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Tomato phenylacetaldehyde reductases catalyze the last step in the synthesis of the aroma volatile 2-phenylethanol

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Abstract

The volatile compounds, 2-phenylacetaldehyde and 2-phenylethanol, are important for the aroma and flavor of many foods, such as ripe tomato fruits, and are also major constituents of scent of many flowers, most notably roses. While much work has gone into elucidating the pathway for 2-phenylethanol synthesis in bacteria and yeast, the pathways for synthesis in plants are not well characterized. We have identified two tomato enzymes (LePAR1 and LePAR2) that catalyze the conversion of 2-phenylacetaldehyde to 2-phenylethanol: LePAR1, a member of the large and diverse short-chain dehydrogenase/reductase family, strongly prefers 2-phenylacetaldehyde to its shorter and longer homologues (benzaldehyde and cinnamaldehyde, respectively) and does not catalyze the reverse reaction at a measurable rate; LePAR2, however, has similar affinity for 2-phenylacetaldehyde, benzaldehyde and cinnamaldehyde. To confirm the activity of these enzymes *in vivo*, *LePAR1* and *LePAR2* cDNAs were individually expressed constitutively in petunia. While wild type petunia flowers emit relatively high levels of 2-phenylacetaldehyde and lower levels of 2-phenylethanol, flowers from the transgenic plants expressing *LePAR1* or *LePAR2* had significantly higher levels of 2-phenylethanol and lower levels of 2-phenylacetaldehyde. The *in vivo* alteration of volatile emissions is an important step toward altering aroma volatiles in plants.

Keywords: *Solanum lycopersicum*; Solanaceae; 2-Phenylethanol; Tomato flavor; Reductases; Flower scent

1. Introduction

The way in which humans perceive flavor is little understood at the molecular level. For example, what we consider to be the flavor of a tomato is the sum of interactions between sugars, acids and over 400 volatile compounds (Buttery, 1993; Baldwin et al., 2000). Of these many volatiles, about 30 are believed to significantly impact flavor (Buttery et al., 1971; Baldwin et al., 2004). Moreover, these volatiles are derived from a diverse set of precursors that include lipids, carotenoids and amino acids. Several of the most important tomato aroma volatiles are derived from phenylalanine (**1**) (Fig. 1) including 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) (Buttery, 1993; Baldwin et al., 2000). Both of these volatiles have fruity/floral properties that are considered desirable, although elevated levels of 2-phenylethanol (**4**) and 2-phenylacetaldehyde (**3**) have also been associated with undesirable flavor in tomato fruit (Tadmor et al., 2002). Additionally 2-phenylethanol (**4**) and 2-phenylacetaldehyde (**3**) are major constituents of scent in many flowers, with the isomers being the major aroma volatile contributing to the scent of rose, while the latter is the major volatile associated with hyacinth and lilac (Knudsen et al., 1993). Indeed, 2-phenylethanol (**4**) is the most used fragrance chemical in cosmetic products (Clark, 1990), as well as being a major contributor to the overall flavor of diverse products including

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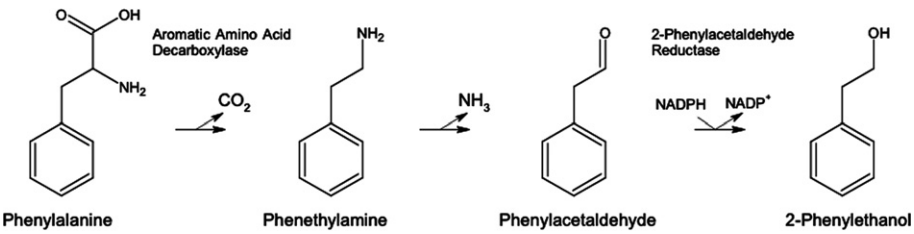

Fig. 1. The biosynthetic pathway for 2-phenylethanol (**4**) from phenylalanine (**1**) via phenethylamine (**2**) and 2-phenylacetaldehyde (**3**) in tomato.

cheese, bread, wine and olive oil. As a consequence, there is much interest in natural sources of 2-phenylethanol (**4**) for the flavor and fragrance industry.

2-Phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) have important biological functions in plants. The latter has long been known to possess antimicrobial properties (Berrah and Konetzka, 1962) and its synthesis by plant reproductive structures may indicate a protective role for flowers and fruits. Both 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) are also potent insect attractants (see http://www.pherobase.com/; Pichersky and Gershenzon, 2002), with each attracting different sets of pollinating and predatory insects (Raguso et al., 2003; Zhu et al., 2005). All of the tomato flavor-associated volatiles are also likely to have major roles in attracting seed-dispersing organisms (Baldwin et al., 1998; Goff and Klee, 2006). These multiple roles, in both defense and reproduction, make regulation of their synthesis critical to plant survival. We are, therefore, interested in elucidating the contributions of 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) to these processes in plants. Therefore, we initiated efforts to identify genes involved in the biosynthesis of these and related phenylalanine-derived volatiles.

Despite the importance of 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) to tomato flavor/aroma and flower scent, the pathway for biosynthesis is not well understood in plants. The yeast, *Saccharomyces cerevisiae*, produces 2-phenylethanol (**4**) from phenylalanine (**1**) via phenylpyruvate and 2-phenylacetaldehyde (**3**) (Vuralhan et al., 2003). Deuterium feeding studies in rose (*Rosa damascena* Mill.), however, indicated the potential for as many as four pathways in their biosynthesis (Watanabe et al., 2002). In addition to the yeast pathway, Watanabe et al. (2002) reported that synthesis can occur via a phenethylamine (**2**)/2-phenylacetaldehyde (**3**) route as well as a *trans*-cinnamic acid/phenyllactate pathway. In earlier work, we had also reported that a tomato enzyme catalyzes the first step in the pathway to 2-phenylethanol (**4**), namely the conversion of phenylalanine (**1**) to phenethylamine (**2**) (Tieman et al., 2006). In the proposed pathway to 2-phenylethanol (**4**), shown in Fig. 1, the final step is the reduction of 2-phenylacetaldehyde (**3**). Herein we show that two tomato (*Solanum lycopersicum*) aldehyde reductases catalyze the final step in the pathway, i.e. the conversion of 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**).

