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A B S T R A C T

A tomato short-chain dehydrogenase-reductase (*SlscADH1*) is preferentially expressed in fruit with a maximum expression at the breaker stage while expression in roots, stems, leaves and flowers is very weak. It represents a potential candidate for the formation of aroma volatiles by interconverting alcohols and aldehydes. The SlscADH1 recombinant protein produced in *Escherichia coli* exhibited dehydrogenase-reductase activity towards several volatile compounds present in tomato flavour with a strong preference for the NAD/NADH co-factors. The strongest activity was observed for the reduction of hexanal (K_m = 0.175 mM) and phenylacetaldehyde (K_m = 0.375 mM) in the presence of NADH. The oxidation process of hexanol and 1-phenylethanol was much less efficient (K_ms of 2.9 and 23.0 mM, respectively), indicating that the enzyme preferentially acts as a reductase. However activity was observed only for hexanal, phenylacetaldehyde, (*E*)-2-hexenal and acetaldehyde and the corresponding alcohols. No activity could be detected for other aroma volatiles important for tomato flavour, such as methyl-butanol/methyl-butanal, 5-methyl-6-hepten-2-one/5-methyl-6-hepten-2-ol, citronellal/citronellol, neral/nerol, geraniol. In order to assess the function of the *SlscADH1* gene, transgenic plants have been generated using the technique of RNA interference (RNAi). Constitutive down-regulation using the 35S promoter resulted in the generation of dwarf plants, indicating that the *SlscADH1* gene, although weakly expressed in vegetative tissues, had a function in regulating plant development. Fruit-specific down-regulation using the 2A11 promoter had no morphogenetic effect and did not alter the aldehyde/alcohol balance of the volatiles compounds produced by the fruit. Nevertheless, SlscADH1-inhibited fruit unexpectedly accumulated higher concentrations of C5 and C6 volatile compounds of the lipoxygenase pathway, possibly as an indirect effect of the suppression of SlscADH1 on the catabolism of phospholipids and/or integrity of membranes.

Keywords:
C5–C6 aroma volatiles
Fruit
Short-chain dehydrogenase-reductase
Solanum lycopersicum

Introduction

The NAD(P)H-dependent interconversion of alcohol and aldehyde group can be catalysed by a wide number of dehydrogenase-reductases commonly named alcohol dehydrogenases (ADH, EC-1.1.1.*x*), which represent one of the most abundant classes of enzymes throughout living world. These enzymes encompass several distinct families of proteins, each characterized by different structural motifs and types of catalysis. They have been classified into two major superfamilies: (i) medium-chain (MDR), whose participation in anaerobic fermentation (Strommer, 2011) and in the reduction of various hydroxyl-cinnamaldehydes has been described (Goffner et al., 1998; Kim et al., 2004) and (ii) short-chain (SDR) whose involvement has been demonstrated in a variety of primary and secondary metabolisms (Tonfack et al., 2011). However the majority of predicted ADH in plant genomes still awaits functional annotation.

Abbreviations: ADH, alcohol dehydrogenase; LOX, lipoxygenase; MDR, medium-chain dehydrogenase/reductase; SDR, short-chain dehydrogenase/reductase.

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In fruit where many aroma volatiles arise from lipids through the lipoxygenase pathway, alcohol dehydrogenases have been involved in the alcohol/aldehyde ratio. Among the ADH families, the expression of medium-chain ADH genes has been associated with the production of aroma volatiles in tomato (Longhurst et al., 1994), melon (Manriquez et al., 2006), mango (Singh et al., 2010), peaches (Zhang et al., 2010), apple (Defilippi et al., 2005) and grapevine (Tesniere et al., 2006). The actual participation of medium-chain ADHs in aroma volatile production *in vivo* has so far only been clearly demonstrated in the case of tomato fruit by over-expressing or down-regulating the *LeADH2* gene (Speirs et al., 1998). The level of alcohols, particularly (*Z*)-3-hexenol and hexanol, were increased in fruit with increased ADH activity and decreased in fruit with low ADH activity (Speirs et al., 1998). Also, down-regulated fruit exhibited an increase in the (*Z*)-3-hexenal: (*Z*)-3-hexenol and 3-methylbutanal/3-methylbutanol ratios (Prestage et al., 1999). In grapevine, over expression or down-regulation of the *VvADH2* gene had small effects on aroma volatile production (Torregrosa et al., 2008). The only change observed was a reduction of benzyl alcohol and 2-phenylethanol in mature berries over-expressing the *VvADH2* gene.

Despite this clear action of medium-chain ADH, other studies suggest that SDRs may also contribute to the biosynthesis of aromas in plants. Tieman et al. (2007) demonstrated that two tomato genes, *LePAR1* and *LePAR2*, expressed in *Escherichia coli* are both capable of catalyzing the conversion of phenylacetaldehyde to the corresponding alcohol. LePAR1 has strong affinity for phenylacetaldehyde while LePAR2 has similar affinity for phenylacetaldehyde, benzaldehyde and cinnamaldehyde (Tieman et al., 2007). Expression of the genes in petunia flowers resulted in higher levels of 2-phenylethanol and lower levels of phenylacetaldehyde, confirming the function of the protein *in vivo* (Tieman et al., 2007). A short-chain ADH *PaADH* shows increased expression in apricot fruit similarly to other genes potentially involved in aroma volatile production, lipoxygenase and alcohol acyl transferase (Gonzalez-Aguero et al., 2009). *CmADH2*, a SDR highly similar to *PaADH* was found to be expressed during melon ripening under the control of ethylene and catalyzes the reduction of several aliphatic aldehydes (Manriquez et al., 2006). Several dehydrogenase-reductases belonging to either the SDR or the MDR superfamilies have been characterized for the synthesis of eugenol in flowers (Koeduka et al., 2006) and monoterpenes in peppermint and spearmint (Croteau et al., 2000). So far their homologues have not been identified in fruits.

As the plant chemical diversity often relies on the diversification of multigenic families and since the aroma of tomato fruit is constituted by approximately 400 molecules (Baldwin et al., 2000) we investigated the potential role of uncharacterized SDRs in aroma biosynthesis and focused on a first candidate, *SlscADH1* that was highly expressed during tomato fruit ripening. The capacity of the recombinant *SlscADH1* to oxidize or reduce various aroma volatiles precursors was investigated *in vitro* and the function of the gene was evaluated *in planta* using a reverse genetic approach consisting in knocking-down *SlscADH1* gene expression by RNAi silencing.

Materials and methods

Plant material and culture conditions

All experiments were performed using *Solanum lycopersicum* L. cv. Micro-Tom, a miniature tomato cultivar. Plants were grown in soil in a culture room with 14 h/10 h light/dark regime, 25 °C day/22 °C night, 80% hygrometry and 250 μmol m^{-2} s^{-1} light intensity.

