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Characterisation of the *FAD2* gene family from *Hiptage benghalensis*: A ricinoleic acid accumulating plant

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ABSTRACT

We have characterised the *FAD2* gene family from *Hiptage benghalensis*, a tropical plant that accumulates high levels of ricinoleic acid in its seeds. Functional characterisation of six *FAD2* gene family members showed that two of them were capable of functioning as Δ12-hydroxylases while the other *FAD2* members were confirmed to be Δ12-desaturases. The Δ12-hydroxylation function of these two genes was confirmed in yeast cells, using C16:$1^{\Delta 9}$ and C18:$1^{\Delta 9}$ monounsaturated fatty acids as substrates. These Δ12-hydroxylases, like the other Δ12-hydroxylases previously cloned from plants *Ricinus communis* (castor), *Physaria fendleri* and fungus *Claviceps purpurea*, also showed some Δ12-desaturase activity. The hydroxylation activity of the two *Hiptage* hydroxylases was further confirmed by their expression in the Arabidopsis *fad2/fae1* double mutant where they were able to produce equivalent or higher levels hydroxylated fatty acids in the seed oil when compared with the other known hydroxylases.

1. Introduction

Cultivated oil crops mainly produce seed oils dominated by five fatty acids, palmitic, stearic, oleic, linoleic and α-linolenic acids. However, wild plants show a greater diversity of fatty acid composition in their seed oils (Badami and Patil, 1980). Many of them have been used or have great potential applications in chemical industry as raw materials, or for pharmaceutical products. For example, hydroxy fatty acids, such as ricinoleic acid (12-hydroxy-octadec-9-enoic acid, or RA), have already been used in a range of industrial applications, in the forms of feedstocks for products such as lubricants, paints, coatings, nylons and resins (Jaworski and Cahoon, 2003). Castor bean (*Ricinus communis*) contains about 90% ricinoleic acid in its seed oil. There is increasing scientific and industrial interest in the biosynthesis of these unusual fatty acids in better adapted oil crops for large scale agriculture, due to the low yield, poor oil content or poor agronomic performance of the wild plants (Carlsson et al., 2011).

In recent years, considerable effort has been focused on identifying the genes responsible for the production of unusual fatty acids and introducing these biosynthetic pathways into model plants or oil crops by genetic engineering (Carlsson et al., 2011; Dyer et al., 2008; Singh et al., 2005). Fatty acid Δ12-modification enzymes, such as hydroxylase, epoxygenase, conjugase and acetylenase isolated so far mainly belong to Δ12-fatty acid desaturase (FAD2) like gene family (Cahoon et al., 1999; Dyer et al., 2002; Lee et al., 1998; van de Loo et al., 1995), but also occur among the cytochrome P450 family, such as *Euphorbia lagascae* P450 Δ12-epoxygenase (Cahoon et al., 2002). The oleate hydroxylase gene responsible for the formation of ricinoleic acid was first cloned from castor bean (van de Loo et al., 1995), subsequently from *Lesquerella fendleri* (*Physaria fendleri*) (Broun et al., 1998), *Physaria lindheimeri* (Dauk et al., 2007) and fungus *Claviceps purpurea* (Meesapyodsuk and Qiu, 2008). All Δ12-hydroxylases characterised so far have been shown to belong to the *FAD2* gene family.

Hiptage benghalensis [L.] Kurz, a member of the Malpighiaceae family, is a vine-like plant native to temperate and tropical Asia, and present in Australia as a destructive weed invading the wet tropics of northern Queensland and remnant bushland in south-eastern Queensland (Vitelli et al., 2009). It typically grows as a high-climbing (50–60 m), twining liana when adjacent to trees or forms a large shrub group to 4 m high when trees are absent. Hiptage is used for medicinal purposes in India to treat scabies, chronic rheumatism and asthma, biliousness, coughing, a burning sensation, and thirst and inflammation (Chenthurpandy et al.,

Abbreviations: AT, acyltransferase; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CX:Y, fatty acid containing X carbons with Y double bonds; DMOX, 2,4-dimethyloxazoline; FAD2, fatty acid Δ12-desaturase; FAH12, fatty acid Δ12-hydroxylase; FAME, fatty acid methyl ester; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; RA, ricinoleic acid; TMSi, trimethylsilyl; TMCS, trimethylchlorosilane.