2. Results and discussion

2.1. Identification of a tomato 2-phenylacetaldehyde reductase

2-Phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) are major contributors to flavor in many fresh and processed food products. They also have important and distinct biological functions in plants as antimicrobial compounds and as insect attractants/repellants. Thus, the balance between the concentrations of these two volatile compounds is critical for attracting appropriate pollinating insects. Although some information on the pathway(s) for synthesis of 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) in plants has recently become available, proteins catalyzing this reductive conversion have not been identified (Kaminaga et al., 2006; Tieman et al., 2006). Knowing that both are major contributors to tomato aroma, we searched for genes encoding enzymes that could potentially catalyze the reduction of the aldehyde (**3**) to the alcohol (**4**). In database searches (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tomato) for tomato genes with homology to known alcohol dehydrogenases, we identified two ESTs whose translated protein sequence indicated significant similarity to putative *Eucalyptus gunnii* and *Vigna unguiculata* cinnamyl alcohol dehydrogenases (Fig. 2, EgCAD1 and VuCPRD14, respectively) as determined by the tBLASTn procedure of NCBI. However, since the cDNA for the first clone (*LePAR1*) was not full-length, its full-length cDNA was next cloned by 5′-RACE. The full-length sequence was then obtained by PCR, and confirmed by DNA sequence analysis (GenBank Accession EF613490). The deduced LePAR1 protein sequence has a calculated molecular mass of 35,908 Da, and has high homology to aldehyde reductases from many plant species (Fig. 2). The cDNA for the second gene (*LePAR2*, GenBank Accession EF613491) was full-length, and the encoded protein had a calculated molecular mass of 35,469 Da. The proteins encoded by the *LePAR1* and *LePAR2* cDNAs were most closely related to a putative cinnamyl alcohol dehydrogenase from *E. gunnii* (Goffner et al., 1998) (81% similar, 72% identical for LePAR1 and 85% similar, 73% identical for LePAR2). They were also closely related to a *Vigna radiata* aldehyde reductase (Guillen et al., 1998) (77% similar, 67% identical for LePAR1 and 84% similar/69% identical for LePAR2) (Fig. 2). Overall, LePAR1 and LePAR2 are more closely related to reductases than

