A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene

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ABSTRACT

Chicory (Cichorium intybus L.), which is known to have a variety of terpene-hydroxylating activities, was screened for a P450 mono-oxygenase to convert (+)-valencene to (+)-nootkatone. A novel P450 cDNA was identified in a chicory root EST library. Co-expression of the enzyme with a valencene synthase in yeast, led to formation of trans-nootkatol, cis-nootkatol and (+)-nootkatone. The novel enzyme was also found to catalyse a three step conversion of germacrene A to germacr-1(10),4,11(13)-trien-12-oic acid, indicating its involvement in chicory sesquiterpene lactone biosynthesis. Likewise, amorpha-4,11-diene was converted to artemisinic acid. Surprisingly, the chicory P450 has a different regio-specificity on (+)-valencene compared to germacrene A and amorpha-4,11-diene.

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1. Introduction

Many sesquiterpenes are of significant importance for the flavour and fragrance industry. Hydroxylation of these terpenes often adds to their sensory properties, and is therefore an essential step in the production of flavour molecules. The regio- and stereoselective introduction of a hydroxyl group into an unactivated organic compound by means of organic synthesis is still challenging [1]. For this reason, hydroxylating enzymes such as cytochrome P450 mono-oxygenases are considered as an alternative way to introduce regioselective modifications [2] and have great potential for application in the production of natural flavour molecules.

(+)-Nootkatone is an important oxidised sesquiterpene for the flavour and fragrance industry. It has a characteristic grapefruit-like flavour and a low odour threshold [3]. Natural (+)-nootkatone can be extracted from grapefruit [4]. Since grapefruit material is limited on the world market, synthetic (+)-nootkatone produced from (+)-valencene is predominantly used in commercial applications, also for the flavour market [5]. Chemical oxidation of valencene requires the use of tert-butyl chromate [6], which is a carcinogenic substance. Alternatively non-carcinogenic tert-butyl peracetate [7] or tert-butyl hydroperoxide can be used which are highly flammable and corrosive compounds.

(+)-Nootkatone may also be produced from (+)-valencene via biotechnological approaches in recombinant organisms using the enzymes involved in its biosynthesis [5]. The biosynthetic route to (+)-nootkatone in grapefruit has not been established experimentally, but has been suggested to start from valencene on which a regioselective allylic hydroxylation results in formation of 2-hydroxyvalencene (or nootkatol), followed by oxidation to (+)-nootkatone [4,5].

Several enzymes have been reported to mediate oxidation of (+)-valencene to (+)-nootkatone or to its precursor nootkatol. Engineered cytochrome P450s from Pseudomonas putida and Bacillus megaterium were shown to convert valencene into nootkatone [8,9]. Recently, a (+)-valencene dioxygenase from Pleurotus sapidus was demonstrated to have this activity. For plants, several cytochrome P450 enzymes capable of oxidising valencene have been described. The prennaspirodiene oxygenase from Hyoscyamus

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2. Materials and methods

2.1. Isolation and cloning of P450 gene from chicory

An expressed sequence tag (EST) database of a cDNA library derived from the taproot of chicory was produced using the Roche 454 sequencing platform. Sequences were assembled using an in-house bioinformatics facility and candidate P450s contigs were identified by sequence homology to known sesquiterpene hydroxylases. RACE PCR (Clontech) was used to obtain the sequence of the identified by sequence homology to known sesquiterpene hydroxylases. RACE PCR (Clontech) was used to obtain the sequence of the bioinformatics facility and candidate P450s contigs were found as reaction products [11]. Plant microsomal extracts, such as the chicory root extract, likely contain a variety of P450 enzymes. The formation of bitter sesquiterpene lactones in root tissue requires several oxygenation steps, at different regions of the sesquiterpene carbon scaffold. Recently, a cytochrome P450 from chicory has been cloned which catalyzes oxidation of germacrene A and amorpha-4,11-diene at the allylic position C12 [12]. Our aim was to clone a chicory cytochrome P450 which is capable of catalyzing the regioselective oxidation of (+)-valencene and which can be used for the biotechnological production of (+)-nootkatone.