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2009). It is another species that rich in ricinoleic acid (70%) in its seed oil (Badami and Kudari, 1970). Here we report the isolation of the *FAD2* gene family from *H. benghalensis*, and their functional characterisation by expression in yeast and Arabidopsis. Two out of six FAD2 family members predominantly show Δ12-hydroxylation activity and low level Δ12-desaturation activity, while the other members show only Δ12-desaturation activity. We also confirmed that these two Δ12-hydroxylases are able to catalyse the synthesis of ricinoleic acid when expressed in Arabidopsis seeds.

2. Results and discussion

2.1. Cloning of the H. benghalensis FAD2 gene family

H. benghalensis contains about 70% of ricinoleic acid in its seed oil and therefore serves as a good resource for isolating genes involved in the synthesis and accumulation of hydroxy fatty acid. We first focused on the possibility that the fatty acid Δ12-hydroxylase in *H. benghalensis* may be a Δ12-desaturase FAD2-like enzyme, as is the case in castor, *Physaria* species, and fungus *C. purpurea.* Early, mid and late stages of the developing seeds were collected. The presence of ricinoleic acid was confirmed by GC and GC–MS from mid and late stages of the developing seeds (data not shown). These materials were used for RNA extraction. Degenerate polymerase chain reaction with oligos targeting the *FAD2*-like gene family followed by library screening, and large scale EST sequencing (13,007 ESTs) resulted in the identification of six *FAD2*-like sequences (GenBank Accession Nos. KC533763–KC533768). These are referred to herein as the *HbFAD2* family. One of them was recovered as a partial sequence, missing 72 amino acid residues at N-terminus when compared to the other *HbFAD2* sequences. This partial sequence was apparently expressed at a low level, based on there being no single hit specific to this clone from the 431,763 reads with mean size of 470 bp in 454 deep sequencing, and failed to clone the full-length sequence in spite of extensive RACE attempts. Sequence homology among these six *FAD2*-like sequences ranged from 68–98%. Their amino acid sequences showed 66–75% identity to castor Δ12-hydroxylase (RcFAH12, U22378), 65–70% identity to *P. fendleri* Δ12-hydroxylase (LfFAH12, AAC32755), 63–70% identity to *P. lindheimeri* Δ12-hydroxylase (PlFAH12, ABQ01458), and 67–78% to *Arabidopsis thaliana* Δ12-desaturase (AtFAD2, P46313), but were divergent from the fungus *C. purpurea* Δ12-hydroxylase (CpFAH12, ACF37070), showing only 32–37% amino acid sequence identity.

2.2. Functional characterisation of HbFAD2 genes in yeast

The five full-length *H. benghalensis FAD2*-like genes were expressed in yeast and compared to the empty vector, or *RcFAH12*, *LfFAH12* and *CpFAH12* genes. In the yeast expression system, the introduced Δ12-desaturase can desaturate both C16:1$^{\Delta 9}$ and C18:1$^{\Delta 9}$ to C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$ products (Graphic abstract). Compared to vector only (Fig. 1A), *HbFAD2* clones 1, 2 and 5 (Fig. 1B–D) behaved only as Δ12-desaturases, producing the dienoic acids C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$, while clone 3 and 4 were Δ12-hydroxylases, producing hydroxy fatty acids 12OH-C16:1$^{\Delta 9}$ and 12OH-C18:1$^{\Delta 9}$, similar to other hydroxylases RcFAH12, LfFAH12 and CpFAH12 (Fig. 1E and F, and data not shown). The chemical structures of the hydroxy fatty acid products were confirmed by GC–MS analysis of the fatty acid methyl ester (FAME)-trimethylsilyl (TMSi) derivatives (see Supplementary Fig. S1). Clones showing only Δ12-desaturase activity were named as HbFAD2-1, HbFAD2-2, HbFAD2-3, while two clones showing predominantly Δ12-hydroxylase activity were named as HbFAH12-1, HbFAH12-2 and, and partial sequence as HbFAD2-4.

