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Distinct cell-specific expression of homospermidine synthase involved in pyrrolizidine alkaloid biosynthesis in three species of the Boraginales.¹

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Abbreviations: DHS, deoxyhypusine synthase; eIF5A, eukaryotic initiation factor 5A; FITC, fluorescein isothiocyanate; HSS, homospermidine synthase; PA, pyrrolizidine alkaloid; PMSF, phenylmethylsulfonyl fluoride;
ABSTRACT

Homospermidine synthase (HSS) is the first specific enzyme in pyrrolizidine alkaloid (PA) biosynthesis, a pathway involved in the plant’s chemical defense. HSS has been shown to be recruited repeatedly by duplication of a gene involved in primary metabolism. Within the lineage of the Boraginales, only one gene duplication event gave rise to HSS. Here, we demonstrate that the tissue-specific expression of HSS in three boraginaceous species, *Heliotropium indicum*, *Symphytum officinale*, and *Cynoglossum officinale*, is unique with respect to plant organ, tissue, and cell-type. Within *H. indicum*, HSS is expressed exclusively in non-specialized cells of the lower epidermis of young leaves and shoots. In *S. officinale*, HSS expression has been detected in the cells of the root endodermis and in leaves directly underneath developing inflorescences. In young roots of *C. officinale*, HSS is detected only in cells of the endodermis, but in a later developmental stage, additionally in the pericycle. The individual expression patterns are compared to those within the Senecioneae lineage (Asteraceae), where HSS expression is reproducibly found in specific cells of the endodermis and the adjacent cortex parenchyma of the roots. The individual expression patterns within the Boraginales species are discussed as being a requirement for the successful recruitment of HSS after gene duplication. The diversity of HSS expression within this lineage adds a further facet to the already diverse patterns of expression that have been observed for HSS in other PA-producing plant lineages, making this PA-specific enzyme one of the most diverse expressed proteins described in the literature.
INTRODUCTION

Pathways involved in the biosynthesis of alkaloids are characterized by an efficient coordination of a large number of enzymes. Molecular approaches have shown that the cellular localization of the alkaloid pathways is remarkably diverse and complex, often including the translocation of intermediates between multiple cell types (Facchini and St-Pierre, 2005; Ziegler and Facchini, 2008). The alkaloids are accumulated in cell types and tissues that are in most cases distinct from those that are involved in alkaloid biosynthesis. In the opium poppy (*Papaver somniferum*), alkaloids are synthesized in sieve elements of the phloem and accumulated in laticifers (Bird et al., 2003). Moreover vindoline, a monoterpenoid indole alkaloid of *Catharanthus roseus*, is accumulated in laticifers and also in idioblasts, but biosynthesis involves at least three different cell types (St-Pierre et al., 1999; Burlat et al., 2004). Nicotine and tropane alkaloids, such as hyoscyamine, are synthesized in roots of certain solanaceous species in at least two cell types and are translocated into the shoots (Nakajima and Hashimoto, 1999; Suzuki et al., 1999a). The same is true for the pyrrolizidine alkaloids (PAs), which *Senecio* species produce exclusively in the roots before their transport to the shoots via the phloem (Hartmann et al., 1989; Witte et al., 1990).

For PAs that are constitutively produced by the plant as part of its chemical defense against herbivores, the only unequivocally identified enzyme involved in their biosynthesis is homospermidine synthase (HSS). HSS catalyzes the formation of the first pathway-specific intermediate, homospermidine, which is incorporated exclusively into the necine base, the characteristic bicyclic structure of PAs (Böttcher et al., 1993; Böttcher et al., 1994). HSS has been shown to be recruited by gene duplication from deoxyhypusine synthase (DHS), an enzyme involved in the posttranslational activation of the eukaryotic initiation factor 5A (eIF5A). Phylogenetic analyses suggest that this recruitment occurred at least five times independently in the various angiosperm lineages that are described to produce PAs, *i.e.*, once within the Boraginales order, the monocots, and the Convolvulaceae (Reimann et al., 2004; Kaltenegger and Ober, unpublished). Within the Asteraceae, two independent gene duplications occurred in the lineages of the Senecioneae and the Eupatorieae (Ober and Hartmann, 1999b; Ober et al., 2003b; Reimann et al., 2004). Kinetic analyses and binding assays have shown that HSS can be interpreted as a DHS that lost its
protein-modifying activity while retaining all other properties, including the former “side activity” of the ancestor to catalyze the formation of homospermidine (Ober et al., 2003b). Thus, in the case of HSS evolution, the loss of DHS activity, i.e., the ability to bind the protein substrate, resulted in an enzyme with a new function.