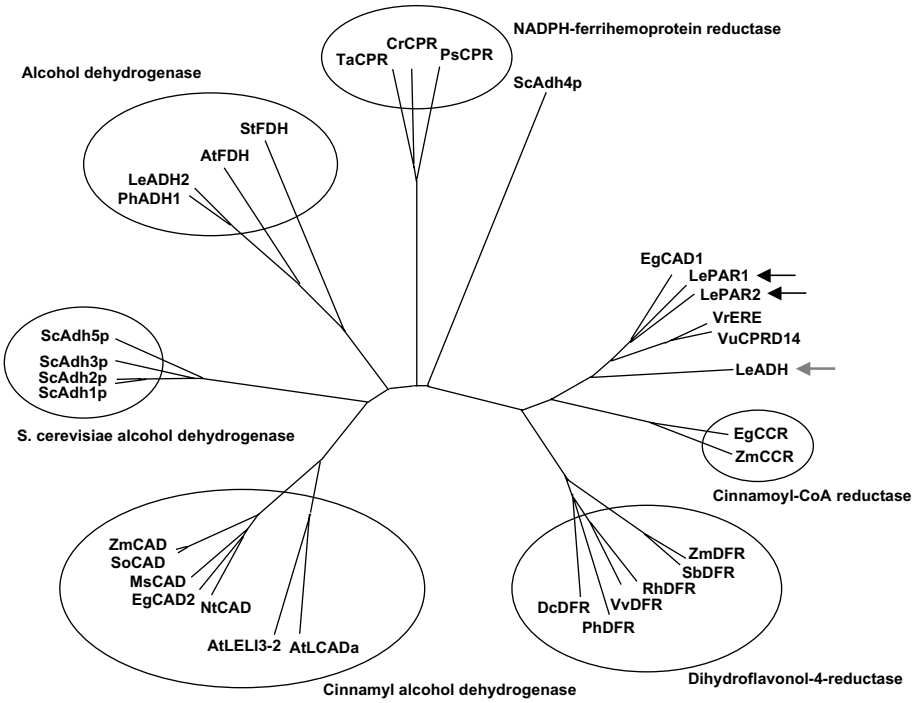

Fig. 2. Relationship between LePAR1, LePAR2 and other related alcohol dehydrogenases and reductases of known function. Sequences were aligned using the ClustalW multiple sequence alignment tool and the phylogenetic tree was generated by the neighbor-joining method. The identifiers and GenBank accession numbers for the proteins included in the tree are as follows: LePAR1and LePAR2, this paper (black arrows); LeADH, unknown function, this paper (gray arrow); EgCAD1, *Eucalyptus gunnii* cinnamyl alcohol dehydrogenase, T10736; VrERE, *Vigna radiata* aldehyde reductase, AAD53967; VuCPRD14, *V. unguiculata* cinnamyl alcohol dehydrogenase, T11610; EgCCR, *E. gunnii* cinnamoyl CoA reductase, T10733; ZmCCR, *Zea mays* cinnamoyl-CoA reductase, CAA66707; TaCPR, *Triticum aestivum* cytochrome P450 reductase, CAC83301; CrCPR, *Catharanthus roseus* cytochrome P450 reductase, Q05001; PsCPR, *Papaver somniferum* cytochrome P450 reductase, O24424; AtLCADa, *Arabidopsis thaliana* cinnamyl alcohol dehydrogenase, T05624; AtELI3-2, *A. thaliana* cinnamyl alcohol dehydrogenase, S28043; NtCAD, *Nicotiana tabacum* cinnamyl alcohol dehydrogenase, S23525; EgCAD2, *E. gunnii* cinnamyl alcohol dehydrogenase 2, P31655; MsCAD, *Medicago sativa* cinnamyl alcohol dehydrogenase, P31656; SoCAD, *Saccharum officinarum* cinnamyl alcohol dehydrogenase, O82056; ZmCAD, *Z. mays* cinnamyl alcohol dehydrogenase, T02990; ScAdh1p, *Saccharomyces cerevisiae* alcohol dehydrogenase, NP_014555; ScAdh2p, *S. cerevisiae* alcohol dehydrogenase, NP_014032; ScAdh3p, *S. cerevisiae* alcohol dehydrogenase, NP_013800; ScAdh5p, *S. cerevisiae* alcohol dehydrogenase, NP_009703; PhADH1, *Petunia hybrida* alcohol dehydrogenase, P25141; LeADH2, *Solanum lycopersicum* alcohol dehydrogenase 2, S51826; AtFDH, *A. thaliana* formaldehyde dehydrogenase, Q96533; StADH, *Solanum tuberosum* alcohol dehydrogenase, T07179; ScAdh4p, *S. cerevisiae* alcohol dehydrogenase, NP_011258; ZmDFR, *Z. mays* dihydroflavonol-4-reductase, P51108; SbDFR, *Sorghum bicolor* NADPH-dependent reductase, T03447; RhDFR, *Rosa hybrida* dihydroflavonol 4-reductase, BAA12723; VvDFR, *Vitis vinifera* dihydroflavonol-4-reductase, P51110; DcDFR, *Dianthus caryophyllus* dihydroflavonol 4-reductase, T10716; PhDFR, *P. hybrida* dihydroflavonol-4-reductase, S07463.

to dehydrogenases. However, their closest relative EgCAD1 has both reductase and dehydrogenase activities, having higher affinity for aldehydes than the corresponding alcohols (Goffner et al., 1998).

2.2. Activity of the tomato 2-phenylacetaldehyde reductase

The putative 2-phenylacetaldehyde reductases (LePAR1 and LePAR2) were expressed in *Escherichia coli* to determine activity and substrate specificity. To do this, the *LePAR1* or *LePAR2* coding regions were cloned into vector pDEST15 containing a GST-tag and transformed into *E. coli* BL21-AI cells for inducible expression. Enzyme activities of the purified recombinant proteins were determined on several substrates by measuring the loss of substrate over time. Maximal activity was observed between pH 6.0 and 7.5 (data not shown), with reductase activities of the purified proteins on 2-phenylacetaldehyde (**3**) and several related substrates determined (Table 1). The highest level of LePAR1 activity was observed with 2-phenylacetaldehyde (**3**) as a substrate ($K_m = 32$ μM), and it also had the highest turnover number (K_{cat}) with this substrate. The K_{cat}/K_m values show that LePAR1 was catalytically the most active with 2-phenylacetaldehyde (**3**) as substrate (Table 1). Lower activities were observed with cinnamaldehyde (**6**) ($K_m = 1179$ μM) and benzaldehyde (**5**) ($K_m = 572$ μM), whereas activity was not detected with salicylaldehyde. Similar LePAR2 activities were observed when the substrate was 2-phenylacetaldehyde (**3**), benzaldehyde (**5**) or cinnamaldehyde (**6**) (Table 1). The LePAR2 turnover numbers (K_{cat}) for each substrate were also similar, but lower than that of LePAR1. Analysis of the reaction products by gas chromatography (GC) confirmed that LePAR1 and LePAR2 converted 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**) (Fig. 3), with product identification confirmed by GC–MS. The conversion of one

Table 1
Kinetic parameters of LePAR1 and LePAR2

Substrate		K_m (μM)	V_{max} (nkatal mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})
2-Phenylacetaldehyde (**3**)	LePAR1	32 ± 10	503	31	0.96
	LePAR2	62 ± 4	33	2	0.033
Benzaldehyde (**5**)	LePAR1	572 ± 256	176	11	0.019
	LePAR2	42 ± 6	17	1	0.025
Cinnamaldehyde (**6**)	LePAR1	1179 ± 84	167	10	0.0088
	LePAR2	44 ± 24	21	1	0.029

Activities of purified recombinant LePAR1 and LePAR2 on 2-phenylacetaldehyde and related substrates were determined by measuring the conversion of NADPH to $NADP^+$ at pH 6.5 at 25 °C. K_m is presented as average ±SE.

molecule of 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**) required the conversion of one molecule of NADPH to $NADP^+$ (data not shown).

We also identified a third protein (Fig. 2, LeADH) closely related to the *E. gunnii* and *V. unguiculata* cinnamyl alcohol dehydrogenases (Fig. 2). However, no reductase activity on either 2-phenylacetaldehyde (**3**), cinnamaldehyde (**6**) or benzaldehyde (**5**) was observed.

LePAR1 and *LePAR2* were first identified in the TIGR tomato EST database as having homology to cinnamyl alcohol dehydrogenases. However, it is clear that such classifications are frequently incorrect and must be validated experimentally. For example, Kim et al. (2004) showed that many Arabidopsis genes annotated as putative cinnamyl alcohol dehydrogenases actually encode proteins with highly varied substrate specificities. The characterization of the recombinant LePAR1 enzyme showed that it has the highest activity upon 2-phenylacetaldehyde (**3**) with much less affinity for benzaldehyde (**5**) and cinnamaldehyde (**6**). As has been observed with other cinnamyl alcohol dehydrogenase/reductase enzymes, the enzymes required NADPH as a cofactor and no activity was detected with NADH. Activity for the reverse reaction with 2-phenylethanol (**4**) as a substrate was not detected (<1% of forward reaction), thus establishing the enzymes as reductases rather than dehydrogenases.

