernatant phase was desalted using Sephadex G-25 columns (Amersham Biosciences) equilibrated with sodium phosphate 50 mM (pH 5.8). Total proteins were quantified according to Bradford (1976). ADH activity was measured as described above.

## Results and discussion

### Sequence analysis of *Cm-ADH1* and *Cm-ADH2* and predicted proteins

*Cm-ADH1* and *Cm-ADH2* encode proteins of 379 and 266 residues, with the predicted molecular weight of 41.0 kDa and 29.0 kDa, and isoelectric points of 6.3 and 8.2, respectively. The *Cm-ADH1* protein belongs to the highly conserved zinc-medium-chain ADHs sub-family (Chase 1999; Table 2 and Fig. 1). Many genes encoding proteins of this sub-family have been characterized in plants. In tomato, *Le-ADH3a* and *Le-ADH3b* are expressed in anthers (Ingersoll et al. 1994) and *Le-ADH2* in ripening fruit (Longhurst et al. 1994) where it plays a role in the biosynthesis of aroma (Speirs et al. 1998). In grape, *Vv-ADH2*, is a ripening regulated gene (Sarni-Manchado et al. 1997; Tesnière and Verriès 2000). The alignment of *Cm-ADH1* with sequences of different plant zinc-medium-chain ADH proteins shows the presence of a large number of conserved domains (Fig. 1), that are typical of this sub-family (Chase 1999). The identity at the amino acid level between *Cm-ADH1* and other plants zinc-medium-chain ADHs sub-family is very high and ranges between 81 and 85% (Table 2). Many very well conserved amino acids that have been implicated in the fixation of zinc are present in *Cm-ADH1*: Cys, His and Cys at the 50, 72 and 181 positions (Fig. 1) and four Cys at positions 102, 105, 108 and 116 (Eklund et al. 1976; Yokoyama and Harry 1993). The Asp in position 230, corresponding to *Cm-ADH1* sequence has been described as implicated in the preference of NAD as co-factor in the dehydrogenase reaction (Eklund et al. 1976; Fan et al. 1991).

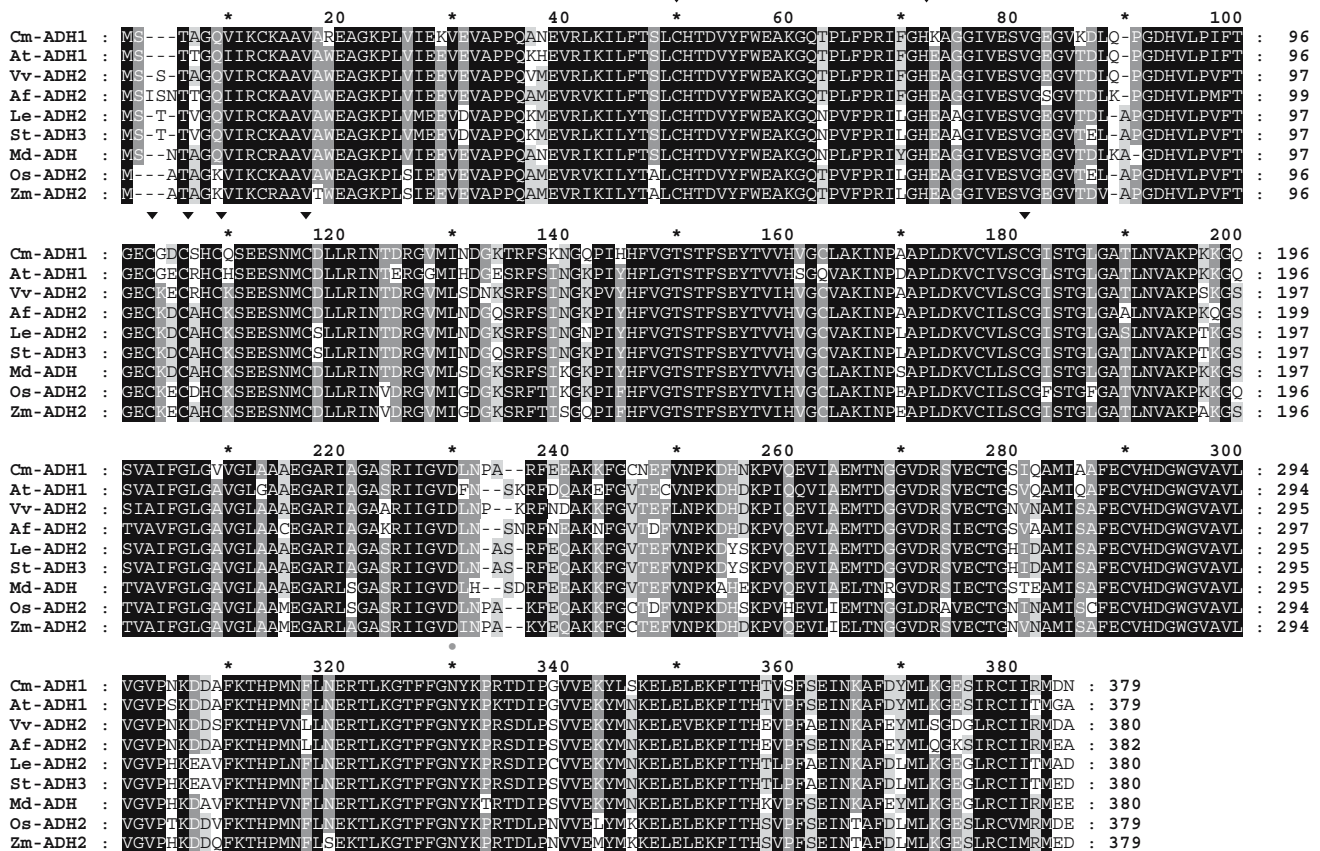
*Cm-ADH2* protein is a member of the short-chain ADH sub-family. Contrary to medium-chain ADHs, the percentage of identity at the amino acid level is highly variable ranging from 21 to 67% (Table 2). The highest

