β-Amyrin Oxidation by Oat CYP51H10 Expressed Heterologously in Yeast Cells: The First Example of CYP51-Dependent Metabolism Other than the 14-Demethylation of Sterol Precursors

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CYP51 has been recognized as a unique CYP family that consists of one isolated molecular species, a sterol 14-demethylase essential for sterol biosynthesis. However, another CYP51 gene classified as the CYP51H subfamily has been identified in higher plants, in addition to a sterol 14-demethylase gene, CYP51G1. To shed light on the function of this “second CYP51”, oat CYP51H10 was introduced into the β-amyrin-producing yeast cells, and the effect of the expressed CYP51H10 on β-amyrin metabolism in the host cells was examined. In the CYP51H10-introduced cells, β-amyrin was converted to a metabolite with 12,13-epoxy and one additional hydroxyl group. Since the 12,13-epoxy group introduced into β-amyrin ring is an essential structure ofavenacin A-1, a triterpene glycoside synthesized from β-amyrin, the present findings indicate the contribution of CYP51H10 toavenacin A-1 biosynthesis from β-amyrin. This is the first study showing a second function of the CYP51 family.

Key words CYP51; P450; sterol 14-demethylation; β-amyrin metabolism; new function

P450 has diversified into huge numbers of monoxygenases through evolution. These variants have been adapted to diversified and specific metabolisms occurring in individual biological species.1–7 P450 classified as the CYP51 family is considered to be unique. It is distributed widely in eukaryotes with conserved function as a sterol 14-demethylase,8–13 and is considered to exist as an isolated molecular species, CYP51A1 in animals, CYP51B1 in bacteria, CYP51E1 in protozoa, CYP51D1 in slime molds, and one of the CYP51F subfamily members in fungi (Nelson DR: http://drlenelson.uthsc.edu/cytochromeP450.html). In higher plants, however, another CYP51 genes classified as the CYP51H subfamily have been identified in addition to the conserved sterol 14-demethylase gene, CYP51G1 (Nelson DR: http://drlenelson.uthsc.edu/biblioD.html). This is an interesting finding that suggests that diversification occurred in the extremely well-conserved CYP51 family.

Recently, it was reported that Sad2, one of the essential genes foravenacin A-1 synthesis in oat, Avena strigosa, is synonymous with CYP51H10.14 Avenacin A-1 is a triterpene glycoside synthesized from β-amyrin, and the Sad2 deletion mutant of A. strigosa was found to accumulate β-amyrin in roots.14 This finding suggests that CYP51H10 is an essential enzyme for the bioconversion of β-amyrin toavenacin A-1. To obtain more direct evidence for this genetically assumed role of CYP51H10, the effect of the heterologous expression of oat CYP51H10 on the metabolism of β-amyrin in β-amyrin-producing yeast cells was examined. The results indicated that CYP51H10 converted β-amyrin to a metabolite having 12,13-epoxy and one hydroxyl group, which might be an intermediate ofavenacin A-1 biosynthesis.