Several examples in the literature show that partitioning of the primary gene function between two daughter genes is common with respect to gene expression (Force et al., 1999; Papp et al., 2003; Zhang, 2003; Bridgham et al., 2008). Comparative analyses of HSS and DHS expression in various PA-producing species have shown that the expression pattern of each enzyme differs, suggesting that, with respect to gene regulation, the gene duplication event was followed by subfunctionalization (Moll et al., 2002; Anke et al., 2004; Anke et al., 2008; Ober and Kaltenegger, 2009). Whereas DHS is expressed uniformly in all analyzed tissues, irrespective of the species, HSS shows highly specific expression patterns. In Senecio vernalis (Asteraceae, Senecioneae), HSS is expressed in groups of specific cells of the endodermis and the adjacent cortex parenchyma that are opposite to the phloem (Moll et al., 2002). The vicinity to the phloem suggests that these cells express not only HSS, but most probably also the complete PA pathway, as the phloem is the tissue by which the PAs are translocated from the roots to the shoots. In contrast, HSS of Eupatorium cannabinum (Asteraceae, Eupatorieae) is expressed in all cells of the root cortex parenchyma, but not in the cells of the endodermis (Anke et al., 2004). This individual expression pattern of the key enzyme of an identical pathway in these two lineages has been interpreted to support the polyphyletic origin of PA biosynthesis within the Asteraceae (Anke et al., 2004). Furthermore, in E. cannabinum, HSS expression has been shown to be dependent on the developmental stage of the plant. HSS expression is highest as long as the plant is growing. As soon as the flower buds open, HSS expression diminishes and is not detectable when the fruits are produced (Anke et al., 2004). This mechanism ensures relatively constant PA levels per biomass, as alkaloids are only produced when the plant is producing biomass by its growth. A similar developmental expression pattern has been observed in the orchid Phalaenopsis, a PA-producing member of the monocots. PA biosynthesis and HSS expression have been detected in mitotically active cells of the apical meristem of aerial roots, most probably providing PAs for the vegetative tissues of the plant, e.g., young leaves and the shoot meristem. As soon as the orchid develops an inflorescence, HSS expression and PA biosynthesis is
additionally detected in young flower buds, providing accessory alkaloids for the developing reproductive tissues. Although HSS expression diminishes with increasing age of the flower buds and is not detectable when the flower opens, PA levels are still high in the open flower, at about 1 mg/g fresh weight (Anke et al., 2008).

With respect to the Boraginales, we have studied three species in detail, i.e., *Heliotropium indicum* (indian heliotrope, perennial), *Symphytum officinale* (common comfrey, perennial), and *Cynoglossum officinale* (houndstongue, biennial). Based on the molecular analyses of this taxon, *H. indicum* is grouped in the family Heliotropiaceae, and the other two species in the Boraginaceae (Gottschling et al., 2001). Phylogenetic analyses of HSS- and DHS-coding cDNA sequences suggest that the gene duplication resulting in HSS occurred early in this lineage, well before the separation of the Heliotropiaceae from the Boraginaceae (Reimann et al., 2004). Tracer feeding experiments have shown that, first, the core pathway for PAs from putrescine and spermidine via homospermidine is the same as the pathway in *Senecio* species and, second, despite the common origin of HSS within the Boraginales lineage, different species differ with respect to their site of PA biosynthesis: PAs are produced exclusively in the shoots of *H. indicum* (Heliotropiaceae), exclusively in the roots of *S. officinale* (Boraginaceae), and in shoots and roots of *C. officinale* (Boraginaceae) (Van Dam et al., 1995; Frölich et al., 2007). To analyze these variations at the cellular level, we have studied the expression patterns of HSS and DHS, which share a common ancestor. Our results show that despite the identical function of HSS in PA biosynthesis, the HSS expression pattern is distinct in all three analyzed species. This observation is discussed with respect to the individual selection pressures that acted after gene duplication during recruitment of HSS for PA biosynthesis in the lineages of the three analyzed species. A comparative analysis of a further member of the Senecioneae lineage (Asteraceae) supports the view that this variability of expression patterns is a unique characteristic of the Boraginales lineage and not a general concept valid for all PA-producing lineages.
RESULTS

To analyze hss and dhs gene expression, we used semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR) to localize the transcript and, in the case of HSS, immunoblots to localize the respective protein. RT-PCR was performed with primers highly specific for the cDNAs of HSS and DHS to test for the presence or absence of the respective transcripts in the various plant tissues and to allow, at best, a rough estimate of transcript levels. Subsequently, the various tissues were tested by immunoblots for the expression of the HSS protein, before those tissues with the highest HSS expression were used for immunolocalization experiments.