**Table 2** Amino acid sequence comparison between the peptides full length *Cucumis melo* (*Cm-ADH1*), *Malus domestica* (*Md-ADH*), *Vitis vinifera* (*Vv-ADH2*), *Lycopersicon esculentum* (*Le-ADH2*), *Arabidopsis thaliana* (*At-ADH1*), *Solanum tuberosum* (*St-ADH3*), *Oryza sativa* (*Os-ADH2*), *Zea mays* (*Zm-ADH2*), and *Ametastegia formosa* (*Af-ADH2*) zinc-medium-chain and *Cucumis melo* (*Cm-ADH2*), *Lycopersicon esculentum* (*Le-ADHs*), *Solanum tuberosum* (*St-ADHs*), *Arabidopsis thaliana* (*At-ADH*), *Citrus sinensis* (*Cs-ADH*), *Ametastegia formosa* (*Af-ADH*), *Oryza sativa* (*Os-ADH*), *Zea mays* (*Zm-ADHs*) and *Datura stramonium* (*Ds-TRR2*) short chain alcohol dehydrogenases proteins

Protein		Amino acid identity (%)	
Name	Size	<i>Cm-ADH1</i>	<i>Cm-ADH2</i>
<i>Cm-ADH1</i>	379	–	15
<i>At-ADH1</i>	379	85	14
<i>Le-ADH2</i>	380	83	12
<i>Md-ADH</i>	380	83	14
<i>Vv-ADH2</i>	380	83	14
<i>St-ADH3</i>	380	83	11
<i>Os-ADH2</i>	379	81	12
<i>Zm-ADH2</i>	379	81	11
<i>Af-ADH2</i>	383	84	13
<i>Cm-ADH2</i>	266	15	–
<i>Le-ADHs</i>	266	16	64
<i>St-ADHs</i>	266	16	64
<i>At-ADH</i>	266	12	53
<i>Cs-ADH</i>	266	15	67
<i>Af-ADH</i>	266	14	59
<i>Os-ADH</i>	277	13	50
<i>Zm-ADHs</i>	253	10	24
<i>Ds-TRR2</i>	260	12	21

For accession numbers see legends of Figs. 1 and 2

percentage of identity to *Cm-ADH2* is for *Cs-ADH* of *Citrus sinensis*. Strikingly, genes of this sub-family have a number of conserved amino acids (around 40) well distributed all over the sequence, but very few conserved domains (Fig. 2). Persson et al. (1991) had highlighted few conserved elements of unknown function among plant short-chain dehydrogenases that are underlined in Fig. 2: (I) ALVTGG(S/T)RGIG, located at the N-terminal region, (II) ILVNNAG, (III) YxaxK and (IV) IRVNxVaP. However, alignment of Fig. 2 shows that none of these domains are well conserved except domains I and IV that show conservation of six amino acids out of 11 and five amino acids out of eight, respectively. Similarly, the three glycine residues at the N-terminal region that have been described by Jörnvall et al. (1995) as implicated in binding NAD in *Drosophila melanogaster* ADH are all present in the *Ds-TRR2* protein at positions 27, 31 and 35 (Fig. 2). However, only Gly 31 is conserved in all other plant ADH sequences shown in Fig. 2. The diversity in amino acid sequence of the short-chain ADHs sub-family can be related to a wide range of biological functions of such proteins that can use a wide range of substrates (Jörnvall et al. 1995) such as tropinone (Nakajima et al. 1993) and 3-oxoacyl-acyl carrier protein (Klein et al. 1992) in higher plants.