Cloning of SlscADH1 for RNAi construct and plant transformation

In order to reduce *SlscADH1* gene expression, the RNAi strategy was employed. A partial clone of *SlscADH1* (400 bp) was cloned into a pGreen0029 binary vector (Hellens et al., 2000) previously modified (Damiani et al., 2005), in sense and antisense orientations, under the transcriptional control of either the Cauliflower mosaic virus 35S or the fruit specific 2A11 promoter. The following primers, forward 5′-AT*CCATGG*AACTGGTGGTGCTAGTGGCA-3′ and reverse 5′-AG*TCTAGA*ATGCCGCATAAGAATGTGGG-3′ were used to amplify the SlscADH1 antisense fragment and forward 5′-AT*CTGCAG*AACTGGTGGTGCTAGTGGCA-3′ and reverse 5′-AT*GAATTC*ATGCCGCATAAGAATGTGGG-3′ for the sense fragment. Restriction sites were added at the 5′ ends of each oligonucleotide (as indicated in italics). Transgenic plants were generated by *Agrobacterium tumefaciens* (strain C58) mediated transformation according to Jones et al. (2002), and transformed lines were first selected on kanamycin (50 mg L^{-1}) to discriminate between different transformation events in the various transgenic lines obtained.

Transient expression of SlscADH1::GFP fusion proteins

The full length coding sequence of *SlscADH1* was used in frame with GFP to build a *SlscADH1::GFP* construct that was cloned into the pGreen0029 vector (Hellens et al., 2000) and expressed under the control of the 35S promoter. The 7-day-old tobacco (*Nicotiana tabacum*). BY-2 cells (2 g) from a suspension culture were transfected according to the method described by Leclercq et al. (2005), using the modified polyethylene glycol method as described by Abel and Theologis (1994). A 200 μL suspension of protoplasts was transfected with 25 μg of salmon sperm carrier DNA (Clontech) and 10 μg of either *35S::SlscADH1-GFP* or *35S::GFP* (control) plasmid DNA. Transfected protoplasts were incubated 12 h at 25 °C. Confocal images of transfected protoplasts were acquired with a confocal laser scanning system (Leica TCS SP2, Leica DM IRBE; Leica Microsystems) equipped with an inverted microscope (Leica) and a 40× water immersion objective (numerical aperture 0.75).

Expression analyses

Total RNA from fruit samples was extracted as described previously (Jones et al., 2002). For leaf, stem, root, and flower material, total RNA was extracted using RNeasy Plant Mini Kit following the manufacturer's recommendations (Qiagen). All RNA extracts were treated with DNAse I (Promega) and cleaned up by phenol–chloroform extraction. DNase-treated RNA (2 μg) was reverse transcribed in a total volume of 20 μL using Omniscript Reverse Transcription Kit (Qiagen). The RT-qPCR was performed with cDNAs (100 ng) in 20 μL reactions using the kit SYBR Green master mix (PE Applied Biosystems) on an ABI PRISM 7900HT sequence-detection system. The following primers, forward 5′-TGTCCCTATTTACGAGGGTTATGC-3′ and reverse 5′-CAGTTAAATCACGACCAGCAAGAT-3′ were used for Sl-actin; forward 5′-GCGATTGAATCAGACGTTCAAA-3′ and reverse 5′-GCGATTGAATCAGACGTTCAAA-3′ were used for *SlscADH1*. The optimal primer concentration was 300 nM. RT-PCR conditions were as follow: 50 °C for 2 min, followed by 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qRT-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 C_t (threshold sample) value was calculated from the amplification curves by selecting the optimal ΔRn (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 C_t method using

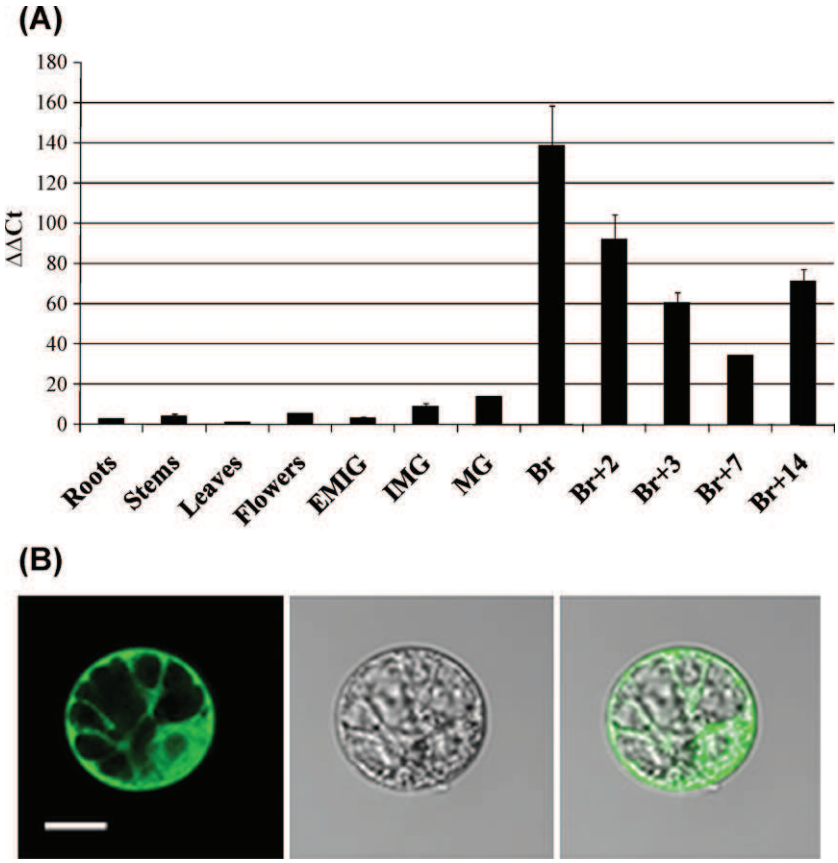

Fig. 1. Expression pattern and sub-cellular localization of SlscADH1. (A) Expression analyses were carried out by quantitative real-time RT-PCR using RNA samples extracted from various tissues of wild type tomato. The experiments were carried out in triplicate. The *X*-axis represents various organs of tomato: roots, stems, leafs, flowers, tomato fruits at early immature green (EIMG), mature green (MG), breaker (BR), and fruits at 2, 3, 7 and 14 days post breaker. $\triangle\triangle C_t$ on the *Y*-axis refers to the fold difference in *SlscADH1*. (B) SlscADH1-GFP protein sub-cellular localization. SlscADH1-GFP fusion protein was transiently expressed in BY-2 tobacco protoplasts and sub-cellular localization was performed by confocal laser scanning microscopy. A green fluorescent picture, the corresponding bright field and an overlay are represented from left to right. The scale bar indicates 15 μm.

