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Cloning of parsley flavone synthase I

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Abstract

A cDNA encoding flavone synthase I was amplified by RT-PCR from leaflets of *Petroselinum crispum* cv. Italian Giant seedlings and functionally expressed in yeast cells. The identity of the recombinant, 2-oxoglutarate-dependent enzyme was verified in assays converting (2*S*)-naringenin to apigenin.

Keywords: *Petroselinum crispum*; Apiaceae; Flavonoid biosynthesis; Flavone synthase I cloning; 2-Oxoglutarate-dependent dioxygenase; Heterologous expression

During the last decade considerable progress has been achieved towards elucidating the mode of action and molecular architecture of 2-oxoglutarate-dependent dioxygenases. These enzymes catalyze diverse reactions, such as the hydroxylation, desaturation, epoxidation or cyclization of substrates, and the activities depend on ferrous iron and molecular oxygen which is reduced during catalysis by two electrons provided by decarboxylation of the cosubstrate (Prescott, 1993; DeCarolis and DeLuca, 1994; Prescott and John, 1996). Although 2-oxoglutarate is the common cosubstrate, some closely related dioxygenases mobilize the electrons from decarboxylation of the substrate itself, e.g. isopenicillin *N*-synthase (Baldwin and Abraham, 1988) and 4-hydroxyphenylpyruvate dioxygenase (Bradley et al., 1986; Rüetschi et al., 1992), or by the oxidation of ascorbate as in ethylene biosynthesis (Zhang et al., 1995; Lay et al., 1996). These latter enzymes may nevertheless classify with the 2-oxoglutarate-dependent dioxygenases stricto sensu in one category of intermolecular dioxygenases. Intermolecular dioxygenases fulfill a variety of pivotal functions in primary and secondary metabolism in bacteria (Omura et al., 1984; Salowe et al., 1990) and fungi, including the cyclization and ring expansion reactions in penicillin/cephalosporin biosynthesis (Baldwin and Abraham, 1988), as well as in mammalian tissues (Lindstedt et al., 1977; Kivirikko et al., 1989; Stenflo et al., 1989). Furthermore, these enzymes catalyze numerous reactions in plants, e.g. in the formation of hydroxyproline-rich glycoproteins (Tanaka et al., 1980), of gibberellins (Hedden and Graebe, 1982) and the secondary metabolites scopolamine (Hashimoto and Yamada, 1986), vindoline (DeCarolis et al., 1990) or of various flavonoids (Forkmann et al., 1980; Britsch et al., 1981; Lukačin and Britsch, 1997; Lukačin et al., 2000 a,b,c).

Five 2-oxoglutarate-dependent dioxygenases have been identified so far from flavonoid biosynthesis, which include the widely distributed anthocyanidin synthase (Menssen et al., 1990), flavanone 3β-hydroxylase (Forkmann et al., 1980; Britsch et al., 1981; Lukačin and Britsch, 1997; Lukačin et al., 2000 a,b,c) and flavonol synthase (Britsch et al., 1981; Holton et al., 1993). Another dioxygenase, catalyzing the 6-hydroxylation of partially methylated flavonols, was reported recently from *Chrysosplenium americanum* (Anzelotti and Ibrahim, 2000), while flavone synthase I, FNS I, appears to be confined to species of the Apiaceae (Britsch, 1990). FNS I had been characterized in 1981 as a soluble enzyme from parsley, in contrast to the microsomal flavone synthase II, FNS II, from other plants (Kochs and Grisebach, 1987; Martens and Forkmann, 1998), and was partially purified through six-steps of fractionation from irradiated cell cultures (Britsch, 1990). This

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enzyme was then employed in kinetic studies aiming at the reaction mechanism, which revealed that synthetic 2-hydroxynaringenin did not compete with flavanone substrates. Accordingly, the 2,3-desaturation of flavanones by FNS I was postulated to proceed by direct abstraction of the vicinal hydrogen atoms (Britsch, 1990), which would assign FNS I to a distinct desaturase subgroup among the 2-oxoglutarate-dependent dioxygenases. An analogous reaction mechanism, exluding the successive hydroxylation and dehydratation, was proposed for the desaturation of alkanes to olefines suggesting a reaction *via* radical intermediates (Mansuy, 1998). The exact course of FNS I catalysis requires further experimental support, but appears to proceed analogous to that of the cytochrome P450-dependent FNS II expressed in many plants except for the Apiaceae. The first full size FNS II cDNAs were recently cloned from *Gerbera hybrida* (Martens and Forkmann, 1999), *Antirrhinum majus* and *Torenia hybrida* (Akashi et al., 1999) by differential display PCR and expressed in yeast cells. As anticipated for a P450-dependent monoxygenase, this FNS II converted labelled flavanones to the corresponding flavones apparently without any intermediate (Martens and Forkmann, 1999).