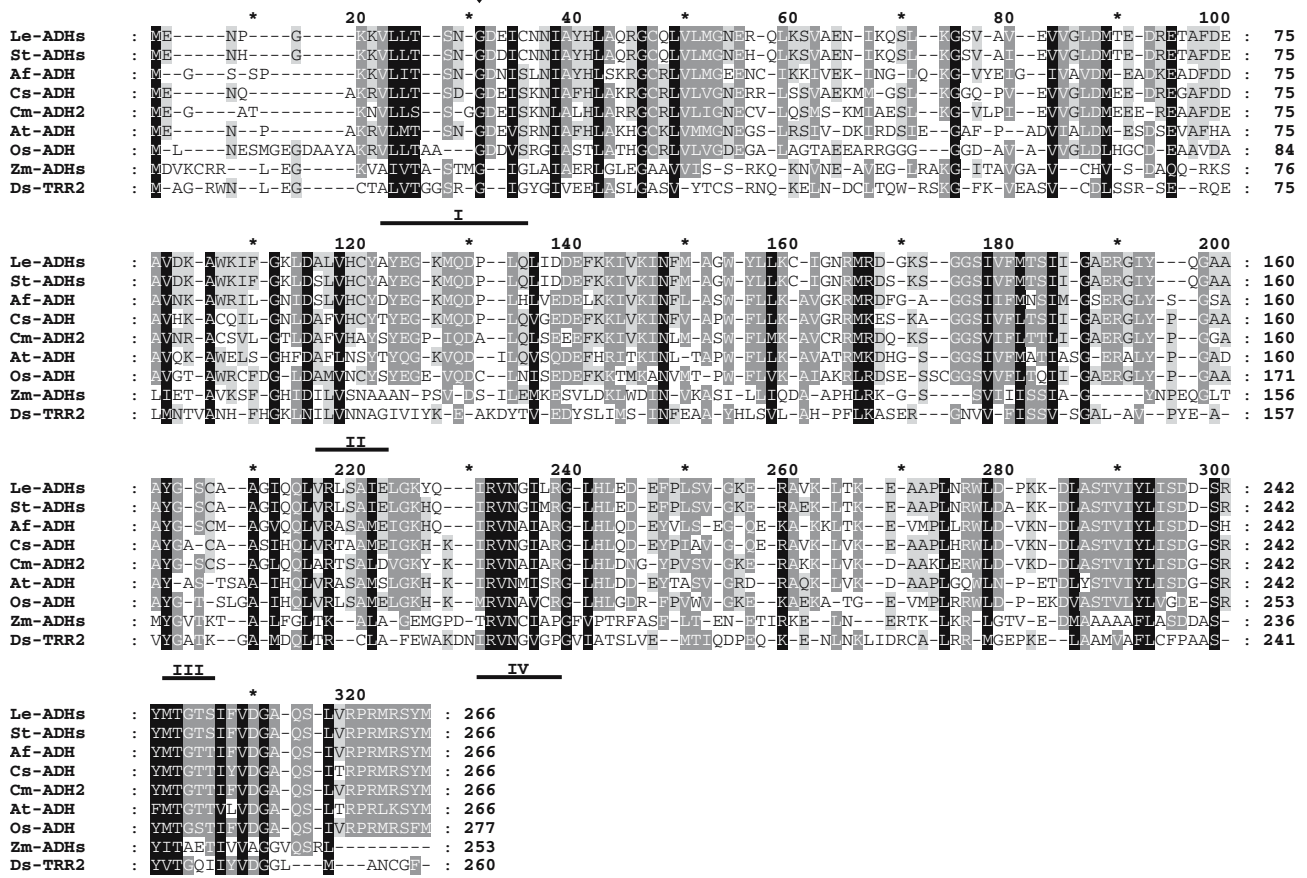
**Fig. 1** Amino acid sequence alignment of *Cucumis melo* Cm-ADH1 (ABC02081<sup>a</sup>) with closely related sequences *Malus domestica* Md-ADH (CAA88271<sup>a</sup>), *Vitis vinifera* Vv-ADH2 (AAL55726<sup>a</sup>), *Lycopersicon esculentum* Le-ADH2 (CAA54450<sup>a</sup>), *Arabidopsis thaliana* At-ADH1 (AAK73970<sup>a</sup>), *Solanum tuberosum* St-ADH3 (AAA33808<sup>a</sup>), *Oryza sativa* Os-ADH2 (AAF34412<sup>a</sup>), *Zea mays* Zm-ADH2 (CAA26001<sup>a</sup>) and *Ametastegia formosa* Af-ADH2 (TC9383<sup>b</sup>) using ClustalX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in seven out of nine of the

sequences and clear grey shading indicates similar residues in five out of nine of the sequences. The black arrows represent the amino acids that have been implicated in the fixation of zinc (Eklund et al. 1976; Yokoyama and Harry 1993). The grey circle represents the Asp that has been described as implicated in the preference of NAD as co-factor in the dehydrogenase reaction (Eklund et al. 1976; Fan et al. 1991). The letters following the accession numbers in the legend of the figure indicate the source database: (a) GenBank and (b) TIGR