Many other plants, such as *Nicotiana* species and *Eucalyptus* species also produce 2-phenylethanol (**4**) in their flowers, leaves or fruits (Hellyer et al., 1966; Oka et al., 1999; Raguso et al., 2003). Cinnamyl alcohol dehydrogenases have been identified in both tobacco and *Eucalyptus*; however, the activity of these enzymes on 2-phenylacetaldehyde (**3**) has not been determined (Knight et al., 1992; Goffner et al., 1998). It would be particularly interesting to examine the activity of the *E. gunnii* cinnamyl alcohol dehydrogenase (Fig. 2, EgCAD1), since phylogenetic analysis groups it most closely with the tomato 2-phenylacetaldehyde reductases (Fig. 2, LePAR1 and LePAR2).

2.3. Expression of *LePAR1* and *LePAR2* in tomato plants

Expression of *LePAR1* and *LePAR2* at the mRNA level was determined for different tissues using quantitative real-time RT-PCR. *LePAR2* mRNA levels were higher than *LePAR1* levels in all tissues examined. *LePAR1* and *LePAR2* mRNA could be detected in all tissues examined with the highest expression occurring in flower buds (Fig. 4). Although expression of the genes was observed throughout fruit development, *LePAR1* and *LePAR2* mRNA levels decreased somewhat during ripening. This highest expression in flowers was consistent with 2-phenylethanol (**4**) emissions, which were highest on a per gram tissue basis from tomato flowers (data not shown).

2.4. Expression of *LePARs* in transgenic petunia

Tomato does not accumulate significant pools of 2-phenylacetaldehyde (**3**). Although we did identify transgenic tomato lines with elevated 2-phenylethanol (**4**) emissions, only one was significantly elevated relative to the control (data not shown), suggesting that synthesis of the substrate, 2-phenylacetaldehyde (**3**), is a limiting step in 2-phenylethanol (**4**) synthesis. In addition, transgenic tomato lines with reduced *LePAR* expression did not have reduced 2-phenylethanol (**4**) levels. These results likely are the consequence of additional proteins with 2-phenylacetaldehyde reductase activity in tomato. In EST database searches, over 50 tomato putative alcohol dehydrogenases were found, suggesting the potential for redundancy in this gene family.

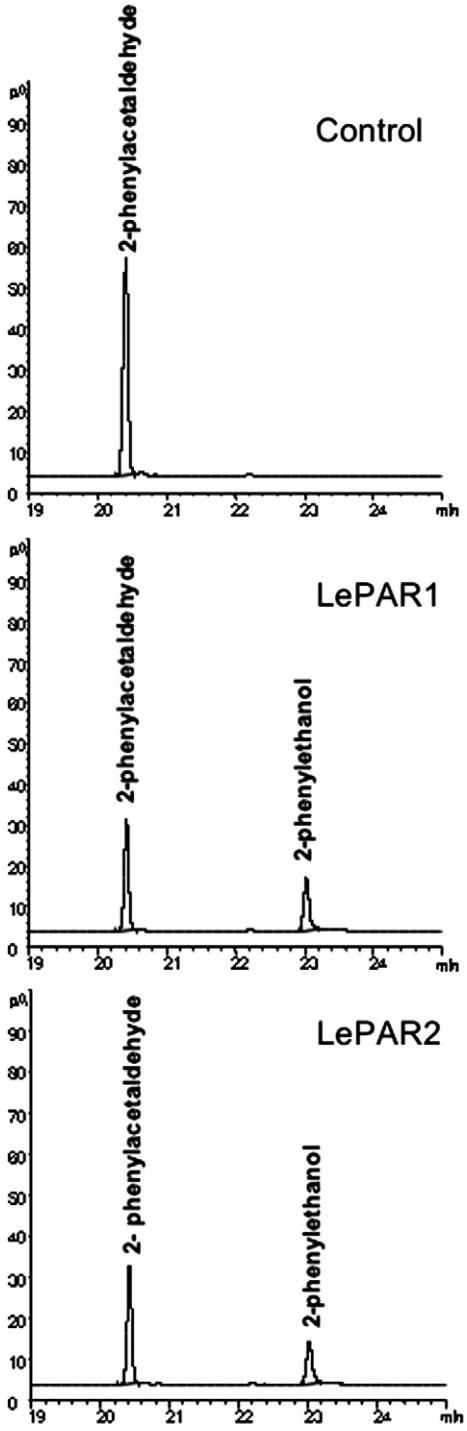

Fig. 3. Conversion of 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**) by LePAR1 and LePAR2. GC analysis of volatile components of the Control (no enzyme), LePAR1 or LePAR2 reaction mixtures. 2-Phenylacetaldehyde (**3**) was converted to 2-phenylethanol (**4**) upon inclusion of LePAR1or LePAR2.

To validate the role of the LePARs in production of 2-phenylethanol (**4**) *in vivo*, the genes were introduced into *Petunia hybrida* plants. We chose petunia as an *in vivo* model because they accumulate high levels of 2-phenylacetaldehyde (**3**), the preferred substrate of LePAR1 *in vitro*. Wild type 'Mitchell Diploid' petunia