the β-actin as an internal standard. In addition, the expression of *SlscADH1* and its close relatives (Solyc12g056610, Solyc10g083170 and Solyc04g0711960) was assayed using semi-quantitative RT-PCR. A 400–600 bp fragment was amplified on different cDNA dilutions with a variable number of cycles (27, 31 and 35) and analysed after agarose gel electrophoresis. For semi-quantitative PCR amplification, the following primers were used: *SlscADH1*-forward 5′-GCGATTGAATCAGACGTTCAAA-3′, *SlscADH1*-reverse 5′-TCCTCCAATAAAACTCCTTTCAAATT-3′, Solyc12g056610-forward 5′-GAGCAAACAGTTATCTATGCCC-3′, Solyc12g056610-reverse 5′-TGTCAAGTACAACACTCCA-3′, Solyc10g083170-forward 5′-AATCACCGCATTCATACACC-3′, Solyc10g083170-reverse 5′-GACTATAACCTCCATCAATCAC-3′, Solyc04g0711960-forward 5′-ATCTCCACCGAAATAATAGCGT-3′ and Solyc04g0711960-reverse 5′-ACATCATCAACCGTCAATTCC-3′.

SlscADH1 expression in Escherichia coli and evaluation of the activity of the recombinant protein

The full length coding region of *SlscADH1* was amplified from cDNA extracted from tomato fruits with forward 5′-ACG*CATATG*GCCACCCCTTCTCTTCA-3′ and reverse 5′-AGT*CTCGAG*AATAAGAATCTGCATAACTTGATTGG-3′ primers. The PCR conditions were as follows: 94 °C for 5 min, 39 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 90 s; then 72 °C for 7 min. The PCR products were cloned in pGEM-T vector using *E. coli* DH5-α as host and the correct sequence was analysed using the "BioEdit Sequence Alignment Editor" software (Hall, 1999). The correct sequence of *SlscADH1* was cloned in *pET15b* vector containing the Histidine tag (Novagen) under the control of T7 promoter. The *pET15b-SlscADH1* and free-*pET15b* (control) DNA plasmids were used to transform *E. coli* strain BL21-Ai (Invitrogen) for inducible protein expression. To purify His-tagged SlscADH1 protein, bacterial cultures were centrifuged at 6000 rpm for 3 min, followed by sonication for 3 × 30 s in Tris–HCl buffer and centrifugation at 21,000 rpm for 60 min. The His-tagged protein was purified from the supernatant by using BD-talon affinity resin (BD Biosciences) according to the manufacturer's instructions. Protein purity was determined by SDS–PAGE followed by staining with Coomassie brilliant blue (Fermentas). The specific activity of the *E. coli*-expressed protein was determined by following the absorbance change corresponding to either oxidation of NADH or reduction of NAD at 340 nm, at 30 °C on a Beckman spectrophotometer equipped with a thermostated cell holder. Each oxidation reaction was performed in a 1 mL reaction mixture containing 100 mM glycine–NaOH buffer (pH 9.6), 5 mM alcohol and 0.25 mM NAD. The reaction mixture for the reduction of NAD comprised 50 mM sodium phosphate buffer (pH 5.8), 5 mM aldehyde, 0.25 mM NADH. In all tests, the reaction was induced by adding 4 μL of protein extract corresponding to 0.6–2 mg of purified enzyme. The reactions were measured for 10–15 min and repeated 3 times. The apparent K_m and V_{max} values were determined using Lineweaver–Burk plots. A control activity assays were performed with cell-free extracts obtained by expression of *pET15b* vector