In order to determine the phylogenetic position of the melon *ADH* genes isolated in this study, a phylogenetic tree was constructed by employing a data set including some of the previously published medium- (Fig. 3A) and short-chain ADHs (Fig. 3B) from both monocots and dicots. The resulting ADH tree roughly consisted in two monophyletic groups ("Clade 1" and "Clade 2") in both A and B sub-families (Fig. 3). Clade 1A and 1B contain *ADH* genes from dicots only including melon ADHs, while Clade 2A and 2B contain *ADH* genes from both dicots and monocots. In medium-chain ADHs (Fig. 3A), proteins show a very low divergence in accordance with the sequence alignments comparisons of Fig. 1. The closest neighbor for Cm-ADH1 is *Arabidopsis thaliana* ADH1. In contrast, short-chain ADHs show a high level of divergence (Fig. 3B).

#### Expression of *Cm-ADH1* and *Cm-ADH2* genes

Real time PCR analysis indicated that the two *Cm-ADH* genes studied here are specifically expressed in fruit. Vegetative tissues (leaves, stems, seeds, roots) and flowers exhibited no or very low expression (Fig. 4) even when treated with ethylene. The pattern of changes in transcript levels during fruit ripening was similar for the two *Cm-ADH* genes with transient and sharp increase at 39 days when ethylene production was maximum (Fig. 4). Both genes exhibited very low expression before and after the peak. In AS melon fruit where ethylene was strongly suppressed by antisense *ACO* mRNA or in WT fruit treated with the ethylene antagonist 1-MCP (for 3 days before harvest at 35 DAP) the transcript levels of both genes were almost undetectable (Fig. 4). However, exposure of AS



**Fig. 2** Amino acid sequence alignment of *Cucumis melo* Cm-ADH2 (ABC02082<sup>a</sup>) with closely related full length sequences of *Arabidopsis thaliana* At-ADH (AAM65725<sup>a</sup>), *Citrus sinensis* Cs-ADH (CX049468<sup>a</sup>), *Ametastegia formosa* Af-ADH (TC19306<sup>b</sup>), *Oryza sativa* Os-ADH (AAO37953<sup>a</sup>), *Datura stramonium* Ds-TRR2 (AAA33282<sup>a</sup>) and ESTs from *Zea mays* Zm-ADHs (AY105662<sup>a</sup>), *Lycopersicon esculentum* Le-ADHs (U213436<sup>c</sup>) and *Solanum tuberosum* St-ADHs (U271654<sup>c</sup>), using ClustalX program. Conserved

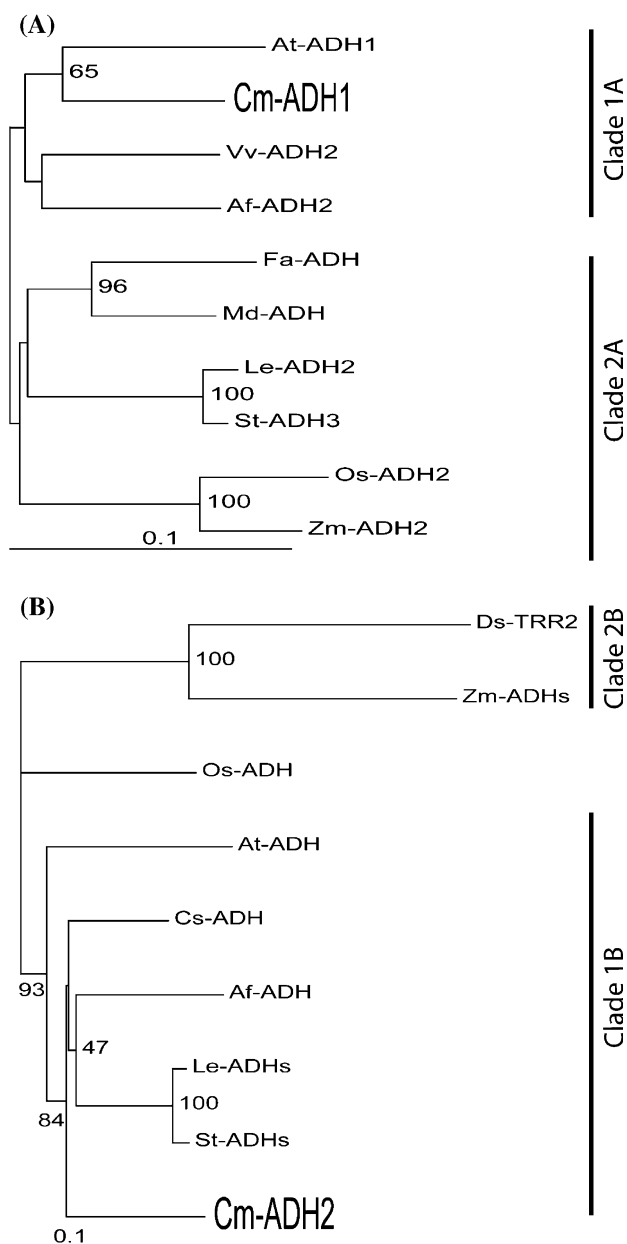
residues are shaded in black. Dark grey shading indicates similar residues in seven out of nine of the sequences and clear grey shading indicates similar residues in five out of nine of the sequences. The underlines represent the conserved amino acids in short-chain ADHs (Persson et al. 1991). The black arrow represents Gly 31 that has been described as implicated in binding NAD (Jörnvall et al. 1995). The letters following the accession numbers in the legend of the figure indicate the source database: <sup>(a)</sup> GenBank, <sup>(b)</sup> TIGR and <sup>(c)</sup> SGN