The common mode of oxygen activation by intermolecular dioxygenases, particularly among the 2-oxoglutarate-dependent enzymes, seems to predict a high degree of homology at the DNA and polypeptide levels. However, only 30% similarity was observed in the polypeptide sequences of, for example, flavanone 3β-hydroxylase from *Petunia hybrida* and hyoscyamine 6β-hydroxylase from *Hyoscyamus niger* (Britsch et al., 1992), and the similarity of prolyl 4-hydroxylase with lysyl hydroxylase from chicken (Myllylä et al., 1991) or of fungal isopenicillin *N*-synthase with desacetoxycephalosporin C synthase ranged only at approx. 20% (Britsch et al., 1993). Nevertheless, superimposing the structural models of the penicillin and cephalosporin synthases revealed an almost identical architecture for these two enzymes (Lloyd et al., 1999), and comparison of the CD spectra of *Petunia* flavanone 3β-hydroxylase and isopenicillin *N*-synthase suggested the same pattern of helical, non-helical and β-sheet motifs for the *Petunia* dioxygenase (Lukačin et al., 2000b). Flavanone 3β-hydroxylase and FNS I both use 2-oxoglutarate as the cosubstrate and depend on the same flavanone substrates (Fig. 1). Accordingly, a thorough examination of the sequential and spatial differences of these two enzymes, together with in vitro mutagenesis studies, might be rather helpful to pinpoint the putative substrate binding sites and to explain the formation of flavones and flavonols, respectively.

Based on alignments of fourteen intermolecular dioxygenase polypeptides from public data bases two conserved sequence motifs were chosen, and, similar to the previous cloning of flavonol synthase (Fig. 1) from *Petunia hybrida* (Holton et al., 1993), degenerate oligonucleotide primers were designed for the cloning of FNS I. In combination with oligo(dT), the primers were employed for RT-PCR amplification of cDNAs from total RNA that had been extracted from young leaflets at four stages of development of flavone-producing *Petroselinum crispum* cv. Italian Giant plants (Martens and Forkmann, 1999). A whole set of intermolecular dioxygenase cDNAs was amplified, and the full-size

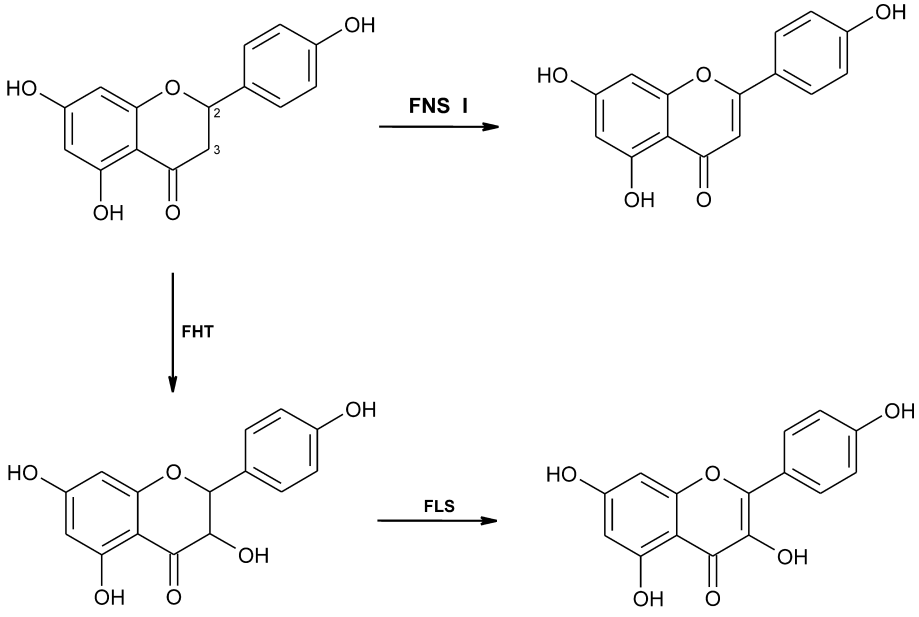

Fig. 1. Reaction catalyzed by flavone synthase I (FNS I), converting (2*S*)-naringenin to apigenin, in comparison to the activities of flavanone 3β-hydroxylase (FHT) and flavonol synthase (FLS), which sequentially convert (2*S*)-naringenin to dihydrokaempferol and kaempferol.