Tissue-Specific Expression of HSS in H. indicum

In H. indicum, transcripts of the hss gene were detectable in all above-ground tissues tested, including young and older stems, leaves, and the flower from the bud to fruit stage (Fig. 1A). This result confirmed previous tracer feeding studies that identified the shoot of H. indicum as the site of PA biosynthesis. Roots were shown to be unable to incorporate tracer into the alkaloid structures, but to synthesize low levels of homospermidine (Frölich et al., 2007). For the tissues taken from the inflorescence, we were unable to exclude a positive signal because of adhering tissue of the inflorescence axis. Therefore, in an additional experiment cDNA was prepared separately from the inflorescence axis, the open flowers, and the fruits. High levels of hss transcript were detectable in the axis and the flowers, but almost no transcript was found in the fruits (Fig. 1B). In contrast, in both experiments, transcript of dhs was detectable in all tested tissues. The presence of the dhs transcript in all tested tissues raised the question as to whether the ability of the H. indicum roots to synthesize homospermidine that was described by (Frölich et al., 2007) was attributable to low levels of HSS in the roots as suggested by the faint signal detected by the PCR containing the cDNA of roots or to the known side activity of DHS (Ober and Hartmann, 1999a; Ober et al., 2003a; Reimann et al., 2004).

Immunoblots showed a more specific pattern of HSS expression (Fig. 1C left panel). Only protein extracts of young leaves revealed an intense signal, a weak signal being detectable in the extracts of young stems and of flower buds. No signal was detectable in roots, older stems, the open flower, the fruits, or older leaves. These
data suggest that HSS expression is dependent on the developmental stage of the leaves. This was tested in an additional immunoblot with extracts of leaves covering all developmental stages (categorized according to leaf size). The strongest signal was detectable in young leaves, steadily decreasing with the increasing size of the leaves (Fig. 1C right panel). This expression pattern of HSS correlates with the tracer feeding experiments by Frölich (2007) showing that PA biosynthesis is most efficient in young leaves. Of note, the epidermis peeled from young stems also showed a distinct signal for HSS expression.

**Immunolocalization of HSS in Shoots of *H. indicum***

The immunoblots suggested that HSS was expressed mainly in young leaves and to a lesser extent in the epidermis of young shoots and flower buds. Therefore, we decided to use young leaves and young stems of *H. indicum* for immunolocalization studies. Figure 2A shows a cross section of a young leaf in the area of the midrib with the longitudinal cells of the palisade parenchyma clearly visible in the upper right part of the section. The elements of the xylem show slight autofluorescence. The label for HSS protein was detectable by UV microscopy exclusively in the cells of the lower epidermis. Within the petiole and the young stem, the epidermis was again the only tissue that was labeled (Fig. 2D-F). The intensity of the label of the epidermis cells was contrasted to the complete lack of label in the single celled trichomes and the guard cells of the stomata, as specialized epidermis cells (Fig. 2B and C). Immunogold labeling of cells of the lower epidermis and detection of gold particles by transmission electron microscopy confirmed the expression of HSS in the cytoplasm of the epidermal cells (Fig. 2G).

**Tissue-Specific Expression of HSS in *S. officinale***

For *S. officinale*, tracer feeding experiments identified the roots as the exclusive tissue of PA biosynthesis, with root-less shoot cultures showing no incorporation of the tracer (Frölich et al., 2007). The RT-PCR experiments confirmed the presence of *hss* transcripts in the roots but also indicate the presence of the *hss* transcript in the leaves and the open flower (Fig. 3A). The transcript of *dhs* was detectable in all tested tissues. At the protein level, the expression of HSS was restricted to the young root. In contrast to the results of RT-PCR, protein extracts of leaves of two developmental stages (4 cm and 12 cm long, respectively) and of the open flower
showed no signal (Fig. 3B). We speculated that the stage of plant development might influence HSS expression. Therefore, we analyzed young terminal leaves that differed in their position on the plant. Type I leaves lay directly beyond a terminal inflorescence with fully opened flowers, type II leaves occurred at the same position, but with flower buds still closed, and type III leaves were on stems without any inflorescences. The extract of the young root served as a positive control. The immuno-blots confirmed the expression of HSS in leaves of type II, whereas leaves of type I and III were devoid of any label. This result supports the idea that HSS expression in the shoot of *S. officinale* depends on the developmental stage of the leaves.

