1. INTRODUCTION

1.1. Background

The sessile nature of plants forces them to cope directly with environmental stresses, such as pathogen attack from, for example, bacteria, fungi, viruses and nematodes, and insect herbivory. To respond to these environmental challenges, plants have developed a variety of chemical compounds, encompassing more than 100,000 known structures, which represent possibly less than 10% of the total number expected in nature (Wink, 1988). These compounds that act predominantly as defence agents against pathogens and herbivores (Harborne, 1993) are products of secondary metabolism. They are not involved in primary processes such as growth and development. One of the most conspicuous features of secondary metabolites is that their occurrence is often restricted to individual species or related groups of species rather than being distributed within the plant kingdom.

Secondary metabolism consists of a large number of diverse processes that are often specific to certain cell types. The large process diversity in secondary metabolism undoubtedly requires an equally diverse set of enzymes responsible for the biosynthesis, storage and deployment of their products. However, little is known about the evolution of the enzymes involved in secondary metabolism. They are believed to be recruited through gene duplication (Pichersky and Gang, 2000) and, as a consequence, are often to be found in gene families. There are several mechanisms by which the evolution of new enzymes can occur. First, as is the case within the chalcone synthase multigene family, new enzymes evolved by diversification of previously existing enzymes with similar properties and similar substrate specificity. The second mechanism is the recruitment of new enzymes whose properties and function are completely different from the pre-existing character of their ancestor (Durbin *et al.*, 2000). An example for the second mechanism, which is also known as evolution by change of function (Ober *et al.*, 2003 b), is the evolution of homospermidine synthase (HSS), the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis, whose gene was recruited from the conserved ubiquitous gene coding for deoxyhypusine synthase (DHS), an enzyme involved in primary metabolism (see 1.1.3).

1.1.1 Pyrrolizidine Alkaloids (PAs)

Pyrrolizidine alkaloids (PAs) are ester alkaloids composed of a necine base moiety and one or more necic acids (Hartmann and Witte, 1995). They are constitutively formed and are not inducible by wounding, herbivory or microbial attack (Van Dam and Vrieling, 1994; Tinney *et al.*, 1998). PAs are known to be liver-toxic for vertebrates (Mattocks *et al.*, 1986; Huxtable, 1989) and mutagenic for insects (Frei *et al.*, 1992). Some PAs that are also strong insect antifeedants, belong to the few plant secondary compound that have been shown to be sequestered by insects for their own defence (Ehmke *et al.*, 1990; Bowers and Williams, 1995; Hartmann *et al.*, 1999). The proposal that PAs represent a complex, but effective, plant defence mechanism is supported by the observation that specialized insects have adopted this plant mediated defence strategy for their own defence during the course of their own evolutionary adaptation to PA-containing plants (Hartmann, 1999; Ober and Hartmann, 2000).

PAs encompass a diverse group of some 360 structures that are found scattered within angiosperm species. More than 95% of the PA-producing species belong to four plant families: Asteraceae (tribes Senecioneae and Eupatorieae), Boraginaceae (many genera), Fabaceae (mainly *Crotalaria*) and Orchidaceae (nine genera). The remaining PA-producing species occur scattered in a few unrelated taxa: Celastraceae, Convolvulaceae, Ranunculaceae, Rhizophoraceae, Santalaceae and Sapotaceae (Hartmann and Witte, 1995).

As typical secondary compounds, PAs seem to be synthesized in a tissue-specific manner. In the Asteraceae, PAs are synthesized as *N*-oxides exclusively in the roots (Toppel *et al.*, 1987; Hartmann, 1994). Likewise, in Orchidaceae, aerial roots, particularly the root tips, have been shown to be the specific site of PA biosynthesis (Frölich, 1996). In contrast to the Asteraceae and Orchidaceae, in the Boraginaceae PA biosynthesis occurs preferably within the shoots and roots, whereas in the Fabaceae they are thought to be synthesized in the shoots (Hartmann and Witte, 1995).

The scattered occurrence of PAs in certain unrelated taxa of the angiosperms provokes an interesting question regarding the origin of the plant's ability to produce PAs. An answer to this question should be possible by analysing the enzymes involved in PA biosynthesis, among which is HSS, which occupies a central position in PA biosynthesis and its regulation.

1.1.2 Homospermidine Synthase, the First Pathway-Specific Enzyme of PA Biosynthesis

Early tracer experiments carried out by Robins (1989) and Spenser (1985) showed that the carbon skeleton of the necine base originates from ornithine or arginine via putrescine and homospermidine. By means of isotope feeding and inhibitor experiments, Hartman *et al*. (1988) have shown that the common pathway of necine base biosynthesis is linked to primary metabolism via putrescine and the aminobutyl moiety of spermidine, both of which are exclusively derived from arginine (Hartmann *et al*., 1988).