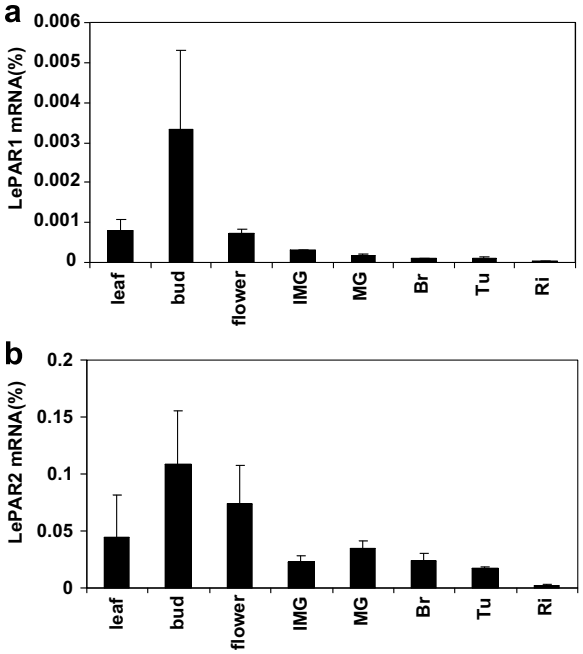

Fig. 4. *LePAR1* and *LePAR2* expression levels in wild type tomato (M82) plants. Tomato fruit RNA was prepared from immature green (IMG), mature green (MG), breaker (Br), turning (Tu) and red ripe (Ri) stages of development. Values are indicated ±SD.

flowers collected at night produce approximately 1 and 0.2 μg gfw^{-1} h^{-1} of 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**), respectively, while ripe tomato fruit only produce 0.01 and 0.1 ng gfw^{-1} h^{-1} of 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**), respectively. The full-length *LePAR* cDNAs were introduced separately into petunia 'Mitchell Diploid' under control of the constitutively expressed figwort mosaic virus 35S promoter. The levels of *LePAR* expression were measured in the transgenic petunia flowers by quantitative real-time RT-PCR (Fig. 5A and B). As expected, wild type flowers had no detectable RNA, indicating that the real-time PCR assay is specific for each *LePAR*, and is not recognizing any petunia *LePAR* homologs. Wild type petunia flowers emit high levels of 2-phenylacetaldehyde (**3**) and relatively lower levels of 2-phenylethanol (**4**). A range of 2-phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) levels was observed in the transgenic lines. The lines with detectable *LePAR1* or *LePAR2* RNA had higher levels of 2-phenylethanol (**4**) and lower levels of 2-phenylacetaldehyde (**3**) emissions than wild type flowers, whereas lines with low expression had lower levels of 2-phenylethanol (**4**) (Fig. 5c and d). This major shift in the ratio of 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**) indicates that the LePAR enzymes are recognizing 2-phenylacetaldehyde (**3**) *in vivo* and converting most of it to 2-phenylethanol (**4**). These data suggest that, in contrast to tomato, petunia 2-phenylacetaldehyde reductase activity is normally limiting, causing 2-phenylacetaldehyde (**3**) to predominate over 2-phenylethanol (**4**) in floral volatile emissions.

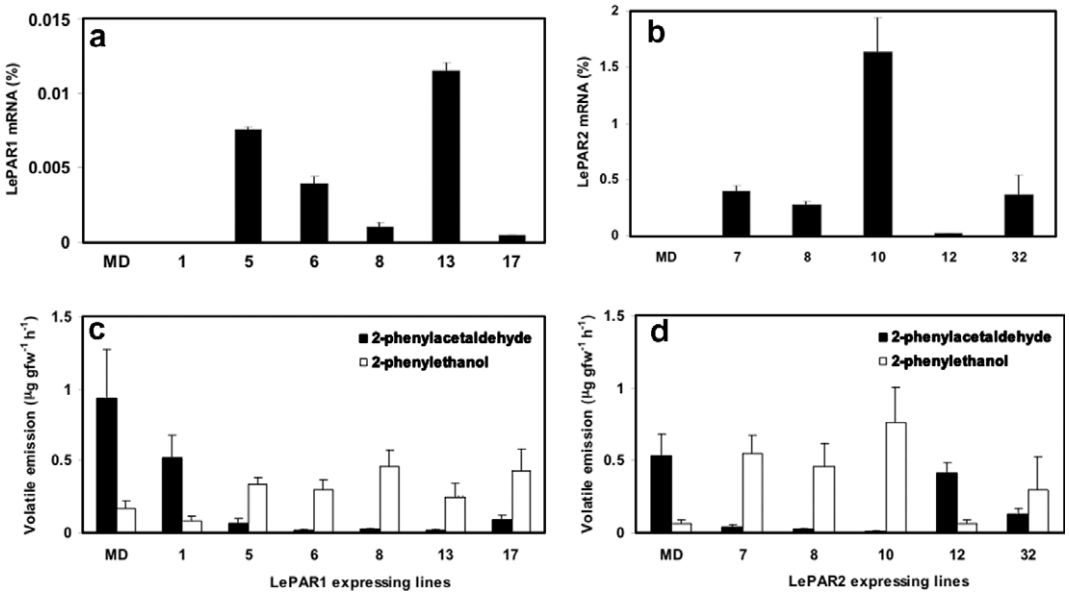

Fig. 5. *LePAR1* and*LePAR2* expression and volatile emissions in transgenic petunia flowers. (a) Expression of *LePAR1* RNA in flowers of wild type (MD) and six independent transgenic petunia lines expressing *LePAR1* (1, 5, 6, 8, 13, 17). (b) Expression of *LePAR2* RNA in flowers of wild type (MD) and six independent transgenic petunia lines expressing *LePAR2* (7, 8, 10, 12, 32). RNA was quantified using Taqman quantitative RT-PCR. (c) 2-Phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) emissions from wild type and transgenic flowers expressing *LePAR1* (±SE). (d) 2-Phenylacetaldehyde (**3**) and 2-phenylethanol (**4**) emissions from wild type and transgenic flowers expressing *LePAR2* (±SE).