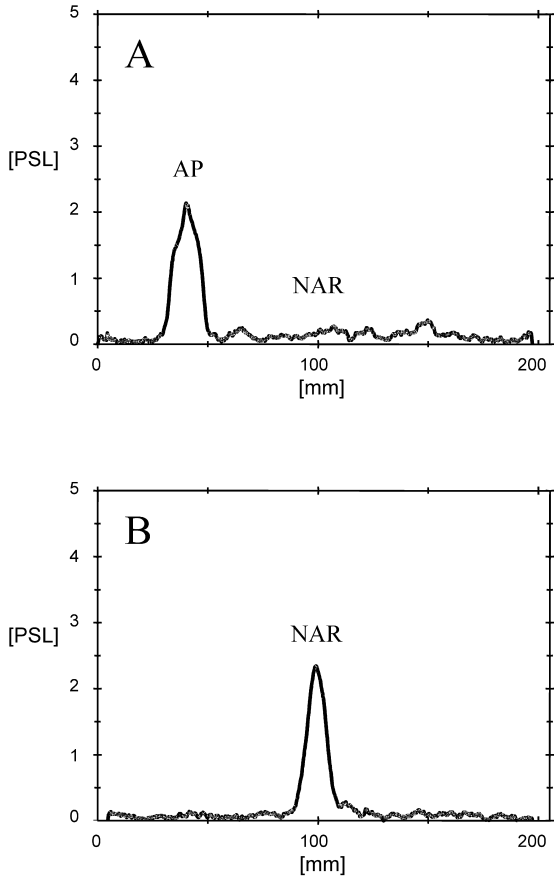

Fig. 2. Catalytic activity of parsley FNS I expressed in yeast cells. Crude extracts from yeast cells expressing the FNS I from the cDNA inserted in the pYES2 expression vector (A) or harbouring the FNS I cDNA in the inverse orientation (B) were incubated with (2*S*)-[4a,6-8-^{14}C]naringenin (NAR) as described for the native plant enzyme (Britsch, 1990). Subsequently, the incubations were extracted with ethyl acetate, the extracts were separated by thin-layer chromatograpy on cellulose in 30% aqueous acetic acid (v/v), and the radioactivity was spotted by a phosphorimager (Martens and Forkmann, 1998). The product was identified by cochromatography with authentic apigenin (AP).

clones were generated by the 5′-RACE technique. In addition to flavanone 3β-hydroxylase, flavonol synthase, 1-aminocyclopropane-1-carboxylate oxidase and two not yet fully characterized 2-oxoglutarate-dependent dioxygenases, FNS I was also recognized among the cDNA clones. The FNS I cDNA was unequivocally identified by expression in yeast strain INVSc1, using the expression vector pYES2 (Invitrogen, Groningen, The Netherlands), and the efficient conversion of naringenin to apigenin by the recombinant enzyme (Fig. 2 A). The identity of the reaction product was confirmed by direct comparison of the retention time on reversed phase HPLC in water/acetonitrile 7:3 and the ESI–MS spectrum with the mobility and fragmentation pattern of authentic apigenin (data not shown). The FNS I polypeptide was also recognized in Western blots by a FNS I polyclonal rabbit antiserum (Lukačin et al., unpublished). Moreover, the yeast strain transfected with the pYES2 vector hosting the FNS I cDNA in the inverse orientation did not express flavone synthase activity (Fig. 2 B). Recombinant FNS I lacked flavonol synthase activity (Fig. 1), and the sequences of these two enzymes differ considerably. Thus, soluble FNS I prevailing in the Apiaceae was cloned for the first time and has become available in quantity for mechanistic studies as well as for the convenient preparative synthesis of radiolabeled flavones which enable further biosynthetic and biotechnological studies. The evolutionary context for the expression of the soluble synthase exclusively in the Apiaceae remains to be established. In addition, the recombinant enzyme may be of value for the production of flavone-nutraceuticals due to their antioxidant and anticancer potentials (Harborne and Williams, 2000).

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