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Functional characterization of SlscADH1, a fruit-ripening-associated short-chain alcohol dehydrogenase of tomato

Hanane Moummou[a,b,c,1], Libert Brice Tonfack[a,b,d,1], Christian Chervin[a,b], Mohamed Benichou[c], Emmanuel Youmbi[d], Christian Ginies[e], Alain Latché[a,b], Jean-Claude Pech[a,b], Benoît van der Rest[a,b,*]

[a] Université de Toulouse, INPT-ENSAT, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole BP 32607, 31326 Castanet-Tolosan, France
[b] INRA, UMR990, Génomique et Biotechnologie des Fruits, Chemin de Borde Rouge, 31326 Castanet-Tolosan, France
[c] Laboratory of Food Science, Faculty of Science Semlalia, University Cadi Ayyad-Marrakech, Morocco
[d] Laboratory of Biotechnology and Environment, Unit of Plant Physiology and Improvement, Department of Plant Biology, Faculty of Science, University of Yaounde 1. PO Box 812, Yaounde, Cameroon
[e] INRA, UMRA 408, Qualité et Sécurité des Produits d'Origine Végétale, Domaine St-Paul, Site Agroparc, 84914 Avignon, France

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ABSTRACT

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.

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.

* Corresponding author at: Université de Toulouse, INP-ENSA Toulouse, Génomique et Biotechnologie des Fruits, Avenue de l'Agrobiopole BP 32607, 31326 Castanet-Tolosan, France. Tel.: +33 5 34 32 38 94; fax: +33 5 34 32 38 72.
E-mail address: benoit.van-der-rest@ensat.fr (B. van der Rest).

[1] These authors contributed equally to this work.

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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′-ATG*AATTC*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