Three of the petunia lines with the highest levels of *LePAR1* transgene expression and 2-phenylethanol (**4**) levels were self-pollinated and homozygous progeny were identified. Volatile emissions from flowers of these lines had significantly decreased levels of 2-phenylacetaldehyde (**3**) and increased levels of 2-phenylethanol (**4**) compared to controls (Fig. 6). Although LePAR1 is active on benzaldehyde (**5**) as a substrate *in vitro* and benzaldehyde (**5**) is present at higher levels than 2-phenylacetaldehyde (**3**), the transgenic petunia plants did not, in general, have

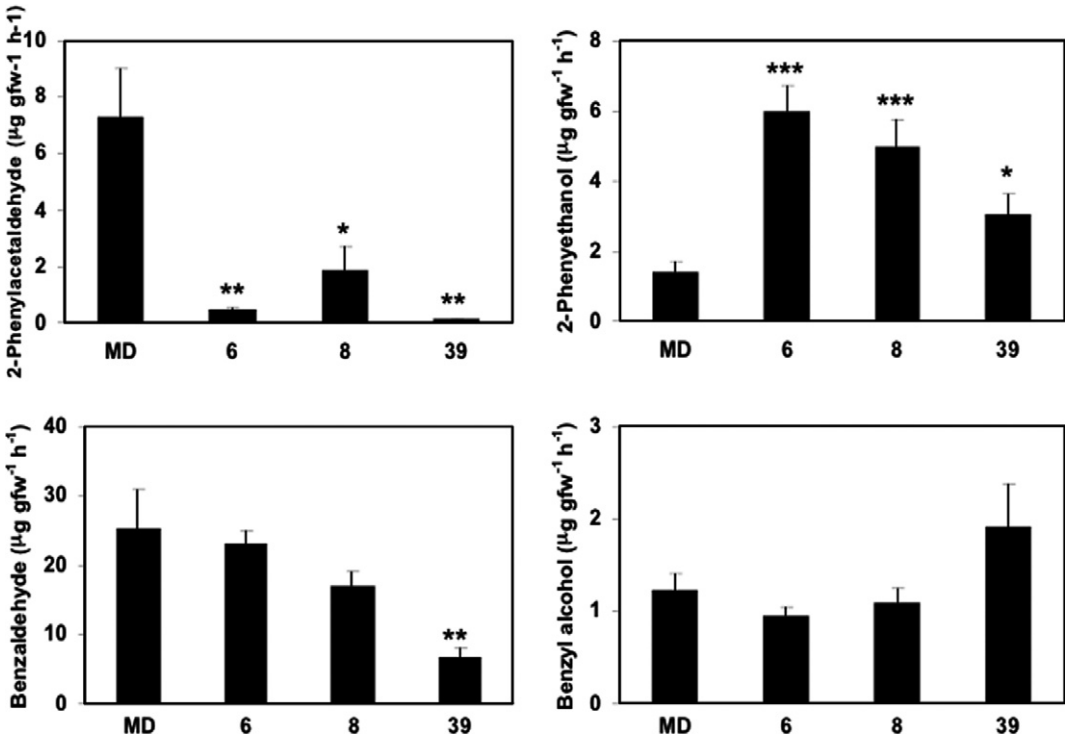

Fig. 6. Petunia floral volatile emissions. Levels of volatiles emitted by flowers from wild type Mitchell Diploid (MD) and three independent homozygous lines (6, 8 and 39) expressing the tomato *LePAR1* cDNA (±SE, $n = 6$). Asterisks (*, ** or ***) indicate results significantly different from wild type ($P < 0.05$, 0.01 or 0.001, respectively).

significantly altered emissions of benzaldehyde (**5**) or benzyl alcohol. Benzaldehyde (**5**) emissions were somewhat lower in one transgenic line than in wild type flowers, although benzyl alcohol levels were not significantly different (Fig. 6, line 39). Thus, the *in vivo* data are consistent with a specific function for LePAR1 in the reduction of 2-phenylacetaldehyde (**3**) to its alcohol (**4**). Overall, these data indicate that the introduction of the *LePAR1* transgene results in the conversion of 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**) in petunia flowers. Although LePAR1 has the highest affinity for 2-phenylacetaldehyde (**3**) as a substrate, it does have activity on benzaldehyde (**5**) *in vitro*. However, as we would predict from the K_{cat}/K_m (Table 1), we saw no significant effect of transgene expression upon benzyl alcohol or benzaldehyde (**5**) levels in the transgenic petunias.

Progeny from three *LePAR2* expressing lines were also examined for altered volatile levels. As in the primary transformants, 2-phenylacetaldehyde (**3**) levels were reduced and 2-phenylethanol (**4**) levels were higher in the transgenic flowers than in control flowers. One *LePAR2* expressing line (Fig. 7, line 7) also had lower levels of benzaldehyde (**5**), although benzyl alcohol levels were not affected. Although the K_m values for 2-phenylacetaldehyde (**3**) and benzaldehyde (**5**) were similar, the levels of benzyl alcohol were not significantly affected in the transgenic petunias. It is possible that the petunia homolog of LePAR1 has higher affinity for benzaldehyde (**5**) and contributes to the conversion of both substrates to their corresponding alcohols. Alternatively, separate enzymes may be present in petunia with preference for either benzaldehyde (**5**) or 2-phenylacetaldehyde (**3**). It is possible than increases in benzyl alcohol are not seen because it is further converted to other compounds. It has been predicted by metabolic flux analysis that benzyl alcohol is further converted to benzyl benzoate in petunia (Boatright et al., 2004).