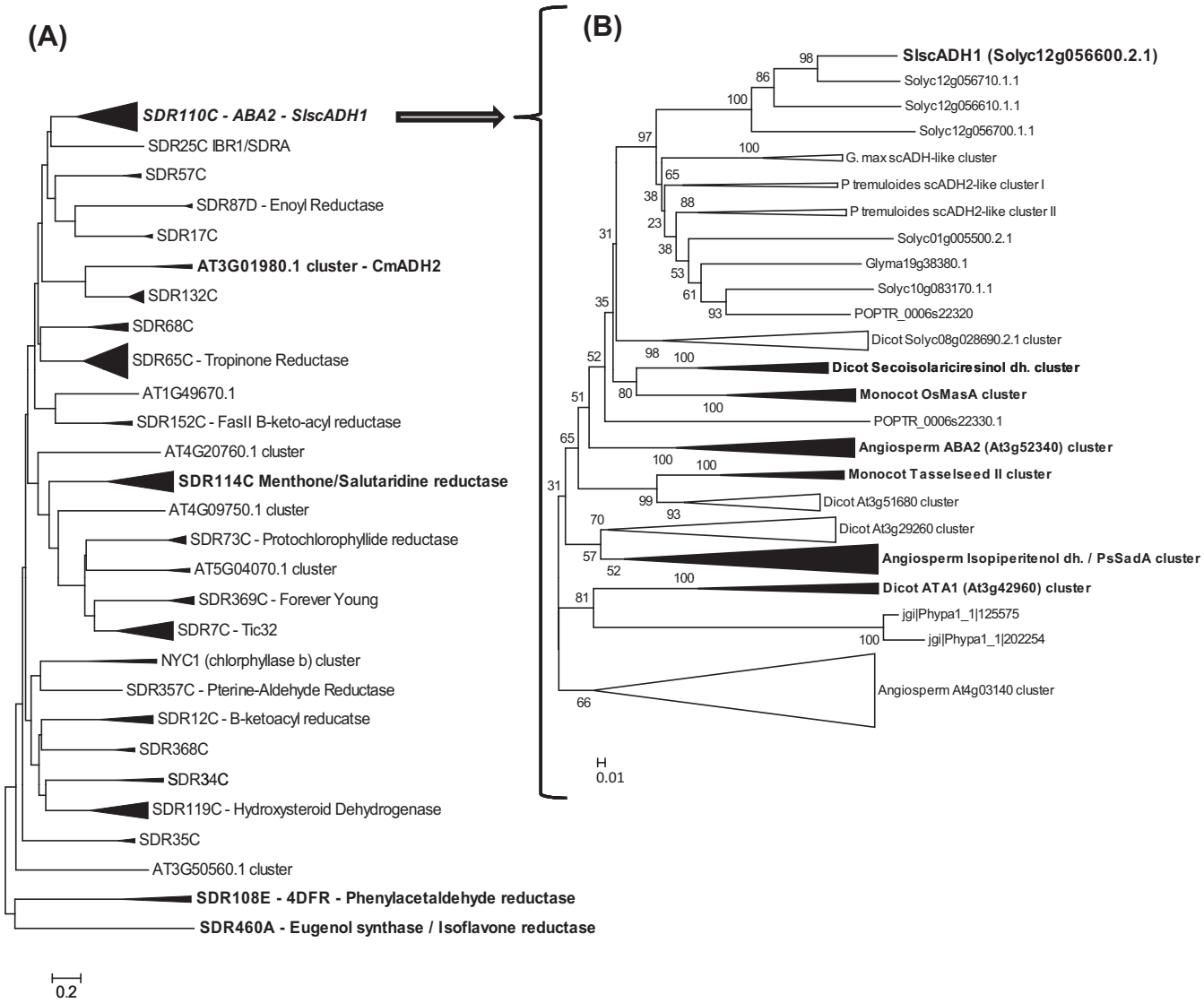

Fig. 2. Relationships between SlscADH1 and plant short chain alcohol dehydrogenases. (A) Overall structure of the SDR superfamily. The structure was deduced from an alignment of SDRs of known functions and *A. thaliana* predicted 'classical' SDRs (Tonfack et al., 2011). Clustering was achieved using the neighbor-joining method. Family identifiers were given in agreement with the SDR nomenclature initiative (Kallberg et al., 2010). Unless related to aroma synthesis, 'extended' and 'atypical' SDRs were omitted in the analysis. (B) Focus on the SlscADH1 (SDR110C) subfamily phylogeny. The different clusters were identified either on the basis of known functions (in bold) or a genomic identifier. The evolutionary history was inferred after alignment of members' sequence from 8 plant genomes (Arabidopsis, poplar, soybean, tomato, rice, sorghum, corn and Physomitrella) using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Analyses were conducted in MEGA5 (Tamura et al., 2011).

without *SlscADH1* gene in *E. coli*. They did not show any activity towards all the substrates tested. Protein concentrations were measured using a Bradford protein determination kit with BSA as a standard (Bio-Rad Laboratories).

Analysis of volatile compounds

Fruits were harvested at breaker +7 days, and the whole of fruits was ground in liquid nitrogen. The aroma compound analyses were run as described previously (Birtic et al., 2009). The breaker stage has been characterized by the change in colour from green to pale orange at about 30% of the surface.

Accession number

Full-length *SlscADH1* sequence has been deposited at Genbank (accession: JQ804996).

Results and discussion

Expression of SlscADH1 in tomato plant organs and subcellular localization

A tomato short-chain alcohol dehydrogenase (*SlscADH1*) was selected as a candidate on the basis of its '*in silico*' expression pattern. The list of the expressed sequence tag (EST) for the SGN-U579700 unigene was found to be highly represented in the Sol Genomic Network database (http://solgenomics.net/, Bombarely et al., 2011) with 54 accessions (Supplemental Fig. S1A). In addition, the cLEG collection, extracted from tomato fruit at the breaker stage, accounted for 21 accessions (Supplemental Fig. S1A), thus suggesting that *SlscADH1* is highly represented during fruit ripening.

The expression pattern deduced from the ESTs was confirmed with qRT-PCR experiments performed on RNA extracted from

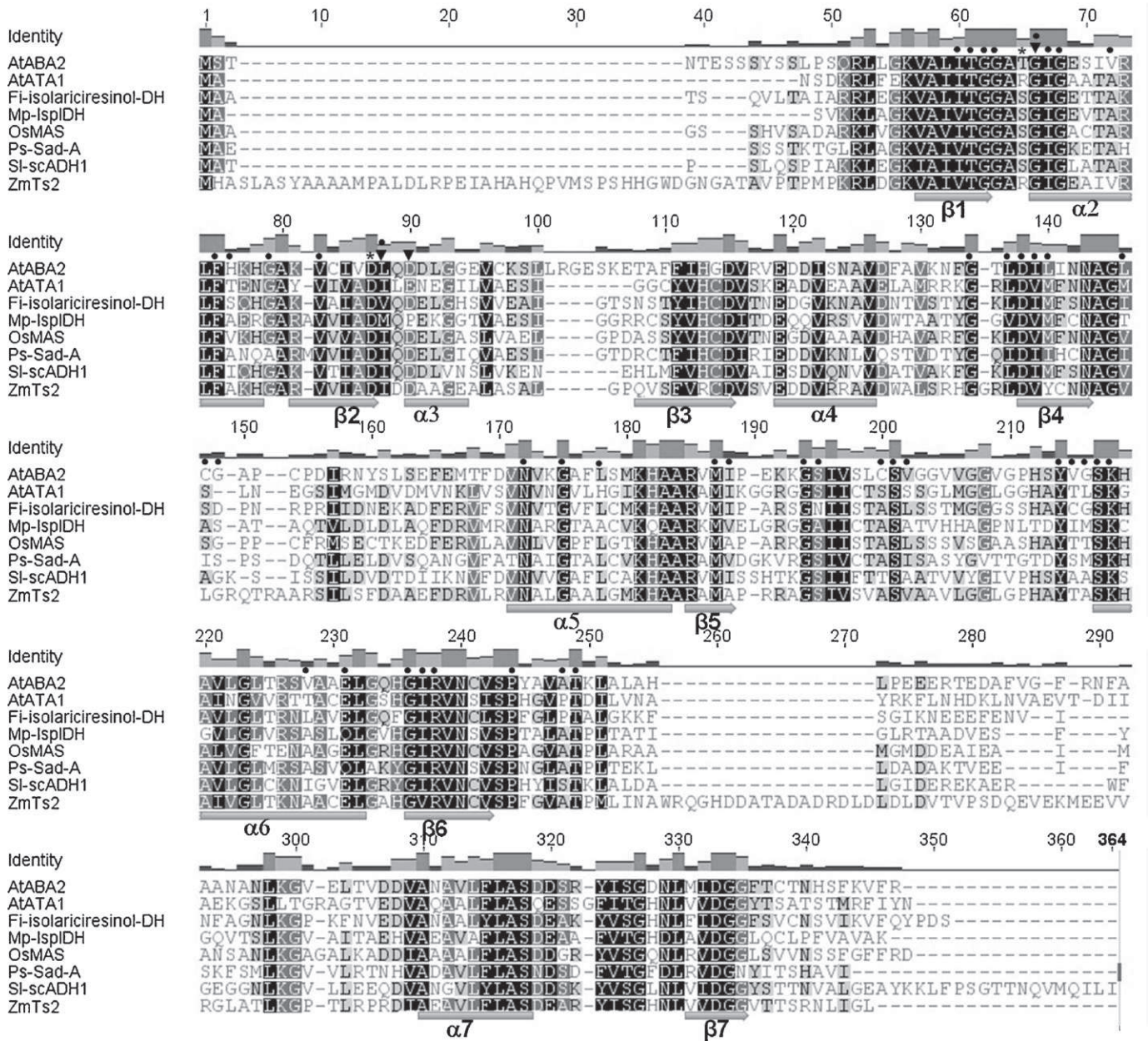

Fig. 3. Amino acid sequence alignment of *Solanum lycopersicum* SlscADH1 (SGN-U2133299) with closely related full length sequences of abscisic acid deficient2 (ABA2) of *Arabidopsis thaliana* (AT1G52340.1), ATA1 of *A. thaliana* (AT3G42960.1), Secoisolariciresinol dehydrogenase of *Forsythia* x *intermedia* (AAK38665.1), IsplDH of *Mintha piperita* (AAU20370.1), Momilactone A synthase of *Oryza sativa* japonica (LOC_Os04g10010.1), Sad-A of *Pisum sativa* (AAF04193.1) and *TASSELSEED 2* of *Zea mays* (GRMZM2G455809_P01). The global alignment with free end gaps was elaborated with the Geneious Pro 5.5.4 software, using the cost matrix Blosum 62 with gap open penalty 12, gap extension penalty 3 and refinement iterations 2. Residues shaded in black colour are 100% similar, in dark grey at least 80% similar, in clear grey 60–80% similar and in white less than 60% similar. The black vertical arrows show the 15, 35 and 37 positions and the stars show the presence of serine (S) and aspartic acid (D) at very characteristic positions.