fruit to ethylene resulted in stimulation of gene expression although the levels of mRNA never reached the values of WT fruit at the peak (Fig. 4).

Our data indicate that ethylene is a major regulator of *Cm-ADH1* and *Cm-ADH2* transcript levels. Partial involvement of ethylene in hypoxic induction of *Arabidopsis thaliana* ADH1 in seedlings has been reported (Peng et al. 2001). In tomato, Van der Straeten et al. (1991) showed that the accumulation of tomato ADH mRNA was related to fruit ripening, with 50 times higher mRNA accumulation in ripe as compared to green fruit. Expression of *Le-ADH2* is strongly induced during fruit ripening (Chen and Chase 1993). However, the induction of expression during ripening is not related to hypoxic conditions in the fruit. Exogenous ethylene stimulated expression, but this appeared as to be indirect because it requires more than 24 h after ethylene treatment (Chen and Chase 1993).

Ethylene was also concluded not to be involved in hypoxic induction of ADH in maize and rice (Morrell and Greenway 1989). In tomato, a partial cDNA clone showing homology to short-chain ADHs is expressed during ripening and expression was greatly reduced in the *rin* mutant (Picton et al. 1993). In pear, the expression of an EST putatively encoding a short-chain ADH increased during ripening in parallel to the expression of the *ACO* gene encoding for a key enzyme of ethylene biosynthesis (Fonseca et al. 2004). In grape, a non-climacteric fruit, ethylene stimulates the expression of *Vv-ADH2* a gene essentially expressed at véraison, the onset of ripening (Tesnière and Verriès 2000). Application of 1-MCP an antagonist of ethylene in berries results in the reduction of the expression of *Vv-ADH2* mRNA (Tesnière et al. 2004). In addition, cell cultures of *Vitis vinifera* treated with 2-chloro ethyl phosphonic acid (CEPA), an ethylene





**Fig. 3** Neighbor-joining bootstrap phylogenetic tree of the two Cm-ADH with ADHs sequences belonging to the medium-chain zinc-binding type (At-ADH1, Vv-ADH2, Af-ADH2, Le-ADH2, St-ADH3, Fa-ADH, Md-ADH, Os-ADH2 and Zm-ADH2) and short-chain type of ADHs (At-ADH, Cs-ADH, Af-ADH, Os-ADH, Ds-TRR2, Zm-ADHs, Le-ADHs, and St-ADHs). The percent bootstrap support for 1000 replicates is shown below each node. Amino acid sequences were aligned using Clustal W. *Fragaria × ananassa* ADH (Fa-ADH) accession number is CAA33613 (GenBank)

regenerating compound, showed a stimulation of the expression of *Vv-ADH2* mRNA as compared to control cells (Tesnière et al. 2004). Furthermore, the promoter of the *Vv-ADH2* gene contains putative ethylene responsive element (ERE) motifs that are probably involved in responsiveness to ethylene treatment (Verriès et al. 2004).

#### Alcohol dehydrogenase activity of Cm-ADH1 and Cm-ADH2 recombinant proteins towards various substrates in vitro

Because endogenous ADH activity was present in yeast, the biochemical characterization of the recombinant Cm-ADH proteins has been performed using a highly purified protein after two successive purifications steps by affinity column chromatography. As aldehyde reductases, the two recombinant enzymes showed a preference for aliphatic aldehydes, mainly acetaldehyde (Table 3). Using acetaldehyde as substrate, Cm-ADH1 shows 4.5 times more activity in the presence of NADPH than in the presence of NADH (Table 3). This was similar to the activity of Vv-ADH2 and Vv-ADH3 that have also strong preference for NADPH (Tesnière et al. 2004). On the contrary, Cm-ADH2 had strong preference for NADH, with an activity of reduction of acetaldehyde that was about six times higher than with NADPH. Beside aliphatic aldehydes, Cm-ADH1 utilized also branched aldehydes as 2 and 3-methylbutyraldehyde, 2-methylpropanaldehyde and aromatic aldehydes such as benzaldehyde, but the activity for this type of substrates is approximately 4–70 times lower than with acetaldehyde both in the presence of NADPH or NADH (Table 3). Cm-ADH2 used almost exclusively aliphatic aldehydes. Activity with branched or aromatic aldehydes was between 20 and 40 times lower in the presence of NADH as a co-factor and not detectable or at trace levels in the presence of NADPH as a co-factor (Table 3).