**Immunolocalization of HSS in Roots of *S. officinale***

Young white roots that developed during the same year were cut and embedded in resin, before being labeled with HSS-specific antibody. Figure 2H+J show two cross sections of a young diarch root, one with an emerging lateral root originating in the pericycle cell layer in front of one of the xylem poles (Fig. 2J). In all such sections, HSS expression was found exclusively in the cells of the endodermis. The Casparian strip characteristic for the radial cell walls within the endodermis exhibited yellow autofluorescence because of suberin incrustation (Fig. 2H, marked with arrowheads). Those cells of the endodermis that were affected by the emerging lateral root did not show the HSS label, suggesting the absence of HSS expression in cells displaced from their original position within the root (Fig. 2J).

**Tissue-Specific Expression of HSS in *C. officinale***

*C. officinale* is the only plant species for which PA biosynthesis was detected by tracer feeding experiments in both shoot and root (Van Dam et al., 1995). Instead, the RT-PCR experiment and the immuno-blots analysis showed that the HSS encoding transcript and HSS protein were found only within the roots, occurring only as traces, if at all, within the other tissues (Fig. 4). This observation was independent of the age of the plant, *e.g.*, whether the samples had been taken off the rosette plant in the first year of development or off the flowering plant in the second year. Again, as in *H. indicum* and *S. officinale*, the *dhs* transcript was detectable in all tested tissues.

**Immunolocalization of HSS in Roots of *C. officinale***
For immunolocalization experiments, young white roots of the rosette plant and the flowering plant in their first and second year of development, respectively, were embedded in resin. For detection with an HSS-specific antibody for *C. officinale*, the various zones of differentiation were analyzed, *i.e.* the region that lies a few millimeters behind the root tip and in which the vascular cylinder is in the procambial stage (Fig. 5A), the region after complete differentiation (Fig. 5B), and the region that is characterized by emerging lateral roots (Fig. 5C+D). The intense green color of the fluorescein isothiocyanate (FITC)-labeled HSS was not detectable before the fate of the cells was determined in the completely differentiated root. Here, expression was restricted to the layer of the endodermis that was unequivocally identified by the presence of the Casparian strip showing yellow autofluorescence. In the region characterized by emerging lateral roots, HSS was detectable not only in the endodermis but also in the pericycle. As shown in Figure 5C, cambial cells were detectable (labeled by asterisks) between the xylem and the phloem tissue indicating that secondary growth started in this region of the root. Figure 5D shows that HSS expression is switched off in the area of an emerging side root, a phenomenon previously described for roots of *S. officinale*. The expression pattern of HSS in the analyzed zones of root differentiation was the same in roots taken from the rosette stage or the flowering stage of the plant in the first and second year of development, respectively (data not shown).

**Comparative Localization of HSS in Roots of *J. vulgaris***

Despite of the monophyletic origin of HSS within the lineage of the Boraginaceae, our data showed individual expression patterns of HSS in the three analyzed species. This observation raised the question as to whether such diversity might also be found within other lineages, for which an independent origin of HSS was reported (Reimann et al., 2004). Therefore, we decided to compare the tissue-specific expression pattern of HSS in two species of the Senecioneae lineage, one of the two lineages within the Asteraceae for which an independent origin of HSS was proposed (Reimann et al., 2004). We selected the species *Jacobaea vulgaris* (syn. *Senecio jacobaea*), which was formerly classified into the genus *Senecio* sect. *Jacobaea*. Recent molecular systematic studies have shown that species that belong to this section form a well-supported cluster, which is only distantly related to other species usually attributed to *Senecio*, such as *S. vernalis* (Pelser et al., 2006; Pelser et al., 2007). For *S. vernalis,*
we have been able, in a previous study, to show that HSS expression is root-specific and restricted to distinct groups of endodermis and neighboring cortex cells located opposite to the phloem (Moll et al., 2002). Figure 6A shows an immunoblot confirming the root-specific expression of HSS for *J. vulgaris*. Figure 5E shows a cross section of a young root of *J. vulgaris*, HSS being expressed in the same cells as described previously for *S. vernalis*, *i.e.*, only in those cells of the endodermis and the adjacent cortex parenchyma opposite to the phloem tissue. The phloem cells are located between the strands of the tetrarch xylem tissue. A longitudinal section of a root of the same age shows that the label runs parallel to the phloem tissue in those cortex cells that are closest to the central cylinder (Fig. 5F).
DISCUSSION

Our research group uses the model system of PA biosynthesis to study the evolution of a pathway in plant secondary metabolism. It is known that the first specific enzyme in PA biosynthesis, namely HSS, originated by duplication of a gene encoding an ancestral DHS, an enzyme that is involved in primary metabolism and that is highly conserved within eukaryotes (Park et al., 1997; Ober and Hartmann, 1999a, b). This duplication event occurred in an almost identical manner several times independently in various plant lineages during angiosperm evolution and, in particular, once early in the Boraginales lineage (Reimann et al., 2004). In consideration of the polyphyletic origin of HSS, the resulting PA-producing pathways are surprisingly identical with respect to the biosynthetic sequences and the structures of the resulting products (Frölich et al., 2007). Although HSS is the only identified and characterized enzyme of PA biosynthesis, convergent evolution is likely to have formed these pathways because of similar selection pressures, i.e., the defense against herbivores.