In our experiments, all of the tested FAH12 hydroxylases could produce Δ12-hydroxylated as well as Δ12-desaturated products from both C16:1$^{\Delta 9}$ and C18:1$^{\Delta 9}$. However, they showed different effectiveness for Δ12-hydroxylating and Δ12-desaturating C16:1$^{\Delta 9}$ and C18:1$^{\Delta 9}$ substrates, resulting in a different hydroxylation to desaturation ratio observed for the FAH12 hydroxylases (Table 1). The production of 12OH-C16:1$^{\Delta 9}$ was previously reported by expressing CpFAH12 (Meesapyodsuk and Qiu, 2008) and RcFAH12 or LfFAH12 (Smith et al., 2003). Unlike plant hydroxylases, the fungal hydroxylase CpFAH12 can also produce low amount of densipolic acid (12OH-C18:2$^{\Delta 9,15}$, Table 1) in yeast cells, due to the week dual function of desaturation at the ω3 position (Meesapyodsuk and Qiu, 2008). The yeast expression data indicated that HbFAH12-2 had similar hydroxylation activity and preference for C18:1$^{\Delta 9}$ substrate when compared to the castor enzyme RcFAH12, while HbFAH12-1 had slightly lower hydroxylation activity than RcFAH12, but with a higher preference for the C18:1$^{\Delta 9}$ substrate in yeast.

2.3. Sequence comparison of HbFAD2 family

H. benghalensis (Malpighiaceae), *R. communis* (Euphorbiaceae) and *Linum usitatissimum* (Linaceae) all belong to a large branch of Malpighiales. The phylogenetic comparison of *H. benghalensis* FAD2 family with previously cloned plant FAH12 and FAD2 from closely related species *R. communis* and *L. usitatissimum* as well as Arabidopsis, showed that they either clustered with FAD2 including RcFAD2 and LuFAD2, or clustered with RcFAH12 (Fig. 2A). It is worth noting that although the bifunctional Δ12-hydroxylase/desaturase from *Physaria* clusters with Arabidopsis AtFAD2 (both are Brassicaceae members), the Hiptage desaturases HbFAD2-3, HbFAD2-4 (partial) cluster with Hiptage hydroxylases HbFAH12-1, HbFAH12-2 and castor hydroxylase RcFAH12. This suggests that in the context of a phylogenetic tree the members of the FAD2-like family does not completely cluster according to their catalytic function.

Alignment of the HbFAD2-like sequences and other FAD2 sequences showed the occurrence of typical conserved three histidine boxes (Fig. 2B). The four key residues that differed between desaturases and hydroxylases (Broadwater et al., 2002) are indicated by an arrow, and can be used for prediction of enzyme activity. HbFAH12-1 and HbFAH12-2 had motif GHECGH at Histidine Box I, same as the Δ12-hydroxylases RcFAH12, LfFAH12 and PlFAH12, while the other HbFAD2 sequences had AHECGH motif, which was same as Δ12-desaturase AtFAD2. Another key residue Ile/Val next to Histidine Box III that was specific to hydroxylases was also found in HbFAH12-1 and HbFAH12-2, while at the same position this residue was Met in other HbFAD2 sequences, as is the case in other plant Δ12-desaturases. The other two key residues in all four HbFAD2 and two HbFAH12 sequences were conserved to other Δ12-desaturases. Although HbFAD2-3 clustered with RcFAH12, HbFAH12-1 and HbFAH12-2 due to overall sequence similarity, the proposed key residues responsible for the partitioning between the desaturation and hydroxylation reaction matched that of the desaturation reaction (Broadwater et al., 2002). Indeed, yeast expression assay confirmed it to be a desaturase.

There is high sequence homology among the *H. benghalensis* HbFAD2 and HbFAH12 sequences, which clustered as a pair (Fig. 2A). The open reading frames of five full-length sequences were 1171 base pairs long, coding for 384 amino acid residues. Comparison of these sequences showed them to be perfectly co-linear with only a few nucleotide changes. Among them, HbFAH12-1 and HbFAH12-2 shared 96.9% sequence identity, with difference in only 21 base pairs, causing a change of 11 amino acid residues. Likewise, HbFAD2-1 and HbFAD2-2 share 94.8% identity at amino acid