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Evolution of pyrrolizidine alkaloids in *Phalaenopsis* orchids and other monocotyledons: Identification of deoxyhypusine synthase, homospermidine synthase and related pseudogenes

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ABSTRACT

In order to study the evolution of pathways of plant secondary metabolism, we use the biosynthesis of pyrrolizidine alkaloids (PAs) as a model system. PAs are regarded as part of the plant's constitutive defense against herbivores. Homospermidine synthase (HSS) is the first specific enzyme of PA biosynthesis. The gene encoding HSS has been recruited from the gene encoding deoxyhypusine synthase (DHS) from primary metabolism at least four times independently during angiosperm evolution. One of these recruitments occurred within the monocot lineage. We have used the PA-producing orchid *Phalaenopsis* to identify the cDNAs encoding HSS, DHS and the substrate protein for DHS, i.e., the precursor of the eukaryotic initiation factor 5A. A cDNA identified from maize was unequivocally characterized as DHS. From our study of *Phalaenopsis*, several pseudogenes emerged, of which one was shown to be a "processed pseudogene", and others to be transcribed. Sequence comparison of the HSS- and DHS-encoding sequences from this investigation with those of monocot species taken from the databases suggest that HSS and probably the ability to produce PAs is an old feature within the monocot lineage. This result is discussed with respect to the recent discovery of structural related PAs within grasses.

1. Introduction

Pyrrolizidine alkaloids (PAs) are typical compounds of plant secondary metabolism and are constitutively produced by the plant as a defense against herbivores (Hartmann and Ober, 2000). We use this class of alkaloids as a system to study the way that plants have recruited complex pathways, i.e., the enzymes catalyzing specific reactions and the regulatory factors that ensure the proper integration of the pathway in plant metabolism during evolution. The occurrence of PAs is restricted to the angiosperms, suggesting that, in evolutionary terms, this pathway is relatively "young". PAs are found in many species belonging to the families of the Asteraceae (the tribes Senecioneae and Eupatorieae), Boraginaceae, Heliotropiaceae, and Apocynaceae, in some species of the Orchidaceae, and within the Fabaceae mainly in the genus *Crotalaria* (Hartmann and Witte, 1995). In some further families (Ranunculaceae, Convolvulaceae, Celastraceae), isolated occurrences have been described in only a few species (Hartmann and Witte, 1995; Jenett-Siems et al., 1993).

We have previously presented evidence that the gene coding for the first specific enzyme in PA biosynthesis, viz., homospermidine synthase (HSS, EC 2.5.1.45), was recruited by duplication of the gene coding for deoxyhypusine synthase (DHS, EC 2.5.1.46) (Ober and Hartmann, 1999b). DHS catalyzes the first of two steps activating the eukaryotic initiation factor 5A (eIF5A), which has been shown to be essential for cell proliferation (Chattopadhyay et al., 2008; Nishimura et al., 2005; Park et al., 1993, 1997). The DHS system is characterized by high sequence conservation related to strongly preserved biochemical functions (Gordon et al., 1987; Kyrpides and Woese, 1998; Magdolen et al., 1994). In plants, activated eIF5A might be involved in processes such as seed germination (Moll et al., 2002), senescence, and apoptosis (Wang et al., 2001, 2003, 2005). In addition to a high degree of sequence identity, HSS and DHS share many biochemical properties (Ober and Hartmann, 1999b; Reimann et al., 2004). HSS catalyzes the formation of homospermidine by the transfer of an aminobutyl moiety of spermidine to putrescine. DHS, which also transfers the aminobutyl moiety of spermidine, modifies a specific lysine residue within the eIF5A precursor protein but is also able to catalyze the formation of homospermidine. This "side-activity" of DHS has been

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proposed as being responsible for small amounts of homospermidine detected in various angiosperm species unable to produce PAs (Ober et al., 2003a, b). Thus, despite their completely different reaction products, HSS and DHS are almost identical with respect to their reaction mechanism with one exception: HSS is unable to bind and modify the eIF5A precursor protein (Ober et al., 2003b). This inability is the distinctive feature between paralogous HSS and DHS (Fig. 1).

A comparison of 23 cDNA sequences coding for HSS and DHS sequences of various angiosperm species has provided evidence that the recruitment of the HSS gene by duplication of the DHS-coding gene occurred at least four times independently during angiosperm evolution (Reimann et al., 2004). Further such recruitments are likely, because HSS sequences have been studied so far only in the Boraginales (one independent origin), Asteraceae (two independent origins within the tribes Eupatorieae and Senecioneae), and the monocots (one independent origin).

Orchidaceae are one of the largest (ca. 30,000 species) and most complex families of flowering plants (Cameron, 2004). According to molecular phylogenies based on the plastid *rbcL* (Cameron et al., 1999; Chase et al., 1994) and *psaB* sequences (Cameron, 2004), members of this family are divided into five subfamilies (Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae, Vanilloideae). Within monocots, PAs have only been found within orchids. They have been described in eight genera, viz., *Liparis*, *Malaxis*, *Cysis*, *Phalaenopsis*, *Vanda*, *Vandopsis* (Hartmann and Witte, 1995), *Pleurothallis* (Borba et al., 2001), and *Cremastra* (Ikeda et al., 2005) belonging to five tribes within the subfamily Epidendroideae (Cameron, 2004).