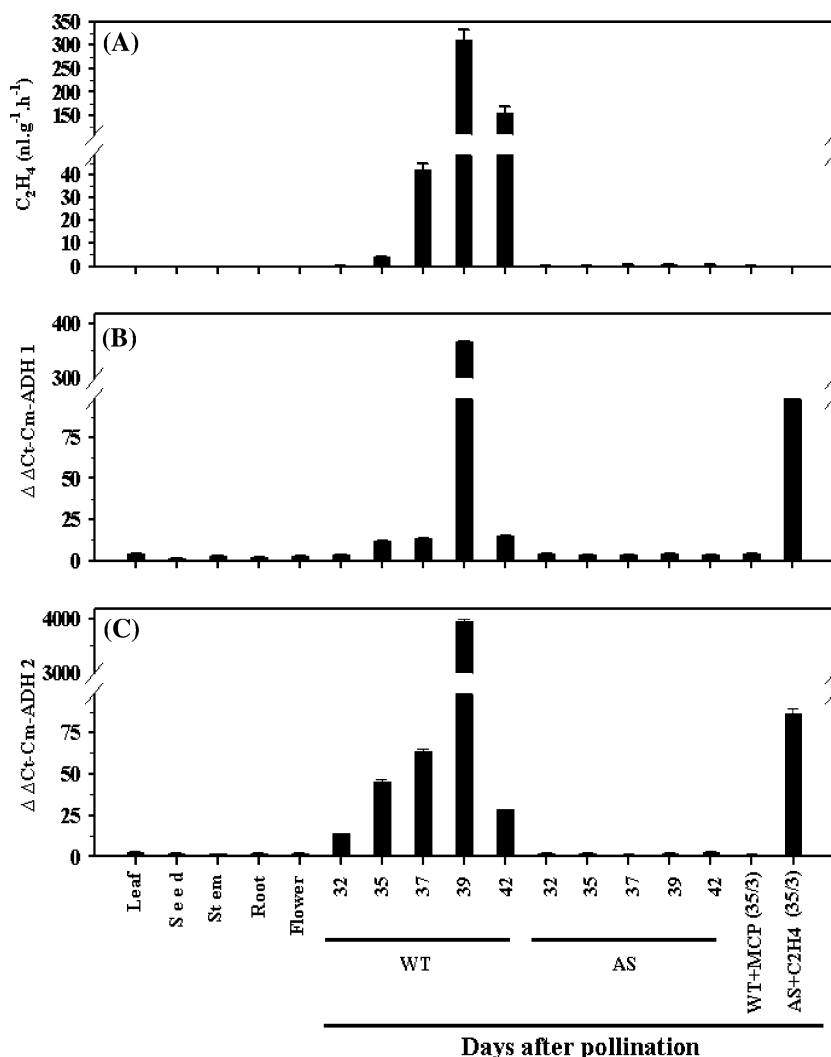
The activity of Cm-ADH1 and Cm-ADH2 towards the oxidation of ethanol was strictly NAD dependant with activities of 8865  $\mu\text{mol mg prot}^{-1} \text{ min}^{-1}$  and 441  $\mu\text{mol mg prot}^{-1} \text{ min}^{-1}$ , respectively. No activity could be detected in the presence of NADP. These results are in agreement with the presence, in Cm-ADH1, of an Asp residue in position 230 (Fig. 1) which is implicated in the fixation of NAD (Eklund et al. 1976; Fan et al. 1991). Information on the NAD binding site in short-chain ADHs is lacking. An Asp residue at the position 130 present in all ADH sequences aligned in Fig. 2, except Ds-TRR2, could correspond to the Asp residue involved in NAD binding.

#### Kinetic parameters of recombinant Cm-ADHs proteins

The kinetic parameters were determined using the preferential substrate and co-factor for the two enzymes operating as either reductases (acetaldehyde/NADPH for Cm-ADH1 or acetaldehyde/NADH for Cm-ADH2) or oxidases (ethanol/NAD for both enzymes). Table 4 shows that Cm-ADH1 had a  $K_m$  for acetaldehyde which was 10 times lower than the  $K_m$  for ethanol (0.25 mM as compared with 2.5 mM). The respective  $V_{\text{max}}$  were of 2500  $\mu\text{mol mg prot}^{-1} \text{ min}^{-1}$  and 5000  $\mu\text{mol mg prot}^{-1} \text{ min}^{-1}$ . The  $K_m$  for