vegetative tissues (roots, leaves, shoots), flowers and fruits in different stages of development and ripening (Fig. 1A). Expression was very low in vegetative organs and flowers. Maximum expression took place at the onset of ripening corresponding to the breaker stage and remained high during all the ripening process (Fig. 1A). The subcellular localization of *SlscADH1* was investigated by transient expression of GFP-fusion proteins in tobacco protoplasts and observation under confocal microscope. Cells transformed with the fusion protein displayed fluorescence throughout the cell with the exception of the vacuole and without any sign of concentration in any organelle (Fig. 1B). It can therefore be concluded that *SlscADH1* is localized in the cytosol.

Analysis of the SlscADH1 sequence and phylogeny

With the recent release of the genome sequence (The Tomato Genome Sequencing Consortium, 2012) the SGN-U579700 UNIGENE was associated with the gene locus Solyc12g056600, located on the chromosome 12 (100% identity). From tomato cDNA, we cloned a complete ORF of 864 bp coding for a SDR protein with a predicted size of 31 kDa. A pfam scan (Finn et al., 2010) of the sequence reveals that it contains motifs characteristic of classical short chain dehydrogenases (pf00106 family). Interestingly, this superfamily contains several enzymes involved in secondary metabolism including aroma synthesis (phenylacetaldehyde

Table 1
Activities of purified SlscADH1 protein towards different aldehydes and alcohols. Recordings were carried out at 5 mM substrate and 0.25 mM cofactor (NADH or NAD^+), at pH 9.6 for alcohol dehydrogenation reaction in 100 mM glycine NaOH buffer and pH 5.8 for aldehyde reduction reaction in 50 mM potassium phosphate buffer. Values representing the mean of three replications are given in μmol of substrate/mg of protein/min ± standard deviation. nd, not detectable; t, trace.

Aldehydes	Cofactor	Activity	Alcohols and ketones	Cofactor	Activity
Hexanal	NADH	1528 ± 11	1-Hexanol	NAD	338 ± 8
Hexanal	NADPH	250 ± 15	1-Hexanol	NADP	t
Phenylacetaldehyde	NADH	1251 ± 6	1-Phenylethanol	NAD	51 ± 1
E-2-hexenal	NADH	800 ± 20	*E*-2-hexenol	NAD	43 ± 3
Acetaldehyde	NADH	772 ± 60	Ethanol	NAD	t
Citral B	NADH	nd	Nerol	NAD	t
Z-2-hexenal	NADH	t	Glycerol	NAD	t
Geranylacetone	NADH	nd	*Z*-2-hexen-1-ol	NAD	t
1-Butanal	NADH	nd	Geraniol	NAD	nd
Benzaldehyde	NADH	nd	1-Butanol	NAD	t
E-3-hexenal	NADH	t	Benzyl alcohol	NAD	t
Z-3-hexenal	NADH	nd	*E*-3-hexenol	NAD	nd
Z-3-nonenal	NADH	t	*Z*-3-hexenol	NAD	t
1-Heptenal	NADH	nd	2-Phenylethanol	NAD	nd
Cinamaldehyde	NADH	nd	*Z*-3-nonen-1-ol	NAD	nd
β-Ionone	NADH	t	1-Heptanol	NAD	nd
6-Methyl-5-hepten-2-one	NADH	nd	Cinnamyl alcohol	NAD	nd
α-Ketoisovaleric acid	NADH	nd	Guaiacol	NAD	t
α-Ketoisocaproic acid	NADH	nd	2-3-Epoxy-propan-1-ol	NAD	nd
E-2-*Z*-nonadienal	NADH	nd	6-Methyl-5-hepten-2-ol	NAD	nd
2-Methyl butyraldehyde	NADH	nd	2-Methyl butanol	NAD	nd
Isobutyraldehyde	NADH	nd	α-Ionol	NAD	t
1-2-Epoxy-octane	NADH	nd	Eugenol	NAD	nd
Syringaldehyde	NADH	nd	Secoisolariciresinol	NAD	nd
Salicaldehyde	NADH	nd			
Matairesinol	NADH	nd			

dehydrogenase, menthone reductase and the melon CmADH2 (Fig. 2A)).

Sequence analyses based on the Hidden–Markov Models developed in the frame of the SDR nomenclature initiative (Kallberg et al., 2010) allowed a refined categorization of SlscADH1 into the SDR110C family of the SDR superfamily (Fig. 2A). A detailed presentation of the SDR110C family (Fig. 2B) shows that it encompasses several dehydrogenases known to participate in plant secondary metabolism or hormone biosynthesis (for review, see Tonfack et al., 2011): *Forsythia intermedia* secoisolariciresinol dehydrogenase (Xia et al., 2001), rice momilactone A synthase (Shimura et al., 2007), ABA2 xanthoxin dehydrogenase (Cheng et al., 2002; González-Guzmán et al., 2002), spearmint isopiperitenol dehydrogenase (Ringer et al., 2005), pea short-chain alcohol dehydrogenase-like protein (P.s.-SAD-A, Scherbak et al., 2011). In addition to AtABA2, other members of the SDR110C were reported for their involvement in plant development despite a lack of information concerning the reaction catalyzed by the enzyme. This is notably the case of the monocot feminization gene *TASSELSEED2* (DeLong et al., 1993; Malcomber and Kellogg, 2006).