MATERIALS AND METHODS

Cloning of the DNA Fragment Encoding CYP51H10 and Its Expression in Yeast Cells DNA fragments corresponding to exons 1 and 2 of CYP51H10 were prepared by polymerase chain reaction (PCR) using the DNA obtained from young leaves ofAvena sativa as a template. The following PCR primers were designed based on the genomic DNA sequence (accession No. DQ680849) and mRNA sequence (accession No. DQ680852) of A. strigosa CYP51H10: exon1-S (5′-cctgcacatgctagcactgatc-3′), exon1-AS (5′-ctcagatcaaagaccagcagtcg-3′), exon2-S (5′-aggagacttgggctttaggg-3′), and exon2-AS (5′-tttctctctctgcgctttg-3′). The PCR products were cloned independently withpBluescript SK+. Then, the DNA fragment covering the coding region of CYP51H10 was constructed in pBluescript KS+ by the ligation of Sad1–Scal fragment and Scal–Nhel fragment from the cloned exons 1 and 2. One nucleotide (208G) of this fragment was substituted with A to match the sequence to A. strigosa CYP51H10.14 To construct the co-expression vector of CYP51H10 and nicotinamide adenine dinucleotide phosphate (NADPH)-P450 reductase, the Sad1–Nhel fragment covering the coding region of CYP51H10 was inserted into the Sad1–Nhel site downstream of the GAL1 promoter of thepESC-LEU vector5 that had been introducedLotus japonicus NADPH-P450 reductase gene at the downstream of the GAL10 promoter. This co-expression vector was introduced into the host yeast cells that could produce β-amyrin owing to the previously introduced expression vector including β-amyrin synthase gene according to the method of Seki et al.15 The transformed yeast was cultivated at 28°C for 24 h. Then, CYP51H10 and NADPH-P450 reductase were induced with galactose according to the method of Seki et al.15 and cultivation was continued for an additional 48 h.
Analysis of β-Amyrin Metabolite  Lipophilic compounds in the culture medium containing yeast cells were extracted with double volume of ethyl acetate. After the removal of colored materials with Chemizorb (MERCK), solvent of the extracts was evaporated off, and the residue was dissolved in a small volume of the chloroform–methanol (2:1). An aliquot of this solution was dried up, trimethylsilylated with trimethylsilylimidazole (TMS-imidazole), and analyzed in a Shimadzu 14B gas chromatograph with TC-17 capillary column (30 m x 0.25 mm; GL Science, Japan). The temperature of the injection port was set at 280°C and the initial column temperature was set at 80°C. After one minute of injection, the column temperature was increased from 80°C to 300°C at a constant rate of 20°C/min, and was held at 300°C for 20 min. GC-MS analysis of trimethylsilylated β-amyrin and its metabolite was carried out in a JEOL JMS-AM SUN200 mass spectrometer and an Agilent Technologies 6890A gas chromatograph equipped with DB-1 capillary column (30 m x 0.25 mm; J&W Scientific, U.S.A.) under the same temperature conditions as above.

RESULTS AND DISCUSSION

The CYP51H10 and NADPH-P450 reductase co-expression vector was constructed and introduced into the yeast cells expressing β-amyrin synthase as described in Materials and Methods. The transformed yeast was cultivated, and CYP51H10 and NADPH-P450 reductase were induced as described in Materials and Methods. Then, lipophilic compounds were extracted from the whole culture medium including the yeast cells, and analyzed with GLC as described in Materials and Methods. The upper and Fig. 1 are the chromatograms of the extracts obtained from the culture media including the CYP51H10-introduced and the non-introduced cells, respectively. It is clear in Fig. 1 that the peak marked by an arrow cannot be observed in the chromatogram of the control culture. The host-vector system used in this experiment

![Fig. 1. Gas-Chromatographic Detection of the β-Amyrin Metabolite Formed in the β-Amyrin-Producing Yeast Cells Expressing CYP51H10 and NADPH-P450 Reductase](image)

![Fig. 2. Analysis of the Metabolite with GC-MS](image)

The lipophilic extracts obtained under the same conditions as for Fig. 1 from the yeast cells expressing CYP51H10 and NADPH-P450 reductase were analyzed with GC-MS as described in Materials and Methods. The mass spectra of peaks A and B are shown as spectra A and B, respectively. The higher m/z region of each spectrum is magnified 20-fold.
was effective for the expression of CYP93E3 (β-amyrin 24-hydroxylase) and production of 24-hydroxy-β-amyrin from β-amyrin produced in the host cells. Therefore, it is highly likely that the compound detected as the peak marked with an arrow was a CYP51H10-dependent metabolite of β-amyrin produced in the host cells. Then, the nature of this compound was examined with GC-MS.