2.2. Co-expression of CYP71AV8 with terpene synthases in yeast

The full length gene was re-cloned, using the NotI/PacI restriction sites, into the yeast expression vector pYEDP80 [17] which was modified to contain PacI and NotI sites at the polylinker. The candidate chicory P450 was co-transformed with either a germacrene A synthase [18], amorpha-4,11-diene synthase [14] or a valencene synthase [19] into the into yeast strain WAT11 expressing Arabidopsis ATR1 NADPH-cytochrome P450 reductase [20]. Germacrene A synthase was cloned into pYEDP80 vector [17] with TRP1 auxotrophic selection marker using BamHI and EcoRI restriction sites. Amorpha-4,11-diene synthase and valencene synthase were both cloned into pYES3/CT yeast expression vector (Invitrogen) with TRP1 selection marker using NotI/BamHI and PacI restriction sites. Amorpha-4,11-diene and (+)-nootkatone were found as reaction products [21]. The second step was diluted to OD600 of 0.05 in 50 ml of Synthetic Galactose minimal medium and incubated at 200 rpm at 30 °C. The culture was overlaid with 5 ml of n-dodecane [21] when the OD600 was in the range from 0.8 to 1 and cultivation was continued for 3 days. The n-dodecane layer was collected and centrifuged at 1200 rpm for 10 min, diluted threefold in ethyl acetate, dried using anhydrous Na2SO4 and then used for GC-MS analysis. In co-expression experiments with amorpha-4,11-diene synthase and germacrene A synthase 7 ml of the yeast culture was further extracted three times with 2 ml ethyl acetate, concentrated, dehydrated using anhydrous Na2SO4 and used for GC-MS analysis. For analysis of artemisinic acid and germacra-1(10),4,11(13)-trien-12-ol, samples were first methylated using diazomethane.

2.3. GC-MS analysis

Analytes from 1 µL samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30 m × 0.25 mm, 0.25 mm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1 ml/min. The injector was used in splitless mode with the inlet temperature set to 250 °C. The initial oven temperature of 45 °C was increased after 1 min to 300 °C at a rate of 10 °C/min and held for 5 min at 300 °C. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard). Compounds were identified by comparison of mass spectra and retention times (rt) with those of the following authentic standards: germacrene A, germacra-1(10),4,11(13)-trien-12-ol, germacra-1(10),4,11 (13)-trien-12-ol [11], amorpha-4,11-diene, artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde, artemisinic acid [22], (+)-valencene, trans-nootkatol, cis-nootkatol (Isobionics) and (+)-nootkatone (Fluka). Quantification of sesquiterpenes was conducted by determination of total ion count (TIC) peak area of the sesquiterpene peaks from three independent fermentation experiments. Absolute concentration of sesquiterpenes was calculated from the peak area by comparison to a standard curve prepared by measuring a dilution series of authentic standards with known concentrations.

3. Results

3.1. Isolation of a novel P450 gene from chicory

A cDNA library was created from root tissue of C. intybus. This library was analyzed by sequencing using the Roche 454 sequencing platform, which resulted in 575 945 EST sequences of on average 400 bp in size. By sequence assembly, 40 847 contigs were formed from which 31 787 singletons remained.

The sequences were combined with 35 973 C. intybus EST sequences from GenBank, and interrogated for sequences with homology to the cytochrome P450 sequences of prenmaspirodiene oxygenase from H. muticus [10] and amorpha-4,11-diene oxidase from Artemisia annua [13,14]. Two sequences with high similarity to both genes were identified, one of which encoded the recently described C. intybus germacrene A oxidase [12]. The second sequence encodes a novel protein CYP71AV8 with 50% sequence identity to prenmaspirodiene oxygenase, 78% to amorpha-4,11-diene oxidase and 81% to the germacrene A oxidase from C. intybus.
3.2. CYP71AV8 is a (+)-valencene oxidase and produces (+)-nootkatone in vivo

To assess its value in a biotechnological production platform, the activity of CYP71AV8 on (+)-valencene was tested by co-expression of the enzyme with a valencene synthase in yeast. In yeast cultures co-expressing valencene synthase and CYP71AV8 the majority of (+)-valencene had disappeared compared to control cultures without P450 (Table 1). As novel product, the predominant peak was identified as trans-nootkatol (rt = 16.53 min). A smaller peak of cis-nootkatol was detected (rt = 16.36 min) (Fig. 1). At a retention time of 17.51 min a small peak of (+)-nootkatone was detected. A

<table>
<thead>
<tr>
<th>Enzymes expressed</th>
<th>Products</th>
<th>Quantity mg/l yeast culture (mean ± S.D., n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS</td>
<td>(+)-valencene</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>VS + CYP71AV8</td>
<td>(+)-valencene</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>trans-nootkatol</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>cis-nootkatol</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(+)-nootkatone</td>
<td>0.04 ± 0.00</td>
</tr>
</tbody>
</table>

VS = valencene synthase.