Alignment of full sequences confirmed the high homologies among the different members of SDR110C families (Fig. 3). All sequences exhibit several motifs typical of classical SDRs, such as a N-terminal segment predicted to bind the NAD–NADH cofactor or the YxxxK catalytical residue motif characteristic of classical SDRs (Fig. 3). In addition, phylogenetic analysis performed after alignment of members of the SDR110C family reveal that, despite high similarities with *Forsythia intermedia* secoisolariciresinol dehydrogenase, rice momilactone synthase or *Arabidopsis thaliana* ABA2, SlscADH1 belong to a clade distinct of the SDR110C proteins previously characterised (Fig. 2). Moreover, the analysis show that on the chromosome 12 *SlscADH1* gene is adjacent to three close homologues (Solyc12g056610, Solyc12g056700 and Solycg056710), suggesting the existence of recent duplication events. Contrary to *SlscADH1*, the ESTs associated with Solyc12g056610 and Solycg056710 were mainly encountered in trichome cDNA libraries and absent in fruit cDNA collections (Figs. S1B and S1C). Similarly to *SlscADH1*, ESTs from vegetative tissues were poorly represented for all the homologues. No EST was associated with Solyc12g056700, suggesting that the gene is barely expressed in most tissues.

Enzymatic characteristics of the recombinant SlscADH1

SlscADH1 protein was produced in the *E. coli* E. BL21Ai bacteria strain, extracted and purified on cobalt resin. SDS polyacrylamide gel electrophoresis revealed the presence of a major protein of 31 kDa molecular mass (Supplemental Fig. S2).

Reductase and dehydrogenase (oxidase) activities of the recombinant protein were assessed using 26 potential substrates containing a carbonyl group (aldehyde, ketone or oxo-acid) and 23 compounds with an alcohol group (Table 1). The purified recombinant protein was able to reduce, with decreasing efficiency, hexanal, phenylacetaldehyde, (*E*)-2-hexenal and acetaldehyde. Low activity was detected as traces for (*Z*)-2 hexenal, the (*E*)-3-hexenal, (*Z*)-3-nonenal and β-ionone. High oxidative activity of the protein was observed using the following alcohols: 1-hexanol, 1-phenylethanol and (*E*)-2 hexenol. A number of alcohols were oxidized at traces levels: ethanol, nerol, glycerol, (*Z*)-2-hexen-1-ol, 1-butanol, benzyl alcohol, (*Z*)-3-hexenol, guaiacol and β-ionol.

The activity of the recombinant protein was strongly dependent on the couple of co-factors, since much lower activity was observed for the reduction of hexanal with NADPH/$NADP^+$ compared to NADH/NAD^+ and very low activity (traces) for the oxidation of 1-hexanol in hexanal (Table 1). The reductase activity was much higher than dehydrogenase (oxidase) activity. The maximum activity (1528 $\mu mol\ mg^{-1}$ prot. min^{-1}) of the protein was observed for the reduction of hexanal in the presence of 0.25 mM NADH while the oxidation reaction of 1-hexanol in the presence of NAD^+ was 5 times lower (338 $\mu mol\ mg^{-1}$ prot. min^{-1}).

The K_m and the apparent turnover (K_{cat}) were determined using the preferred substrates and co-factor as either reductase or oxidase. Table 2 shows that the lowest K_m for aldehydes is for hexanal (0.175 mM) followed by phenylacetaldehyde (0.375 mM), *E*-2-hexenal (0.879 mM) and acetaldehyde (9.43 mM). For alcohols, the K_ms are much higher with 2.9 for hexanol and 23.0 for phenylacetaldehyde. The K_m for the NADH co-factor (36 μM) was more

Table 2
Estimated kinetics parameters (K_m and apparent K_{cat}) of SlscADH1 protein for different substrates.

	K_m (mM)	K_{cat} (s^{-1})
NADH (hexanal, 5 mM)	0.036	940
Hexanal (NADH)	0.175	1240
Phenylacetaldehyde (NADH)	0.375	660
Acetaldehyde (NADH)	9.43	745
NAD (hexanol, 100 mM)	0.065	490
Hexanol (NAD)	2.9	486
1-Phenylethanol (NAD)	23.0	136

than two times lower than for NAD (65 μM). The preference for aldehyde reduction is also confirmed by the catalytic efficiency (corresponding to the K_{cat}/K_m ratio) which is 42-fold higher for hexanal as compared to 1-hexanol (Table 2). This preference of scADHs for the reduction direction was also established by other authors (Manriquez et al., 2006; Tieman et al., 2007; Polizzi et al., 2007).

The optimum pH of the reaction was 5.8 for aldehyde reduction reactions and 9.6 for alcohol dehydrogenation reactions. The two optima pH are similar to those of other scADHs (Manriquez et al., 2006; Polizzi et al., 2007; Wu et al., 2007).

Effects of constitutive and fruit-specific down-regulation of SlscADH1 on the phenotype

In order to obtain RNAi-mediated silencing of *SlscADH1*, a construct under the control of the constitutive cauliflower mosaic virus (CaMV) 35S was introduced into tomato plants *via* the means of *Agrobacterium tumefaciens*. Constitutive down-regulation of *SlscADH1* in all tissues resulted in a strong dwarf phenotype with a reduced number of fruits and flowers (Fig. 4A). Such a growth inhibition could be surprising since the Micro-Tom tomato variety used in the present study has already a dwarf phenotype due to a mutation in brassinosteroid biosynthesis gene (Marti et al., 2006). However, supplemental plant growth inhibition of Micro-Tom tomato plants has already been observed upon introgression of a gibberellic acid synthesis mutation indicating that the development of Micro-Tom plants can still be altered by other hormones (Campos et al., 2010). The strong growth reduction effect of inhibiting *SlscADH1* was also unexpected seeing as the gene is weakly expressed in vegetative tissues. A possible explanation is that *SlscADH1* plays a crucial role in the biosynthesis of certain hormones. There is no indication that short-chain dehydrogenases participate in the biosynthesis of gibberellins (Sponsel and Hidden, 2004) but *SlscADH1* belongs to a family comprising a number of genes identified as involved in plant growth regulation, notably AtABA2 (AT1G52340.1) that catalyzes the conversion of xanthoxin into ABA-aldehyde (Cheng et al., 2002; González-Guzmán et al., 2002) and the grasses flower feminization protein TASSELSEED2 (DeLong et al., 1993; Malcomber and Kellogg, 2006) that displays 3β/17β-hydroxysteroid dehydrogenase and carbonyl/quinone reductase activities (Wu et al., 2007). Alternatively, other members of the SDR110C family intervene in different branches of secondary metabolism, such as rice momilactone A synthase (diterpenoid phytoalexin synthesis, Shimura et al., 2007), secoisolariciresinol dehydrogenase (lignan synthesis, Xia et al., 2001) or pea short-chain alcohol dehydrogenase-like protein (quinone reduction, Scherbak et al., 2011). Thus, the possibility that the dwarf phenotype arises as a consequence of the accumulation of a toxic precursor cannot be ruled out.