As the ability to synthesize PAs evolved independently in various angiosperm lineages, PA biosynthesis offers an instructive system for studying comparatively the repeated evolution of this pathway. Here, we have compared the cell-specific expression patterns of HSS in three species belonging to the Boraginales and show that, in all three species, HSS shows a unique and specific expression pattern (Fig. 7). In contrast, DHS transcription is detectable in all analyzed tissues, an observation that has been described previously for other tested species (Moll et al., 2002; Anke et al., 2004; Nurhayati and Ober, 2005; Anke et al., 2008; Ober and Kaltenegger, 2009). In H. indicum, HSS has been detected in the lower leaf epidermis and the epidermis of the petioles and the stem. Specialized epidermal cells, such as unicellular trichomes and guard cells, do not express HSS. Furthermore, the expression of HSS is dependent on the developmental stage of the leaf with the highest levels occurring in young tissues. This observation supports previous results of tracer feeding experiments that have shown that, in young leaves, the incorporation of tracer into PAs is the highest (Frölich et al., 2007). For S. officinale and C. officinale, the roots have been identified as the dominant and exclusive site of HSS expression. Of note, the expression of HSS is restricted to young roots, i.e., roots that have emerged in the year in which the root samples have been harvested. The loss of HSS expression in older roots is attributable to the extensive physiological and anatomical
modifications in the course of secondary growth. The pericycle develops to a periderm and subsequently all tissues outside of the pericycle will be lost (Esau, 1965), including the tissues of HSS expression. These are found, in the case of *S. officinale*, only in the endodermis, and in *C. officinale*, in the endodermis and pericycle. Expression of HSS within the pericycle of *C. officinale* is only detectable in the phase that is characterized by emerging lateral roots and has never been observed in the roots of *S. officinale*. Fundamental reprogramming of the cells in front of the lateral root that emerges from the pericycle is the most likely the reason for the loss of HSS expression in these tissues in *S. officinale* and *C. officinale* (Fig. 2J and 5D), an observation that has previously been described for HSS expression in *E. cannabinum* (Asteraceae) (Anke et al., 2004).

RT-PCR studies entail the risk of false positive signals that are caused by contamination with genomic DNA or by the presence of transcribed processed pseudogenes. Processed pseudogenes are devoid of introns. Therefore, the PCR products of a specific cDNA under study and a related processed pseudogene are only discriminable by sequencing. Recent studies of HSS and DHS in species of the genera *Crotalaria* (Fabaceae) and *Phalaenopsis* (Orchidaceae) have shown the presence of processed pseudogenes with a high similarity of HSS and DHS sequences, some of which are transcribed (Nurhayati and Ober, 2005; Nurhayati et al., 2009). Of the three Boraginales species in this study, we have never identified a processed pseudogene related to HSS or DHS. Nevertheless, we have found differences between the results of RT-PCR and immunoblot, namely, not all RT-PCR signals have also been detected by immunoblot. For example, the detection of *hss* transcript in open flowers of *H. indicum* by RT-PCR is not supported by the detection of the encoded protein in the immuno-blot (Fig. 1). These differences might be attributed to the higher sensitivity of RT-PCR in comparison with the immunoblot technique. Furthermore, the samples used for RT-PCR and immunoblot are not identical. Differences in sampling might be critical; indeed, we have shown in previous studies that HSS expression can be limited to specific developmental phases (Anke et al., 2004; Anke et al., 2008). Further investigations will be necessary to test for HSS expression in the flowers of *H. indicum* and to determine whether the young leaves of *S. officinale* do indeed only express HSS or the full pathway of PA biosynthesis.
Remarkably, all the types of tissue in which we have identified HSS expression in the three species of the Boraginales have previously been described as the site of expression for enzymes involved in other alkaloid-producing pathways. For example, the epidermis of *C. roseus* has been shown to express all genes involved in the pathway of the monoterpenoid indole alkaloids (Murata et al., 2008). In *Thalictrum flavum*, the transcripts of all genes involved in the biosynthesis of benzylisoquinoline alkaloids have been localized in the pericycle and the adjacent cortical cells (Samanani et al., 2005). Moreover, putrescine *N*-methyltransferase and hyoscyamine 6β-hydroxylase, which catalyze the first and the last steps in the biosynthesis of the tropane alkaloid scopolamine, respectively, have been localized to the pericycle in the roots of *Atropa belladonna* and *Hyoscyamus niger* (Hashimoto et al., 1991; Kanegae et al., 1994; Suzuki et al., 1999a; Suzuki et al., 1999b). Tropinone reductase I, another specific enzyme of this pathway, is expressed in the endodermis and cells of the inner cortex (Nakajima and Hashimoto, 1999). The same set of cells express HSS in PA-producing *S. vernalis*, but only those cells that are opposite to the phloem strands in the central radial vascular bundle (Moll et al., 2002). This expression pattern seems to be characteristic for the Senecioneae lineage within the Asteraceae, as has been confirmed in this study for *J. vulgaris*.