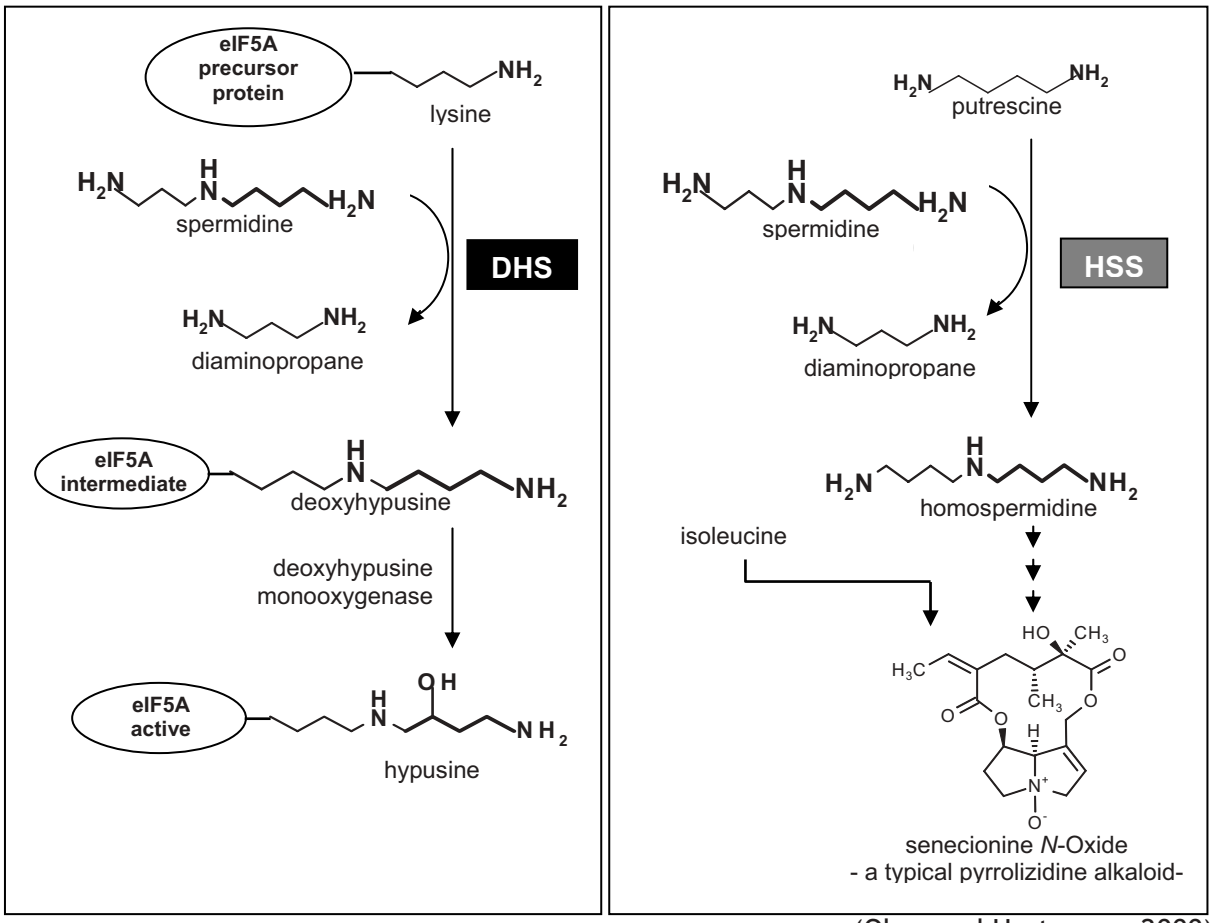
Homospermidine was identified as the first pathway-specific intermediate of PA biosynthesis (Khan and Robins, 1985) and is produced in a reaction catalyzed by homospermidine synthase (HSS). HSS (HSS spermidine-dependent, EC 2.5.1.45) catalyzes the transfer of the aminobutyl group from spermidine to a putrescine molecule in the presence of NAD^+ (Böttcher *et al*., 1993; Böttcher *et al*., 1994; Tholl *et al*., 1996; Ober *et al*., 2000). Once homospermidine is synthesized, it is exclusively used for the biosynthesis of PAs (Sander and Hartmann, 1989; Böttcher *et al*., 1993). Thus, HSS has a crucial role in the regulation of substrate flow from primary to secondary metabolism.