The trace of total ion monitor (TIC of Fig. 2) in the GC-MS analysis of the extracts from the CYP51H10-introduced cells showed two distinct peaks corresponding to β-amyrin and the metabolite, observed in the gas-chromatogram (Fig. 1). The mass spectra of peaks A (β-amyrin) and B (the metabolite) are shown as spectra A and B, respectively (Fig. 2). The molecular ion [M⁺] of the metabolite was observed at m/z 602, and the characteristic fragment ions assigned as M⁺−CH₃, M⁺−O-TMSH and M⁺−(CH₃+O-TMSH) were observed at m/z 587 [M⁺−15], m/z 512 [M⁺−90] and m/z 497 [M⁺−(15+90)], respectively (spectrum B of Fig. 2). Formation of these three fragments in EI-MS is a well-known characteristic of the trimethylsilylated derivatives of circular triterpenoids such as lanosterol and β-amyrin. Actually, the mass spectrum of trimethylsilylated β-amyrin (spectrum A of Fig. 2) showed them at m/z 483 [M⁺−15], m/z 408 [M⁺−90] and m/z 393 [M⁺−(15+90)]. These findings suggested that the metabolite might be a circular triterpenoid with a molecular mass of 602 in its trimethylsilylated form. The molecular mass of the trimethylsilylated metabolite was 104 higher than that of trimethylsilylated β-amyrin, and this difference corresponds to the sum of an oxygen atom and a trimethylsilylated hydroxyl group. Accordingly, the metabolite was considered to be formed by the addition of one oxygen atom and one hydroxyl group to β-amyrin.

The most abundant fragment ion of trimethylsilylated β-amyrin was observed at m/z 218 (Fig. 2, spectrum A).
formed by the cleavage of ring C owing to the presence of a double bond at C-12.\textsuperscript{16} However, the corresponding fragment ion was not observed in spectrum B of Fig. 2, suggesting the absence of a 12,13-double bond in the metabolite. The absence of this double bond in the metabolite was also supported by the smaller $M^+\text{-}(O\text{-TMSH})$ peak ($m/z$ 497) compared with the $M^+\text{-}($CH$_3$O$\text{-}$TMS-H$)$ peak ($m/z$ 512), because the $M^+\text{-}($CH$_3$O$\text{-}$TMS-H$)$ peak of $\beta$-amyrin ($m/z$ 393) was markedly higher than its $M^+\text{-}(O\text{-TMSH})$ peak ($m/z$ 408) owing to extensive 14-demethylation due to the 12,13-double bond existing at its $\alpha$-position. It can thus be concluded that the metabolite might be the 12,13-epoxide of $\beta$-amyrin having one additional hydroxyl group. Although the existence of one additional hydroxyl group in the metabolite was also supported by the smaller $M^+\text{-}($CH$_3$O$\text{-}$TMS-H$)$ peak ($m/z$ 422) (Fig. 2B), its exact position in the metabolite has not yet been determined.

Figure 3 shows the time-dependent change of the metabolite/$\beta$-amyrin ratio in the extracts from CYP51H10-expressing yeast cells. Since the host cells produced $\beta$-amyrin with the constitutively expressed $\beta$-amyrin synthase,\textsuperscript{15} it was expected that the metabolite/$\beta$-amyrin ratio should be increased after the initiation of CYP51H10 induction and reach a steady state, if the metabolite was formed by the expressed CYP51H10. As expected, the metabolite/$\beta$-amyrin ratio linearly increased up to 9.5h after CYP51H10 induction, and then reached a plateau (Fig. 3).

Taking all observations together, it can be concluded that CYP51H10 catalyzes the conversion of $\beta$-amyrin to its 12,13-epoxide with one additional hydroxyl group (Fig. 4). Since the introduction of 12,13-epoxide and a few hydroxyl groups into the $\beta$-amyrin ring is necessary for avenanin A-1 biosynthesis (Fig. 4), the present findings confirm the contribution of CYP51H10 to avenanin A-1 biosynthesis as assumed from the genetic analysis by Qi et al.\textsuperscript{14}

This is the first example showing the occurrence of "second CYP51" catalyzing a reaction other than sterol 14-demethylation. Interestingly, the reactions catalyzed by CYP51H10 and CYP51G1 are situated at comparable positions in the branched pathways of triterpenoid metabolism to form avenanin A-1 and phytosterols (Fig. 4). This is the first example showing the functional diversification of CYP51, the most conserved P450.

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REFERENCES