LePAR1 and LePAR2 can convert 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**) in transgenic petunia flowers. Petunia is an excellent system for the study of the action of this enzyme *in vivo*, since the flowers emit both metabolites. Transgenic plants expressing the tomato gene have flowers that emit much reduced levels of 2-phenylacetaldehyde (**3**) and higher levels of 2-phenylethanol (**4**), providing *in vivo* confirmation of the action of LePAR1. Interestingly, although the levels of 2-phenylethanol (**4**) are higher in the transgenic *LePAR1*-expressing petunia flowers, the increases are not equivalent on a molar basis to the reductions in 2-phenylacetaldehyde (**3**). We cannot exclude a feedback system in which 2-phenylethanol (**4**) negatively regulates flux through the pathway. Alternatively, a portion of the 2-phenylethanol (**4**) produced in the transgenic petunia flowers may be converted to 2-phenylethanol β-D-glucopyranoside. Glucosylation occurs in rose flowers administered deuterium-labeled phenylalanine. In rose flowers, 2-phenylethyl-β-D-glucopyranoside accumulates before flower opening and declines after flower opening and 2-phenylethanol (**4**) evolution. An increase in β-glucosidase activity was observed upon flower opening, consistent with the emitted 2-phenylethanol (**4**) being released from the 2-phenylethyl-β-D-glucopyranoside (Watanabe et al., 2002;

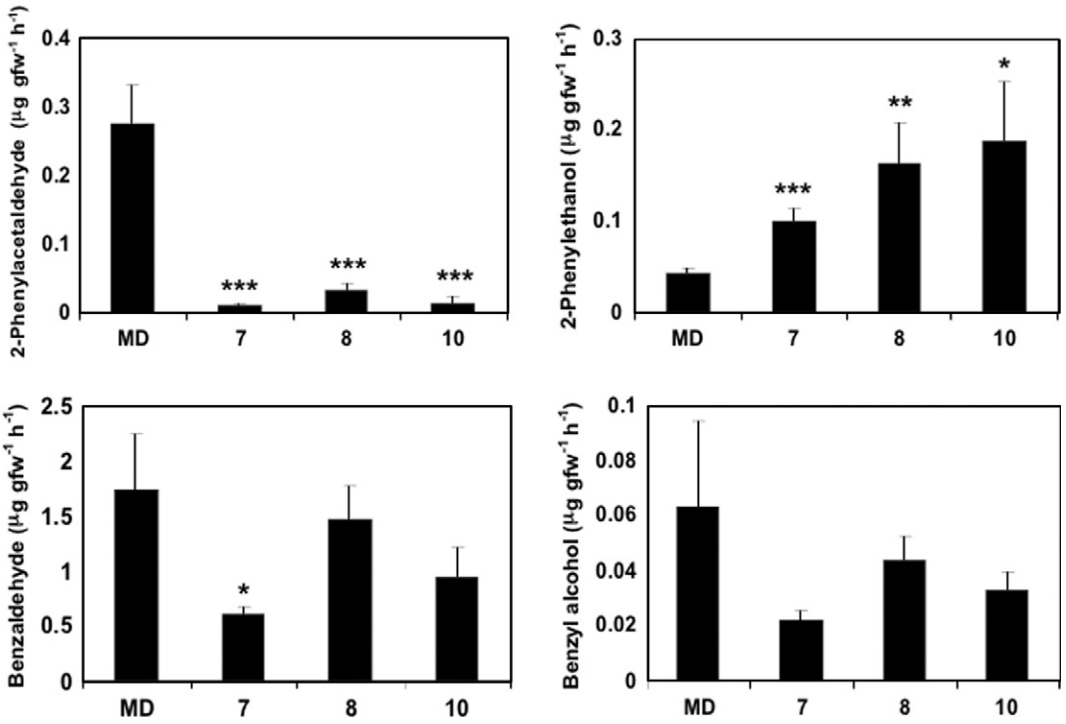

Fig. 7. Petunia floral volatile emissions. Levels of volatiles emitted by flowers from wild type Mitchell Diploid (MD) and progeny from three independent lines (7, 8 and 10) expressing the tomato *LePAR2* cDNA (±SE, $n = 6$). Asterisks (*, ** or ***) indicate results significantly different from wild type ($P < 0.05$, 0.01 or 0.001), respectively.

Oka et al., 1999; Hayashi et al., 2004). We have detected significant quantities of 2-phenylethyl-β-D-glucopyranoside in petunia flowers (unpublished results).

LePAR1 is a member of the short-chain dehydrogenase/reductase (SDR) family. This is a large and diverse family of over 3000 enzymes from all kingdoms with broad substrate specificities that include many aromatic compounds (Persson et al., 2003). The *Arabidopsis thaliana* genome contains 138 members of this diverse family. Phylogenetic analysis groups LePAR1 with a *E. gunnii* enzyme called a cinnamyl alcohol dehydrogenase; however, the *Eucalyptus* gene does not group with other characterized cinnamyl alcohol dehydrogenases (Fig. 2), and has a strong preference for aldehyde substrates over their corresponding alcohols. The *Eucalyptus* enzyme has a broad substrate specificity but 2-phenylacetaldehyde (**3**) was not tested as a potential substrate (Goffner et al., 1998). Another closely related aromatic aldehyde reductase from *V. radiata* was shown to reduce the fungal toxin eutypine (Guillen et al., 1998). Again, this enzyme was not tested with 2-phenylacetaldehyde (**3**) as a substrate. Since many plants have the capacity to synthesize 2-phenylethanol (**4**), it is likely that a number of the genes annotated as cinnamyl alcohol dehydrogenases actually encode enzymes that reduce 2-phenylacetaldehyde (**3**) to 2-phenylethanol (**4**).

2.5. Concluding remarks

The identification of genes encoding enzymes in the pathways to flavor and aroma volatiles should enable researchers to manipulate fruit flavor and flower scent by conventional breeding or metabolic engineering. Indeed, expression of *LePAR1* or*LePAR2* in transgenic petunias does significantly alter the volatile profile of flowers. It will be interesting to determine the effects of the altered volatile emissions on attraction of pollinating insects as well as human preferences. This is an step toward elucidation of the pathway(s) to the flavor and aroma volatile, 2-phenylethanol (**4**).

3. Experimental

3.1. Plant material

Tomato (*S. lycopersicum* cv. M82) plants were grown in the greenhouse under standard conditions (Tieman et al., 2001), whereas petunia plants (*Petunia* × *hybrida*, cv. Mitchell Diploid) were greenhouse grown as described earlier (Underwood et al., 2005).