Most of the PAs found in plants are characterized by a 1,2-double bond that, amongst others, is responsible for the liver toxicity of PAs (Fu et al., 2004; Stewart and Steenkamp, 2001). They are usually synthesized, translocated, and stored as *N*-oxides (Hartmann and Witte, 1995). In contrast, the PAs found in the Orchidaceae belong to the phalaenopsine type of PAs as classified by Hartmann and Witte (1995). They are characterized by a 1,2-saturated necine base that is esterified with an aryl, aralkyl, or alkyl necic acid. Furthermore, only 40–60% of the PAs of *Phalaenopsis* occur in their *N*-oxide form (Frölich et al., 2006). This putative structural simplicity, the lack of liver toxicity, and the assumed restricted occurrence of these phalaenopsine-type PAs in only certain genera of the orchids might be interpreted as being indications for a more basal defense system. This interpretation and the observation that HSS, as the first enzyme of PA biosynthesis, has an independent origin within monocots, suggest that the ability of monocots to produce PAs is a young feature, probably restricted to only some orchid genera.

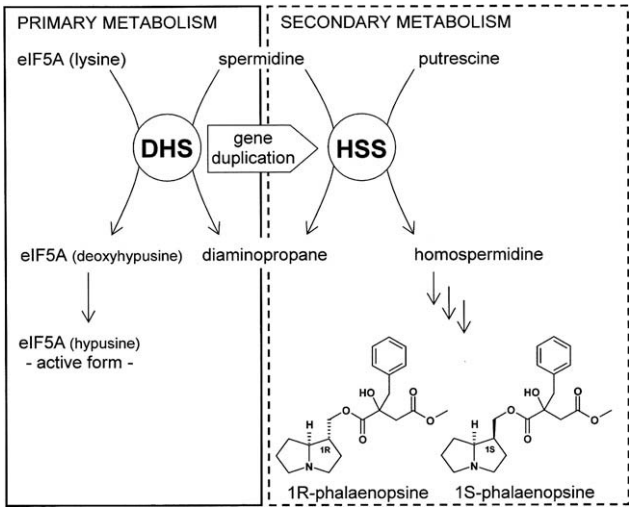

Fig. 1. The role of the paralogous enzymes DHS and HSS in the activation of eIF5A and the biosynthesis of PAs, respectively. Both enzymes catalyze the transfer of an aminobutyl moiety of spermidine, releasing diaminopropane, to modify a specific lysine residue within the eIF5A prescursor protein to deoxyhypusine (in the case of DHS) and to transform putrescine to homospermidine (in the case of HSS). Homospermidine is the first specific intermediate in the biosynthesis of PAs. The two isomers 1R-phalaenopsine and 1S-phalaenopsine are the only PAs found in *Phalaenopsis*.

In the present paper, we describe the identification of the cDNAs encoding DHS in *Phalaenopsis* and maize and of the eIF5A substrate protein of *Phalaenopsis*. The encoded proteins have been heterologously expressed in *Escherichia coli* and used for comparative biochemical characterization with HSS and several HSS-related pseudogenes of *Phalaenopsis*. A phylogenetic analysis of these sequences in addition to sequence data available from the databases suggest that the gene duplication creating the HSS was not a recent event within monocots.

2. Results

2.1. Cloning, expression, and identification of recombinant *Phalaenopsis* HSS

The identification of the open-reading frame (ORF) of the cDNA coding for PA-specific HSS from the total RNA of aerial root tips of an interspecific hybrid of *Phalaenopsis* has been briefly described (Reimann et al., 2004). The ORF codes for 371 amnio acids and is flanked by a 5'-untranslated region (UTR) of 50 bp and a 3'-UTR of 206 bp. To simplify the purification of the recombinant enzyme (HSS-*Ph*), the cDNA in this study was inserted into the pET23a expression vector, which added a hexahistidine tag at the C-terminal end of the protein during expression. The recombinant protein was characterized after metal chelate affinity chromatography. The protein showed only HSS activity (5.04 nkat/mg, Table 1) but no DHS activity, having the characteristics of most identified HSS of plant origin (Ober et al., 2003b).

To rule out the possibility that this sequence codes for a DHS that is just unable to accept the eIF5A precursor protein of *Senecio vernalis* used in the assay as a substrate, we identified, cloned, and expressed an eIF5A protein of *Phalaenopsis* in *E. coli*. This protein, termed eifph1, was accepted as a substrate by the DHS of *S. vernalis* (Ober and Hartmann, 1999b) to almost the same degree as its native substrate, the eIF5A of *S. vernalis* (data not shown). After purification by metal chelate affinity chromatography, eifph1 was used as a substrate for all further DHS assays. The enzyme protein HSS-*Ph* encoded by the identified sequence of *Phalaenopsis* again showed only HSS activity but no DHS activity, supporting the classification of the protein as HSS (Table 1).

2.2. Identification of pseudogenes with homology to the hss gene

The same set of degenerate primers used for the identification of the HSS-coding cDNA of *Phalaenopsis* was used in various combinations to identify the cDNA coding for DHS. For this

Table 1
Specific enzyme activities of affinity-purified recombinant HSS and DHS from *Phalaenopsis* and of DHS of maize. Enzyme assays for DHS were performed with 20 μM eIF5A substrate protein of *Senecio vernalis* (eifsv1), of *Phalaenopsis* (eifph1), and of *Zea mays* (eifzm1), respectively. nd, not detectable, –, not analyzed.

	HSS *P. amabilis*	DHS *P. amabilis*	DHS of *Z. mays*
HSS assay	5.04 nkat/mg	34.6 pkat/mg	198.5 pkat/mg
DHS assay			
With eifsv1	nd	8.9 pkat/mg	–
With eifph1	nd	36.4 pkat/mg	–
With eifzm1	–	–	202.2