As our initial objective was to check the *SlscADH1* role in aroma metabolism, it was decided to perform a new set of constructs and transformed lines, using a fruit specific promoter. The new series of transformations was undertaken using the fruit-specific 2A11 promoter. Several lines were generated among which two showed

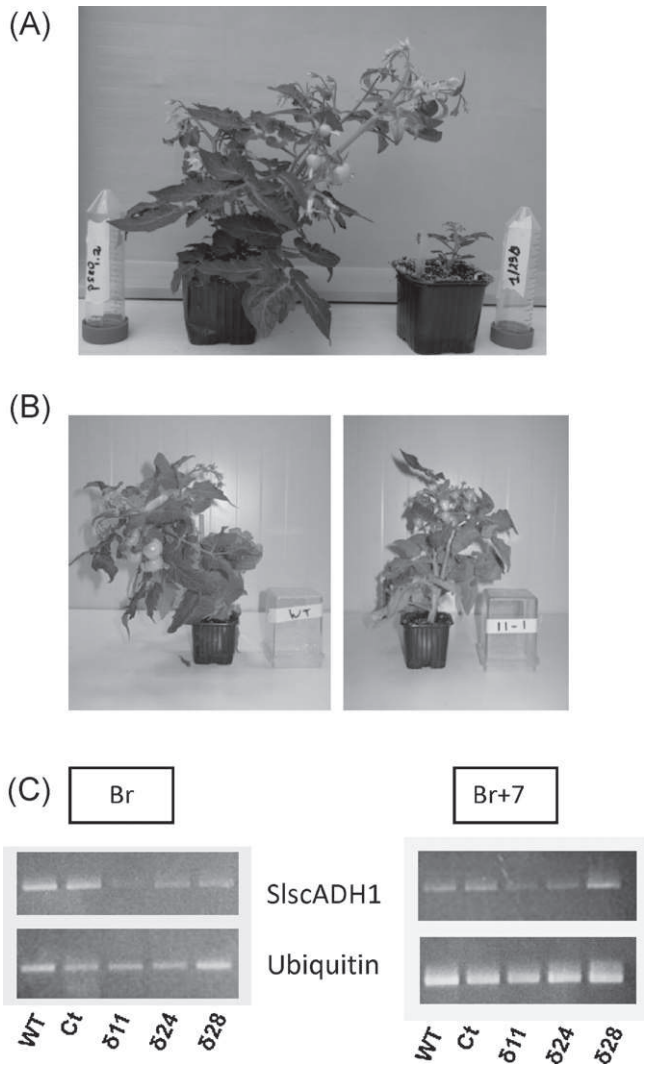

Fig. 4. Transgenic plants with RNAi-mediated silencing of *SlscADH1*. (A) Phenotype of a two-month old plant with altered *SlscADH1* expression under the control of the CaMV 35S constitutive promoter (right) compared to WT plant (left). (B) Phenotype of a two-month old plant with altered *SlscADH1* expression under the control of the 2A11 fruit-specific promoter (right) compared to WT plant (left). (C) RT-PCR analysis of *SlscADH1* transcript accumulation in wild-type and 2A11-RNAi transgenic lines of Micro-Tom (C_t, δ11, δ24 and δ28) tomato fruits at two ripening stages of development: Breaker (Br) and 7 days after breaker (Br +7). Control plants (Ct) correspond to plants transformed with the pSR01 plasmid devoid of the *SlscADH1* insert.

strong (δ11) or moderate (δ24) down-regulation (Fig. 4C). Down-regulation was somewhat higher at the breaker than at the breaker +7 days in agreement with previous observations of the efficiency of the 2A11 fruit-specific promoter (Van Haaren and Houck, 1993). The down-regulated plants now exhibited no alteration of the phenotype (Fig. 4B).

The specificity of the RNAi construct was further tested on two genes presenting high homologies to *SlcsADH1* (Fig. 2B): Solyc12g056610, a very similar SDR (82% identity in the mRNA targeted by RNA interference) and Solyc04g0711960 (58% identity), the SDR displaying the strongest homology to the Arabidopsis *AtABA2*. While the expression of *SlABA2* was not affected by the RNA interference (Fig. 5), the Solyc12g056610 down-regulation was comparable to *SlscADH1*. The expression of Solyc10g083170 was also assessed, but no mRNA was detected either in WT or in transgenic lines (data not shown). Therefore, we can reasonably assume that the RNAi mediated inhibition affects the four tandemly-duplicated genes found on chromosome 12 (Solyc05600,

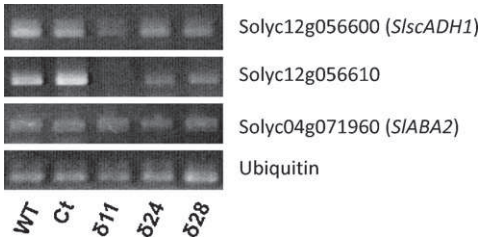

Fig. 5. RT-PCR analysis of transcripts from *SlscADH1* and two homologues (Solyc12g056610, Solyc04g071960) in wild-type and 2A11-RNAi transgenic lines of Micro-Tom (C_t, δ11, δ24 and δ28) tomato fruits at the Breaker (Br) stage. Control plants (Ct) correspond to plants transformed with the pSR01 plasmid devoid of the *SlscADH1* insert. In the region targeted by RNA interference, Solyc12g056610 and Solyc04g071960 nucleic sequences display respectively 82% and 58% of identity towards *SlscADH1*.

Solyc12g056610, Solyc056700 and Solyc12g056700) which exhibit very similar mRNA sequences (from 73 to 82% identity) whereas the expression of remote members of the SDR110C family remains unaffected by RNAi-silencing.

Analysis of the ripening rate and aroma volatile production of SlscADH1 down-regulated fruit

There was no difference in the rate of fruit development, time from anthesis to breaker, between wild type and fruit of the δ11 and δ24 lines in which the SlscADH1 was down-regulated (data not shown). However, GC–MS analyses of volatile compounds indicated that more 5- and 6-carbon compounds (C5–C6) accumulate in fruit from the down-regulated lines while no difference was observed as far as the other compounds were concerned (Supplemental Table S1). Data on C5–C6 compounds are represented in Fig. 6 showing that pentanal, 1-penten-3-one, hexanal, (*E*)-2-pentenal, 1-penten-3-ol, (*E*)-2-hexenal, 1-pentanol and (*E*)-2-pentenol are more abundant in the two transformed lines. For three of the C5–C6 compounds (1-pentene-3-one, (*E*)-2-pentenal and 2-pentyl furan), there is a highly significant difference ($P < 0.05$). The trend is the same for the other C5–C6 compounds with slightly higher probabilities (P up to 0.116). None of the