**Fig. 4** Ethylene production and *Cm-ADH* genes expression during fruit ripening and various organs of melon. (A) ethylene production, (B) and (C) levels of *Cm-ADHs* transcripts assessed by real time quantitative PCR. The experiments were carried out in triplicate. The X-axis represents various organs of melon (leaf, seed, stem, root) and flower; wild-type (WT) and antisense (AS) melon fruit at different days after pollination; WT (35 DAP) fruit exposed to 1-MCP (1  $\mu\text{l l}^{-1}$ ) for 3 days, and AS fruit treated with ethylene (50  $\mu\text{l l}^{-1}$ ) for 3 days.  $\Delta\Delta\text{Ct}$  in the Y-axis of each figure refers to the fold difference in *Cm-ADH1* and *Cm-ADH2* expression relative to seeds and to wild-type melon fruit treated 3 days with 1-MCP, respectively


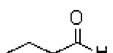

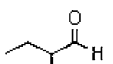
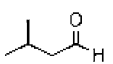

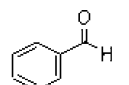


NADPH was about 3.5 times lower than for NAD (0.07 mM and 0.25 mM, respectively). The *Cm-ADH2* protein had a  $K_m$  for acetaldehyde which was about 18 times lower than for ethanol (0.24 mM and 4.52 mM). The corresponding  $V_{\max}$  were 588.2  $\mu\text{mol mg prot}^{-1} \text{min}^{-1}$  and 370.4  $\mu\text{mol mg prot}^{-1} \text{min}^{-1}$ , respectively (Table 4). The  $K_m$  for NADH was lower (0.02 mM) than for NAD (0.37 mM). When compared with data obtained for other ADHs, it appeared that the  $K_m$  values for acetaldehyde of *Cm-ADH1* and *Cm-ADH2* (0.25 mM and 0.24 mM) were similar with the  $K_m$  for acetaldehyde of the grape *Vv-ADH2* (0.45 mM) reported by Tesnière et al. (2004). However, the  $V_{\max}$  for acetaldehyde of *Vv-ADH2* (300  $\mu\text{mol mg prot}^{-1} \text{min}^{-1}$ ) was closer to *Cm-ADH2* (588.2  $\mu\text{mol mg prot}^{-1} \text{min}^{-1}$ ) than to *Cm-ADH1* (2500  $\mu\text{mol mg prot}^{-1} \text{min}^{-1}$ ). Low  $K_m$  values for the co-factors have also been observed by Tesnière et al. (2004) for the recombinant proteins of grape, *Vv-ADH2* and *Vv-ADH3*, (0.02 mM and 0.04 mM, respectively). In addition,

Salas and Sanchez (1998) have also described a very low  $K_m$  for NADPH of an ADH purified from olive fruit.

The lower  $K_m$  for the acetaldehyde substrate of the two melon ADHs as compared to ethanol suggest that these two enzymes operate preferentially as reductases of aldehydes into alcohols rather than oxidases of alcohols into aldehydes. This observation is confirmed by the catalytic efficiency (corresponding to the  $k_{\text{cat}}/K_m$  ratio) which is 5-fold (*Cm-ADH1*) to 30-fold (*Cm-ADH2*) higher for acetaldehyde as compared to ethanol (Table 4). Although these data have been obtained with recombinant proteins, it can be assumed, however, that the preferential production of alcohols also occurs in the fruit in vivo. Alcohols such as ethanol, butanol, hexanol and 3-methylbutanol are indeed substrates for alcohol acyl-transferases (AAT) implicated in the aroma biosynthesis in melon fruit (Yahyaoui et al. 2002; El-Sharkawy et al. 2005). Therefore, the mode of action of the two ADHs of melon supports a role for the two enzymes in the biosynthesis of aroma in melon fruit.

**Table 3** Activities of purified recombinant Cm-ADH1 and Cm-ADH2 proteins towards different aldehydes (5 mM) and NADH or NADPH (0.25 mM)

Aldehydes	Cm-ADH1		Cm-ADH2	
	NADH	NADPH	NADH	NADPH
 Acetaldehyde	474 ± 96	2216 ± 228	487 ± 38	80 ± 6
 Butyraldehyde	316 ± 41	980 ± 33	273 ± 41	60 ± 8
 Capronaldehyde	285 ± 56	978 ± 63	241 ± 36	57 ± 1
 2-methylbutyraldehyde	24 ± 13	41 ± 5	7 ± 1	ND
 3-methylbutyraldehyde	129 ± 30	483 ± 32	14 ± 3	TR
 2-methylpropionaldehyde	76 ± 6	88 ± 11	16 ± 2	TR
 Benzaldehyde	15 ± 2	30 ± 2	6 ± 2	ND

Activity is expressed in  $\mu\text{mol mg prot}^{-1} \text{ min}^{-1}$  as the mean  $\pm$  SE of three replications

However, none of the melon recombinant ADHs were capable of converting different ketones such as acetone, 3-pentanone, 1-pentene-3-one,  $\beta$ -ionone and 6-methyl-5-heptene-2-one into the corresponding alcohols (data not shown) while some of these compounds are present in aroma volatiles of melon (Aubert and Bourger 2004). Since some short-chain ADHs characterized in plants are capable of reducing ketones (Chase 1999), it can be concluded that

other ADH proteins may, therefore, be present and expressed in melon for the synthesis of such compounds.