As the first pathway-specific enzyme of PA biosynthesis, HSS is interesting not only for understanding pathway regulation, but also for answering the question of its evolutionary origin. Recently, HSS has been successfully cloned and characterized from *Senecio vernalis* (Ober and Hartmann, 1999 b). Sequence comparison has revealed a high amino acid identity of HSS to an enzyme of primary metabolism, deoxyhypusine synthase (DHS, EC 2.5.1.46), indicating a close relationship between

the two enzymes (Ober and Hartmann, 1999 b, 2000). This interesting, almost unexpected finding provides the rare chance to understand the evolutionary origin of a secondary pathway. In the case of PAs, this pathway is believed to be, in phylogenetic terms, a relatively young pathway, because PAs are only found in angiosperms.

1.1.3 Evolutionary Origin of HSS from DHS

Because of its sequence homology, HSS undoubtedly evolved from DHS (Ober and Hartmann, 1999 b, 2000), an essential enzyme of primary metabolism. DHS catalyzes the first step of the post-translational activation of the eukaryotic initiation factor 5A (eIF5A), which is essential for eukaryotic cell growth and proliferation, although the mode of action is still not clearly understood. Despite the close genetic relationship between these enzymes, DHS and HSS are functionally unrelated that involved in two different areas of plant metabolism, one in primary and the other in secondary metabolism. Notwithstanding their totally different working areas DHS and HSS possess identical catalytic reactions mechanisms as presented in Fig. 1-1. In NAD^+-dependent reactions, HSS and DHS transfer the aminobutyl moiety of spermidine onto their second substrate, i.e. putrescine in the case of HSS and a specific lysine residue of the eIF5A precursor protein in the case of DHS to form homospermidine and deoxyhypusine, respectively (Ober and Hartmann, 2000). In contrast to HSS, which is unable to use the eIF5A precursor protein as its second substrate, DHS can also use putrescine as the second substrate to form homospermidine (Ober and Hartmann, 1999 b). The ability of DHS to produce homospermidine should result in the occurrence of homospermidine in all plants. This, however, is not the case, homospermidine has been reported as a rare polyamine from only few plant sources (Ober *et al.*, 2003 a). Recent studies have shown that trace amounts of homospermedine are detectable in all investigated plants selected from several unrelated plant families. These studies have suggested a possible role of the ubiquitous DHS in synthesizing homospermidine as a by-product (Ober *et al.*, 2003 a).



(Ober and Hartmann, 2000)

Fig. 1-1 The analogous reactions catalysed by HSS and DHS in their different metabolic environments.

DHS and HSS are homotetramers with a sub-unit size of about 45 kDa and have a pH optimum of about 9.5 (Ober *et al.*, 2003 b). These similar kinetic properties in addition to the genetic and biochemical similarities between the enzymes have prompted the hypothesis that HSS evolved from the ubiquitous DHS by gene duplication and recruitment for PA biosynthesis (Ober and Hartmann, 1999 b, 2000). Furthermore, the evolution of HSS from DHS is regarded as evolution by change of function; following gene duplication, HSS may simply have lost its ability to bind the eIF5A protein (Ober and Hartmann, 2000; Ober *et al.*, 2003 b) as shown in Fig. 1-2.