The expression of HSS in the vicinity of the phloem tissue of species belonging to the Senecioneae matches functional requirements, as PAs are translocated in *Senecio* from the roots as the site of synthesis to the shoot via the phloem (Hartmann et al., 1989). The correlation of HSS expression with young and growing tissues and the finding that, in *S. officinale*, in addition to the roots, young leaves express HSS that are closely beneath an inflorescence should be interpreted as the outcome of selection processes that have optimized the protection of reproductive tissues. Growth correlation of HSS expression and the boost of PA content by additional PA biosynthesis within developing flower buds have previously been described for other species and seem to be a repeatedly used pattern for PA biosynthesis (Anke et al., 2004; Anke et al., 2008).

Plant secondary metabolism is well-known for its diversity concerning the huge array of chemical structures that are produced. Our studies show that secondary metabolism is also diverse with respect to its regulation. Although HSS catalyzes an identical reaction in PA biosynthesis, it is one of the most diverse enzymes described
so far for plant metabolism with respect to its expression pattern. Furthermore, we have demonstrated that, despite the monophyletic origin of an enzyme, its expression pattern can be highly specific in different species and thus does not provide a robust argument for conclusions about the evolutionary origin of this specific gene (Anke et al., 2004). This has previously proposed for the enzymes that are involved in benzylisoquinoline alkaloid biosynthesis; these enzymes have been localized to different tissues in the opium poppy and T. flavum, and a monophyletic origin early in the evolution of the Angiosperms has been proposed for them (Liscombe et al., 2005; Samanani et al., 2005). For the evolution of HSS within the Convolvulaceae, we have been able to show that the duplication of the dhs gene was followed by a time of strong purifying selection on both copies, most likely because of protein-protein interactions involved in the mechanism of DHS and HSS (Kaltenegger and Ober, unpublished). These involve the binding of the eIF5A precursor protein as the substrate of the DHS and the interaction of subunits, as both enzymes are homotetramers. Mutations within one of the gene copies would have detrimental effects on the essential function of DHS, resulting in a strong purifying selection on both copies. Subfunctionalization with respect to tissue-specific expression has been postulated to precede the diversification of the structural gene. Within the Convolvulaceae, positive Darwinian selection has been detected to have shaped one of the copies to become a HSS later in evolution, most probably after subfunctionalization of the gene copy regulation (Kaltenegger and Ober, unpublished). A similar scenario is likely within the Boraginales, suggesting that speciation events might have preceded the lineage-specific modification of the regulatory elements of one of the gene copies, i.e., the gene that developed to encode the HSS. The high cell specificity of HSS expression might be a tool for a straightforward approach for the identification of new enzymes and regulatory elements involved in PA biosynthesis by differential or cell-specific approaches. The knowledge of these additional elements is essential if we are to shed light on the independent evolution of the pathway in various plant lineages.
MATERIAL AND METHODS

Plant Material

H. indicum plants were grown in the greenhouse at approx. 25°C and were propagated by shoot cuttings. S. officinale and C. officinale plants were grown in the institute garden, some of them being potted to allow easy sampling of the roots.

RNA Isolation and Semiquantitative RT-PCR

Samples of various plant tissues were pulverized in liquid nitrogen in a mortar before total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen). For each sample, 1 µg of total RNA was used as a template for reverse transcription with an oligo(dT)17 primer (Supplemental Table 1) by using SuperScript II Reverse Transcriptase (Invitrogen). PCR with Taq DNA polymerase was performed with the following primer pairs (and annealing temperatures): P1/P3 (60°C) for HSS and P4/P6 (60°C) for DHS of H. indicum, P7/P9 (60°C) for HSS and P10/P12 (60°C) for DHS of S. officinale, P13/P15 (57°C) for HSS and P16/P18 (57°C) for DHS of C. officinale. Aliquots were taken after 30 and 35 cycles to ensure sampling before saturation of PCR product formation. Identity of the PCR products was confirmed by sequencing. To test for reproducibility of RNA quantification, an aliquot of 1 µg of each RNA sample was separated together with the PCR products on a 1% (w/v) agarose gel.