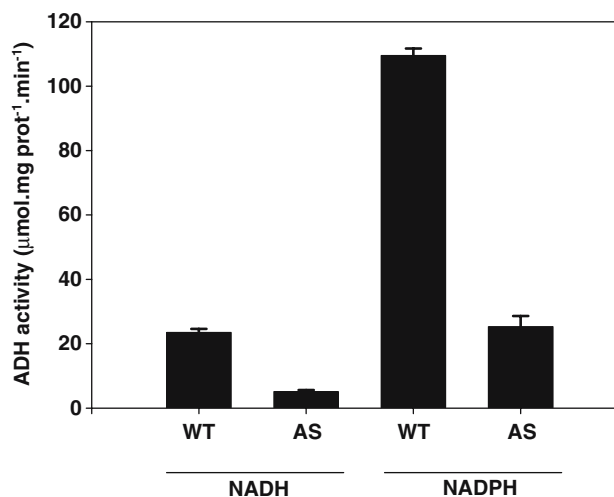
#### ADH activity in fruit

ADH activity was measured in fruit extracts of untransformed melon (WT) and antisense *ACC* oxidase melon (AS) with acetaldehyde as a substrate in the presence of

**Table 4** Kinetic parameters of purified recombinant Cm-ADH1 and Cm-ADH2 proteins

		$V_{\max}$ ( $\mu\text{mol mg prot}^{-1} \text{ min}^{-1}$ )	$K_m$ (mM)
Cm-ADH1			
Acetaldehyde	NADPH (0.25 mM)	2500.0	0.25
NADPH	Acetaldehyde (1.25 mM)	3333.3	0.07
Ethanol	NAD (0.25 mM)	5000	2.5
NAD	Ethanol (20 mM)	12,500	0.25
Cm-ADH2			
Acetaldehyde	NADH (0.125 mM)	588.2	0.24
NADH	Acetaldehyde (1.25 mM)	555.6	0.02
Ethanol	NAD (0.125 mM)	370.4	4.52
NAD	Ethanol (20 mM)	526.3	0.37

**Fig. 5** ADH activity in WT and AS fruit (42 DAP). Enzyme activity was measured with 5 mM acetaldehyde as a substrate in the presence of 0.25 mM NADH or NADPH. Activity is expressed in  $\mu\text{mol mg prot}^{-1} \text{min}^{-1}$  as the mean  $\pm$  SE of three replications



NADH and NADPH (Fig. 5). ADH activity was always higher in WT than in AS fruit with both co-factors although activity in the presence of NADPH was around four times higher. In considering that *Cm-ADH1* has higher activity in the presence of NADPH, it is likely that this enzyme accounts for most of the ADH activity in fruit. However, *Cm-ADH1* gene expression is much lower than *Cm-ADH2* which has preference for NADH. This suggests that other *Cm-ADH* genes may be expressed in ripening melon fruit. Nevertheless, Fig. 5 clearly shows that the suppression of ethylene production in AS fruit results in a strong reduction of ADH activity. This is in agreement with gene expression studies of Fig. 4 showing that both *Cm-ADH1* and *Cm-ADH2* gene expression were almost totally inhibited in ethylene suppressed AS fruit. Previous studies on in vivo bioconversion assays on fruit disks had also shown that ADH activity of fruit was strongly regulated by ethylene (Flores et al. 2002). Residual activity in AS melon fruit suggests the presence of ethylene-independent ADH(s). In tomato, there is a strong increase in ADH activity during ripening (Speirs et al. 2002) which is stimulated by ethylene treatment and correlated with the level of *Le-ADH2* gene expression (Chen and Chase 1993). In non-climacteric fruit such as grape and strawberry where ethylene is not supposed to play an essential role, ADH activity also increases during ripening (Tesnière and Verriès 2000; Moyano et al. 2004). However, when the grape berries were treated with the ethylene action inhibitor 1-MCP, ADH activity was significantly reduced (Tesnière et al. 2004). Altogether these data indicate that in both climacteric and non-climacteric fruit, some ADHs are regulated by ethylene, others are not.

In conclusion, this paper shows that two highly divergent *ADH* genes are specifically expressed in ripening melon and are up-regulated by ethylene. They encode proteins that operate preferentially as aldehyde reductases.

However, the two proteins have differential substrate and co-factors preference indicating that they probably play specific roles in providing substrates to the downstream alcohol acyl-transferases (Yahyaoui et al. 2002; El-Sharkawy et al. 2005).

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