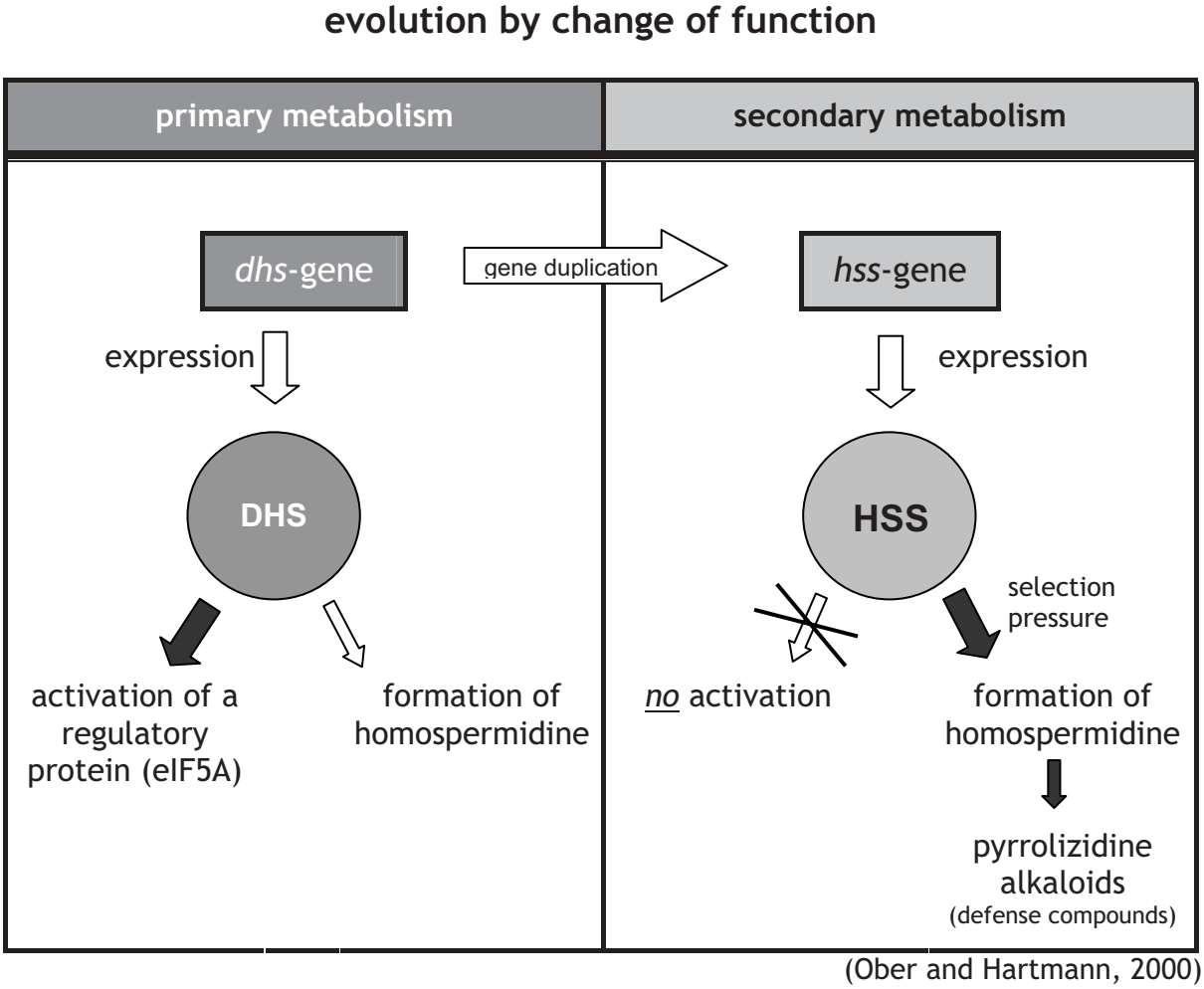


Fig. 1-2 Hypothetical evolutionary origin of HSS by duplication of the DHS-coding gene followed by the loss of DHS activity.

1.1.4 Current Knowledge of the Specific Expression of HSS

DHS as a typical enzyme of primary metabolism is conserved and found ubiquitously in all eukaryotic and archaebacterial organisms. The existence of DHS within plants has been shown for tobacco and *Senecio vernalis* (Ober and Hartmann, 1999 a, 1999 b). *S. vernalis* has been intensively investigated as a model for PA-producing plants. It has been used to study the first pathway-specific enzyme of PA biosynthesis, HSS, which has, for the first time, been isolated and cloned (Ober and Hartmann, 1999 b) from this plant. In accordance with the typical expression of enzymes involved in secondary metabolism, reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot studies conducted by Moll *et al*. (2002) have shown that HSS is exclusively present in the roots of *S. vernalis*, whereas DHS is

found in all plant organs of *S. vernalis* and of tobacco, a plant devoid of PA biosnythesis. Furthermore, by means of imunolocalization techniques, Moll *et al.* have successfully demonstrated the HSS expression more precisely in the root of *Senecio vernalis* in groups of spezialied cells of the endodermis and the neighbouring cortex located in the direct vicinity of the phloem.

Long before HSS from *S. vernalis* was intensively studied, the specific site of PA synthesis was identified as being in the roots of *S. vernalis*, with the phloem being identified as the vascular tissue for the translocation of PAs to the shoots (Hartmann *et al.*, 1989; Witte *et al.*, 1990).

1.2. Research Strategies and Objectives

The scattered occurrence of PAs in several unrelated families within the angiosperms has provoked the question as to whether the ability of plants to synthesize PAs occurred repeatedly and independently during evolution (polyphyletic) or whether all PA-producing plants share a common ancestor (monophyletic). The close relationship of HSS, as the first pathway-specific enzyme of PA biosynthesis, to the conserved and ubiquitous DHS, an enzyme involved in primary metabolism, has opened the way to answer this question about the evolutionary origin of PAs by studying the evolution of HSS in angiosperms.

With the aim of answering this evolutionary question, molecular cloning of DHS and HSS from two unrelated PA-containing species of *Phalaenopsis* (Orchidaceae) and *Crotalaria* (Fabaceae) has been carried out in the work presented here. Sequence comparison of DHS and HSS cDNA from these PA-containing species should provide useful information for answering this question. Furthermore, the typical expression of HSS in these plants has also been studied at the RNA level. As an extension of the analyses, the genomic structure of DHS and HSS has been determined in order to refine phylogenetic relationships among angiosperms.