Recombinant Protein Expression

To generate and to test the specificity of polyclonal antibodies against HSS of H. indicum, S. officinale, C. officinale, and J. vulgaris, recombinant HSS and DHS of each species were expressed and purified. The cDNAs encoding HSS and DHS of all four species were amplified and cloned into pET3a/pET3amod expression vectors according to Reimann et al. (2004) but with modified reverse primers that encoded a 6xhis tag directly in front of the stop codon (Supplemental Table 1). The expression vector pET23a was used only for expression of HSS and DHS of J. vulgaris. Expression constructs were introduced into Escherichia coli BL21(DE3) and expressed as described previously (Ober and Hartmann, 1999a). E. coli cells were harvested by centrifugation, suspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole), and broken up by sonication. His-tagged proteins were purified with nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) according
to the manufacturer’s instructions. A cobalt-containing resin (Talon Metal Affinity Resin, Clontech) was used only in the case of the HSS of *S. officinale*.

**Polyclonal Antibody Preparation and Affinity Purification**

Recombinant HSS of *H. indicum, S. officinale, C. officinale*, and *J. vulgaris* were used to raise polyclonal serum in rabbits (Bioscience, Göttingen, Germany). For affinity purification, 1-5 mg of purified recombinant HSS was coupled to CNBr-activated Sepharose 4B (GE Healthcare). The resulting matrix was used to purify the polyclonal antibodies as described previously (Anke et al., 2004). The antibodies used in this study against HSS of *H. indicum, S. officinale, C. officinale*, and *J. vulgaris* were tested in immunoblots for their cross-reactivity to DHS of the same species. Two gels for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared, each with identical amounts of purified recombinant HSS and DHS of *H. indicum, S. officinale, C. officinale*, and *J. vulgaris* respectively. After the blotting step, one membrane was used for protein staining with Indian ink to ensure equal loading of HSS and DHS protein samples, and the other was developed as an immunoblot with the antibody against HSS of the respective species. In all analyses, the antibody against HSS was also able to bind to the DHS of the same species, but with a 5- to 10-fold (*H. indicum*), 5-fold (*S. officinale*), 10-fold (*C. officinale*), and 5-fold (*J. vulgaris*) lower affinity than to HSS.

**Protein Gel-Blot Analysis**

Various tissues were sampled and immediately frozen in liquid nitrogen and stored in -80°C until use. For protein extraction, the samples were pulverized in a mortar in the presence of liquid nitrogen and extracted twice in phosphate-buffered saline supplemented with 5% (m/v) polyvinylpyrrolidone and 2.5% (w/v) sodium ascorbate. Addition of polyvinylpyrrolidone and sodium ascorbate reduced protein precipitation during sample preparation by polyphenols present in large amounts in tissues of Boraginaceae plants. Protein separation by SDS-PAGE, semidry blotting, and immunodetection was carried out as described in Anke et al. (2008). To estimate the mass of protein fragments, the protein molecular weight marker 14.4 to 116 kDa (Fermentas) was used.
Immunocytochemical Localization

Small segments of various plant organs (0.5-1.0 cm) were immersed for 2 h under reduced pressure in ice-cold buffered fixative according to Anke et al. (2004). To avoid protein precipitation by polyphenols, sodium ascorbate (2.5%, m/v) was added as an antioxidant to the fixative just before the samples were added. Following dehydration in a graded ethanol series, the tissues were embedded in Technovit 7100 resin (Hereaus-Kulzer, Hanau, Germany) for light microscopy analyses or in Unicryl resin (Plano, Wetzlar, Germany) for transmission electron microscopy, according to the manufacturer’s instructions. Sections of 3 to 4 µm in thickness were cut by using a microtome (HM355S, Microm) and mounted on glass slides coated with Teflon (Roth, Karlsruhe, Germany) or on adhesion microscope slides (SuperFrost, Menzel, Braunschweig, Germany). Blocking of the sections with protein, incubation with antibodies, and detection was performed as described previously (Anke et al., 2008) with the modification that, for the UV detection step, a goat-anti rabbit AlexaFluor488 antibody (1:100, Molecular Probes, Invitrogen) was used as secondary antibody. The specificity of the label was confirmed by the incubation of successive sections with HSS-specific antibody of the respective plant to which increasing amounts of soluble HSS were added. These preincubations resulted in a decreasing intensity of the label. Preincubations with soluble DHS and bovine serum albumin (BSA) had no effect on labeling intensity, excluding any cross-detection of DHS and any non-specific protein labeling (Supplemental Fig. 1-5).