Fig. 1. GC-MS analysis of in vivo hydroxylation of (+)-valencene by CYP71AV8 to nootkatol and (+)-nootkatone. Chromatogram of the n-dodecane layer from (a) yeast strain WAT11 producing (+)-valencene (1) is compared to (b) the yeast strain WAT11 expressing valencene synthase and CYP71AV8 where (+)-valencene (1) is transformed into cis-nootkatol (2), trans-nootkatol (3) and trace amounts of (+)-nootkatone (4). Inserts in the chromatograms zoom in on the region of 17.2 to 17.7 min of the chromatograms. The y-axis scales of the chromatograms are identical. Mass spectra for (+)-nootkatone (c), cis-nootkatol (e) and trans-nootkatol (g) produced by yeast and (+)-nootkatone (d), cis-nootkatol (f) and trans-nootkatol (h) standards are shown.
observed. Thus CYP71AV8 can also function as a germacrene A synthase, catalyzing a three-step conversion of germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid, as was previously shown for the chicory germacrene A oxidase [12]. The authors characterized a plant P450 enzyme that preferentially hydroxylates the C2 position of (+)-valencene in the trans-orientation. A small portion of nootkatol is further oxidised to (+)-nootkatone. Likely this results from the poor production of (+)-valencene and other precursors. Extensive engineering of yeast producing artemisinic acid resulted in production levels of up to 100 mg/L culture [13].

Surprisingly, the same enzyme which oxidises the C2 position in (+)-valencene, also oxidises at the C12 position, when amorpha-4,11-diene or germacrene are supplied as substrates (Fig. 3). This observation may provide novel insights in the regiospecificity of plant P450 enzymes. One of the few reports discussing factors affecting regiospecificity is the pioneering work of Takahashi and coworkers [10]. The authors characterized H. muticus premaspirodiene oxygenase, which introduces an oxygen exclusively in the C2 regio-position of the A ring of both spirodecanes (e.g. premaspirodiene) and eremophilanes (5-epi-aristolochene, epi-eremopheline and (+)-valencene). In the recently published work by Nguyen et al. [12], the chicory germacrene A oxidase is shown to selectively oxidise at the C12 position of both germacrene A and amorpha-4,11-diene. Thus it would seem that two categories of sesquiterpene modifying P450 enzymes can be distinguished in the plant kingdom: those that act on the A-ring, such as the H. muticus premaspirodiene oxygenase, and those that act on the allylic C12 position, such as the chicory germacrene A oxidase and the A. annua amorpha-4,11-diene oxidase. Therefore, finding that a single P450 can oxidise different regio-positions on such structurally related molecules as germacrene A and (+)-valencene was unexpected.

In the past, De Kraker et al. [11], reported that a microsomal extract of chicory could indeed perform both regiospecific reactions. In the latter work, it was postulated that chicory germacrene A oxidase also mediates C-12 oxidation of amorpha-4,11-diene, but that the formation of (+)-nootkatone is catalyzed by a different monoxygenase. This latter mono-oxygenase would possibly catalyse a later step in bitter sesquiterpene lactone biosynthesis, the conversion of costunolide into leucodin. Notably, the authors describe that formation of (+)-nootkatone was inhibited up to 90% by the addition of germacrene A, an effect which would not be in accordance with their two-enzyme hypothesis, but is in accordance with a single cytochrome P450 mediating both (+)-valencene to (+)-nootkatone conversion, as well as germacrene A to germacra-1(10),4,11(13)-triene-12-oic acid conversion. Still, this leaves open the question why two different positions on the sesquiterpene carbon scaffold can be oxidised by the same enzyme. Likely this is the result of a different orientation of (+)-valencene on one hand, and germacrene A to amorpha-4,11-diene on the other hand, in the substrate binding pocket of the P450 enzyme.

Regiospecificity of P450 enzymes is important for the production of fine chemicals. This work clearly shows that regiospecificity of plant P450 enzymes is poorly predictable from the carbon atom
Fig. 3. Reactions catalyzed by CYP71AV8. CYP71AV8 catalyses (A) conversion of (+)-valencene to (+)-nootkatone via trans- and cis- nootkatol, (B) a three-step conversion of germacrene A to germacr-1(10),4,11(13)-tien-12-ol, and (C) a three step conversion of amorph-4,11-diene to artemisinic acid. The preferred position of hydroxylation is marked in bold.

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References