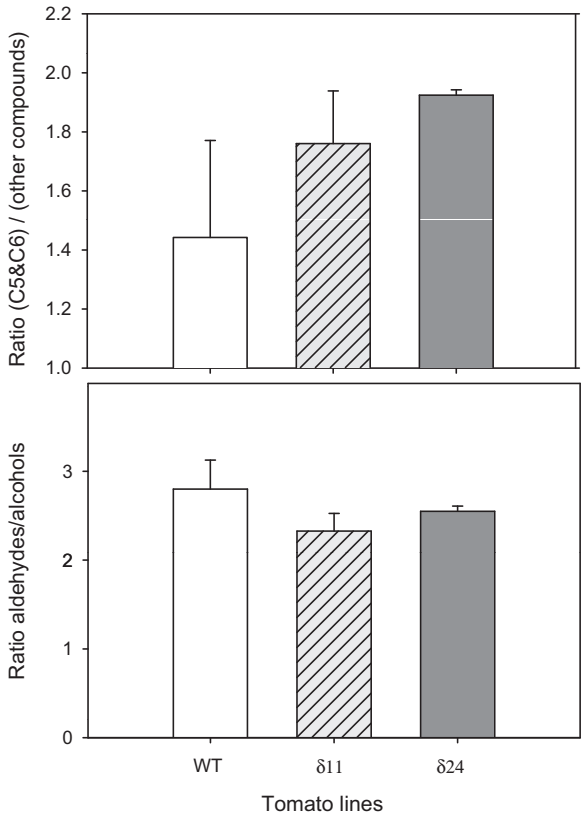

Fig. 7. (A) Ratios of 5- and 6-carbon compounds *vs* total volatiles and (B) aldehydes *vs* alcohols in wild type fruit (WT) and fruit of two independent *SlADH1* down-regulated RNAi lines (δ11 and δ24). The analyses were performed by GC–MS and individual compounds were quantified by reference to an internal standard as described in "Materials and methods". Error bars show standard errors, $n = 3$ biological replicates.

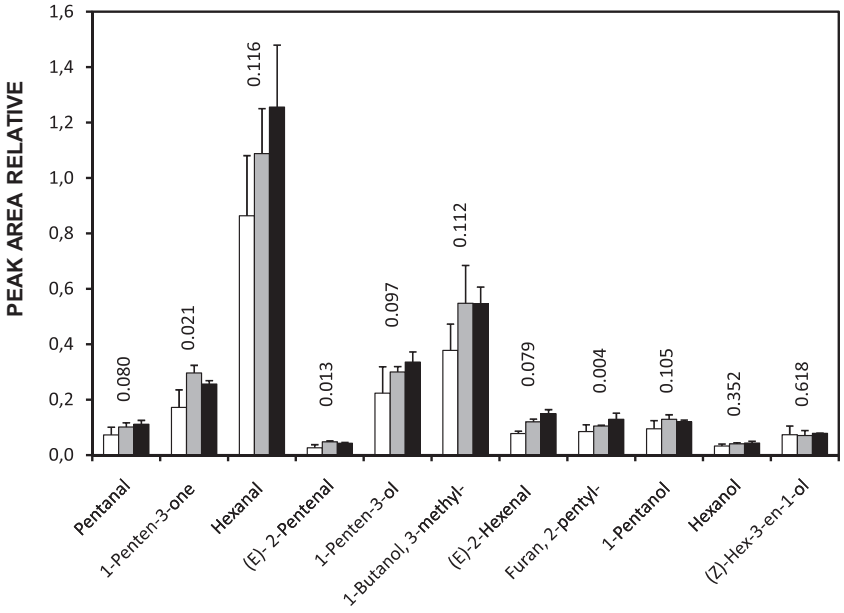

Fig. 6. Comparison of the amount of 5- and 6-carbon volatile compounds in wild type fruit (white bars) and fruit of two independent *SlADH1* down-regulated RNAi lines, δ11 (grey bars) and δ24 (black bars). Bars represent the means of three biological replicates, with standard deviation values as error bars. Numbers above the histograms represent the *P* values of statistical difference between WT and RNAi lines.

other compounds present in the aroma volatile analysis show the same trend (Supplemental Table S1). The overabundance of C5–C6 compounds is also illustrated by the fact that the ratio of C5–C6 compounds *vs* all other compounds was 29% and 36% higher in δ11 and δ24 mutant fruit than in wild type, respectively (Fig. 7A). However there was no difference in the aldehyde/alcohol ratios between ADH mutants and wild type (Fig. 7B).

The C5 and C6 compounds arise from the degradation of lipids (Pech et al., 2008), indicating that the down-regulation of *SlscADH1* has probably resulted in a stimulation of the lipoxygenase pathway. Interestingly, the leaves of an *Arabidopsis* mutant deficient in ADH activity also produced higher amounts of C6 volatile compounds (Bate and Rothstein, 1998). In this work C5 volatiles were not mentioned. They derive from the LOX-dependent C-5, 13-cleavage of α-linoleic (Fisher et al., 2003). The mutated ADH was a medium-chain rather than a short chain ADH. Nevertheless the mutation or down-regulation of the two ADH genes both result in a stimulation of the LOX-derived volatiles. As suggested by Bate and Rothstein (1998) the overabundance of (*E*)-2-hexenal, an immediate product of HPL activity would result in positive feedback on HPL activity and gene expression. In our conditions the level of hexenal was higher in the down-regulated fruit tissues (Fig. 6). The enhanced production of LOX-derived volatiles could also be considered as an indirect effect of the suppression of SlscADH1 on the catabolism of phospholipids and/or integrity of membranes. The mechanisms involved remain unknown.

Conclusions

The *SlscADH1* gene studied here is a short-chain ADH which is strongly expressed in tomato fruit and had not been characterized so far. Functionally, scADHs are defined as NAD(P)(H)-dependent oxidoreductases. Some scADHs have been described as involved in the primary metabolism pathways but a large number are thought to be involved in major functions like hormone synthesis or synthesis of metabolites (Tonfack et al., 2011). The capacity of the recombinant SlscADH1 to catalyse the inter-conversion of aroma volatiles was evaluated and it appeared that major compounds known to participate in the flavour of tomato fruit were recognised as substrates, but not all. The SlscADH1 activity was stronger in the reduction direction at physiological pH (aldehyde → alcohols), and this activity was also stronger with NADH than with NADPH.

A strong inhibition of plant development with very limited fruiting has been obtained after RNAi-mediated silencing of *SlscADH1* under the control of the 35S constitutive promoter which prevented studying the effect of *SlscADH1* on fruit ripening. For this reason, the 2A11 promoter was used to down-regulate the target gene specifically in fruit tissues. This strategy resulted in the generation of plants showing normal vegetative phenotypes and normal fruit development thus rendering possible the evaluation of aroma volatile production. A higher production of C5 and C6 volatiles was observed in *SlscADH1*-inhibited fruit although the alcohol to aldehyde ratio remained unaffected. As the C5 and C6 volatile compounds derive from the lipoxygenase pathway, their increased accumulation in *SlscADH1*-deficient tomatoes may result from an indirect effect of the altered protein activity on the catabolism of phospholipids and/or integrity of membranes. An alteration of membranes could give a unified explanation for the impact of *SlscADH1* on plant development and on lipoxygenase-derived volatiles, but this deserves further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2012.06.007.

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