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Analysis of the enzymatic formation of citral in the glands of sweet basil

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Abstract

Basil glands of the Sweet Dani cultivar contain high levels of citral, a mixture of geranial and its *cis*-isomer neral, as well as low levels of geraniol and nerol. We have previously reported the identification of a cDNA from Sweet Dani that encodes an enzyme responsible for the formation of geraniol from geranyl diphosphate in the glands, and that these glands cannot synthesize nerol directly from geranyl diphosphate. Here, we report the identification of two basil cDNAs encoding $NADP^+$-dependent dehydrogenases that can use geraniol as the substrate. One cDNA, designated *CAD1*, represents a gene whose expression is highly specific to gland cells of all three basil cultivars examined, regardless of their citral content, and encodes an enzyme with high sequence similarity to known cinnamyl alcohol dehydrogenases (CADs). The enzyme encoded by *CAD1* reversibly oxidizes geraniol to produce geranial (which reversibly isomerizes to neral via keto–enol tautomerization) at half the efficiency compared with its activity with cinnamyl alcohol. CAD1 does not use nerol and neral as substrates. A second cDNA, designated *GEDH1*, encodes an enzyme with sequence similarity to CAD1 that is capable of reversibly oxidizing geraniol and nerol in equal efficiency, and prolonged incubation of geraniol with GEDH1 in vitro produces not only geranial and neral, but also nerol. GEDH1 is also active, although at a lower efficiency, with cinnamyl alcohol. However, *GEDH1* is expressed at low levels in glands of all cultivars compared with its expression in leaves. These and additional data presented indicate that basil glands may contain additional dehydrogenases capable of oxidizing geraniol.

Keywords: Citral; Geraniol; Nerol; Geraniol dehydrogenase; Cinnamyl alcohol dehydrogenase; Sweet basil; Secondary compounds; Plant biochemistry; EST databases; Evolution

Citral, the name given to the mixture of the monoterpene aldehydes geranial and neral, imparts a strong "lemony" scent and is known to be emitted or accumulated in such herbs as lemon grass [1], ginger [2], and some varieties of sweet basil [3,4]. Citral is a valuable flavor and scent reagent that is heavily used in the food and perfume industries [5,6]. Several previous investigations have reported that citral is synthesized from geraniol or nerol by an alcohol dehydrogenase [7,8] or alcohol oxidase [9,10], but an enzyme capable of catalyzing such a reaction has not yet been purified and characterized.

Previously, we showed that sweet basil cultivar Sweet Dani is particularly rich in citral, which is localized in the oil sac attached to the peltate glands on the surface of the leaves [11]. In glands of this variety, geranial and neral are the main terpene constituents, while small amounts of geraniol and nerol are also observed. We have previously shown that Sweet Dani glands synthesize geraniol from geranyl diphosphate (GPP)[1] in a reaction catalyzed by geraniol synthase (GES).

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[1] *Abbreviations used:* GPP, geranyl diphosphate; GES, geraniol synthase; SD, Sweet Dani; GEDH, geraniol dehydrogenase; GC, gas chromatography; EST, expressed sequence tag; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase.

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Furthermore, these glands do not express a terpene synthase that can convert GPP to nerol [11].

Here, we report the isolation of two basil cDNAs encoding dehydrogenases capable of oxidizing geraniol to geranial, which then undergoes tautomerization to neral, yielding the mixture of geranial and neral (i.e., citral). We functionally expressed these cDNAs in *Escherichia coli* and biochemically characterized the enzymes to determine their kinetic parameters and cofactor requirements. Moreover, we examined the expression level of the genes encoding these enzymes in Sweet Dani glands and leaves as well as in glands and leaves of two other basil cultivars that do not produce citral. The analysis of the combined data indicates that while basil plants have at least one dehydrogenase that is highly specific for geraniol, this enzyme is expressed at low levels in the glands as compared to its expression in non-gland leaf tissue. On the other hand, a gland-specific dehydrogenase that has a higher specificity to cinnamyl alcohol than to geraniol nonetheless appears to make an important contribution to the production of citral in the glands.

Materials and methods

Plant material

Plants of the three basil cultivars Sweet Dani (SD), EMX, and SW were grown in the green house under controlled illumination as described in previous reports [11,12].

Glands' crude enzyme extraction

Glands were isolated following the procedures previously described by Gang et al. [16]. Crude enzyme preparations from these glands were prepared as previously described [11].

Isolation of cDNAs and expression in E. coli

The construction of EST databases from the peltate glands of cultivars SD, EMX, and SW was previously reported [12]. BLAST searches revealed numerous ESTs with sequence similarity to alcohol dehydrogenases. Putative ADH cDNAs were assembled into contigs. Full-length cDNA from each contig (obtained by 5′ RACE when necessary) was cloned into the pCRT7/CT-TOPO TA vector (Invitrogen, Carlsbad, CA) and expressed in the *E. coli* expression system. *CAD1* cDNA was also ligated into the expression vector pET28-(a) to produce a protein with an N-terminal His tag. These plasmids were transformed into Codon Plus cells (Invitrogen). *E. coli* cultures carrying alcohol dehydrogenase expression constructs were induced by adding 0.5 mM IPTG with 10 μM $ZnCl_2$ and grown at 18 °C for 18 h. Cells were then harvested by centrifugation, resuspended in lysis buffer, and sonicated as previously described [12].

Purification of heterologously expressed GEDH1

All purification steps were carried out at 4 °C unless stated otherwise. The supernatant of the bacterial lysate (20 mL) after induction of GEDH1 protein in *E. coli* was loaded onto a DEAE–cellulose column (8 mL of DE53; Whatman, Clifton, NJ) pre-equilibrated with a buffer containing 100 mM Tris–HCl, pH 7.5, 20% glycerol [v/v], and 10 mM β-mercaptoethanol. After the sample was loaded, the column was washed with 30 mL of the pre-equilibration buffer. GEDH1 protein did not bind to DE53 as most of the recombinant protein eluted in the wash fractions. Next, 150 μL of this enzyme solution was loaded onto a size-exclusion column (10 × 300 mm) packed with Superose 12 (Pharmacia Biotech), and the active fractions were isocratically eluted with 100 mM KCl in Buffer A at 0.5 mL/min. Fractions (0.5 mL each) were collected and protein purity was estimated by SDS–PAGE, followed by Coomassie brilliant blue staining or silver staining. The fraction with the highest degree of purity was used for further characterization. Protein concentrations were measured by the Bradford method or by staining intensity on SDS–PAGE gel compared with bovine serum albumin concentration standards.

Purification of heterologously expressed CAD1

His-tagged basil *CAD1* cDNA was expressed in *E. coli* as described above for the *GEDH1* cDNA and purified using Ni–NTA affinity columns as previously described [13].

Sequence analysis

Alignment of multiple protein sequences was performed using the ClustalX program [14]. Sequence relatedness by the neighbor-joining method was determined using the protocol included in the ClustalX package. The phylogenic tree was drawn using the TREEVIEW program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) [15].

Dehydrogenase enzyme assays

Oxidative dehydrogenase activity was assayed by incubating 5 μL of the enzyme sample in a final volume of 100 μL buffer containing 100 mM glycine–NaOH, pH 9.5, 1 mM $NADP^+$, and 1 mM substrate. Assays were carried out at 25 °C for 15–30 min and time courses were measured by monitoring the formation of the generated NADPH (or NADH) in a spectrophotometer at 340 nm. The molar extinction coefficient (ε_{340}) used for NADPH