3.2. Volatile collection

Petunia volatiles were collected from fully open flowers with nonyl acetate as an internal standard as described by Schmelz et al. (2003). Flowers were harvested in the evening after sunset when volatile levels were high. Flowers were enclosed in glass tubes, air filtered through a hydrocarbon trap (Agilent, Palo Alto, CA) flowed through the tubes for 1 h with the aid of a vacuum pump connected to a Super Q column. Volatiles collected on the Super Q column were eluted with CH_2Cl_2, with the volatiles separated on an Agilent (Palo Alto, CA) DB-5 column and analyzed on an Agilent 6890N gas chromatograph; retention times were compared to known standards. Identities of volatile peaks were confirmed by GC–MS as described by Schmelz et al. (2001). GC standards were purchased from Sigma–Aldrich (St. Louis, MO).

3.3. LePAR expression in E. coli

A partial *LePAR1* cDNA was obtained from the TIGR database. The full-length 2-phenylacetaldehyde reductase cDNA was cloned by 5′-RACE from tomato fruit cDNAs using primer 5′TCCTTGGCCCCACCAAGAGAAAGCAAGTGCTGCGT. Following sequence analysis of the 5′-RACE products, the full-length cDNA was obtained by PCR. The full-length *LePAR2* gene sequence was obtained from the TIGR database. The *LePAR2* coding region was amplified from cDNA with forward primer 5′CACCATGGCGATGAGAACAGTATGTGTAACAG and reverse primer 5′TCAGTAAAACTTCTTCTCTTTCAAGCTTTCAGC. The *LeADH* coding region was amplified from cDNA with forward primer 5′CACCATGGAAAGTAAGAATATTGGAG and reverse primer 5′TTAAATGTGGAGGAAGTTC. The sequences of the *LePAR1*, *LePAR2* and *LeADH* cDNAs have been deposited in Genbank (Accession Nos. EF613490, EF613491, and EF613492, respectively). The coding regions of both *LePARs* were cloned into vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA). The coding region was then recombined into vector pDEST15 containing a GST tag (Invitrogen, Carlsbad, CA), and transformed into *E. coli* strain BL21-AI (Invitrogen, Carlsbad, CA) for inducible protein expression. To purify GST-tagged LePAR proteins, bacterial cultures were centrifuged at 5000*g* for 5 min, followed by sonication for 1 min in 1× PBS buffer and centrifugation at 10,000*g* for 15 min. The GST-tagged protein was purified from the supernatant by using Glutathione-Uniflow resin (BD Biosciences, Mountain View, CA) according to the manufacturer's instructions followed by removal of glutathione with PD-10 desalting columns (Amersham Biosciences, Piscataway, NJ). Protein purity was determined by SDS–PAGE followed by staining with Coomassie brilliant blue. Enzyme activity of *E. coli*-expressed proteins was determined by the method of Larroy et al. (2002) using 2-phenylacetaldehyde (**3**), cinnamaldehyde (**6**) or benzaldehyde (**5**) as a substrate. Assays were performed in triplicate at 25 °C in a 100 μl reaction. The reaction was monitored at 365 nm, and an extinction coefficient of $3.4 \times 10^3\ M^{-1}\ cm^{-1}$ was used to calculate the conversion of NADPH to $NADP^+$. The apparent K_m and V_{max} values were determined using Lineweaver–Burk plots. Protein concentrations were

measured using a Bradford protein determination kit using BSA as a standard (Bio-Rad Laboratories, Hercules, CA). Dehydrogenase activity of the reverse reaction was measured using 2-phenylethanol (**4**) as a substrate, and the conversion of $NADP^+$ to NADPH was monitored. For GC analysis of reaction products the reaction mixture containing nonyl acetate as a control for recovery was extracted twice with an equal volume of CH_2Cl_2. Volatiles were separated on an Agilent DB-5 column and analyzed on an Agilent 6890N gas chromatograph with a flame ionization detector and retention times compared to known standards. GC–MS was performed on an Agilent 6890N gas chromatograph with an Agilent 5975 mass selective detector. The reaction mixtures produced the characteristic 2-phenylethanol (**4**) mass-spectral fragments of *m/z* 102, 91, 77, 65, 51 as well as the parent ion of $m/z = 122$.

3.4. RNA expression analysis

Total RNA was extracted using a Qiagen (Valencia, CA) total RNA extraction kit followed by DNase treatment to remove any contaminating DNA. *LePAR* mRNA levels were measured by real-time quantitative RT-PCR using Taqman One-Step RT-PCR reagents and a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probe for real-time PCR were as follows: LePAR1 Taqman probe, 5′6-FAM-CCATCAGTAAAACGAGTTGTTTTAACGTCTTCCA-3′BHQ; LePAR1 forward primer, CGGGTCATGTGCCAAAGC; LePAR1 reverse primer, GGCTGACCACTGTAAGCAACTG; LePAR2 Taqman probe, 6-FAM-TCCCTGGATGGAGCTAAGGAGAGACTCC-3′BHQ; LePAR2 forward primer, ATGATCAAAAAAAGGTAGATCACTTGAC; LePAR2 reverse primer, CCAGTAGGTCCGCTTTGAACA. For absolute quantification of RNA, a standard curve was constructed from *LePAR1* RNA. *LePAR1* RNA was synthesized by *in vitro* transcription of the coding sequence as described previously (Tieman et al., 2001).

3.5. Production of transgenic petunia plants

The full-length 2-phenylacetaldehyde reductase cDNAs were cloned into a vector under the control of the figwort mosaic virus 35S promoter (Richins et al., 1987) and followed by the *Agrobacterium* nopaline synthase (*nos*) 3′ terminator. The transgene was introduced into *P. hybrida* cv. Mitchell Diploid by the method of Jorgensen et al. (1996) with kanamycin resistance as a selectable marker. Transgenic plants were grown to maturity in a greenhouse and self-pollinated to obtain seed.

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