Detection of Unspecific Proteinases in Protein Preparations for Immunoblots

In some of our immunoblots, mainly of root tissue, incubation with HSS-specific antibody resulted not only in the detection of the HSS signal of approx. 44 kDa, but also in the detection of two additional bands of 18 and 26 kDa (Fig. 3B, 4B, 6A). We speculated that these two bands might be the result of cleavage of the HSS protein, e.g., by proteolytic activity from contamination of residual soil adhering to the fine young roots harvested for the analyses or by an endogenous regulatory process within the root. The latter idea resulted from the observation that, in initial experiments, we observed the cleavage mainly in samples containing older roots. To test these hypotheses, we used a root tissue culture of J. vulgaris grown under axenic conditions, divided the sample into three aliquots and extracted one aliquot according to the standard protocol. To the extraction buffer of the second aliquot, soil
material was added that had been taken from the field in which the *J. vulgaris* plants of this study had been grown. The third aliquot was extracted under the same conditions as the second aliquot but with addition of the proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF, final concentration 0.2 mM). Figure 6B shows a strong signal of HSS at 44 kDa. Cleavage of HSS was found in the second aliquot by detection of an additional 26 kDa signal. Cleavage was efficiently inhibited by the addition of PMSF in the third aliquot, supporting our hypothesis that degradation of HSS was attributable to contamination from soil material.

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Figure Legends:

Figure 1. Tissue-specific expression of HSS and DHS in *H. indicum*. Semiquantitative RT-PCR was performed with primers specific for HSS and DHS of *H. indicum*, with an RNA quantification control of various plant tissues (A) and of a dissected inflorescence (B). One microgram total RNA was used as template for reverse transcription and for RNA quantification control on a 1% agarose gel. Immunoblot of proteins extracted from various tissues and from leaves of various sizes with antibody specific for HSS of *H. indicum* (C). Twenty-microgram aliquots of each extract were applied to the gel, and the protein weight marker was stained separately with Indian ink. The 45-kD band is indicated by an arrowhead.

Figure 2. Immunolabeling of HSS in cross sections of the shoot of *H. indicum* and of *S. officinale*. Cross sections of a young leaf (A and B), of a petiole (C and E), and a young stem (D and F) of *H. indicum* and of young roots of *S. officinale* (H and J). Sections of 3 µm in thickness were incubated with antibody against HSS of *H. indicum* (A-G) and against HSS of *S. officinale* (H and J) before detection with AlexaFluor488 secondary antibody (A-C, H and J) or with a gold (18 nm)-labeled secondary antibody followed by silver enhancement and visualization by bright-field (E and F) or dark-field microscopy (D). Figure B is a higher magnification of A. Figure G, transmission electron micrograph of HSS in a cell of the lower leaf epidermis. H, Section of a primary diarch root. The xylem and the Casparian strip (arrow heads) show yellow autofluorescence. J, Section as in A but with an emerging lateral root. Size bars, A-D: 10 µm, E and F: 50 µm, G: 1 µm, H and J: 20 µm. CO, cortex parenchyma; CU, cuticle; CW, cell wall; EN, endodermis; EP, epidermis; GC, guard cell; LR, lateral root; MR, midrib; PH, phloem; PP, palisade parenchyma; SM, spongy mesophyll; T, trichome; XY, xylem.

Figure 3. Tissue-specific expression of HSS and DHS in *S. officinale*. Semiquantitative RT-PCR was performed with primers specific for HSS and DHS of *S. officinale*, with an RNA quantification control of various plant tissues (A). Immunoblots of proteins extracted from various tissues (B) and from leaves at different developmental stages (C) with antibody specific for HSS of *S. officinale*. The 45-kD band is indicated by an arrowhead.
Figure 4. Tissue-specific expression of HSS and DHS in *C. officinale*. Semiquantitative RT-PCR was performed with primers specific for HSS and DHS of *C. officinale*, with an RNA quantification control of various plant tissues (A). Immunoblot of proteins extracted from various tissues (B) with antibody specific for HSS of *C. officinale*. The 45-kD band is indicated by an arrowhead.

Figure 5. Immunolabeling of HSS in root sections of *C. officinale* (A-D) and of *J. vulgaris* (E and F). A, Cross section of a young root a few millimeters behind the root tip, in which the vascular cylinder is in the procambial stage. B, Cross section after complete differentiation. C and D, Cross sections of emerging lateral roots. The Casparian strip is labeled by arrow heads. Cambial cells between the xylem and the phloem that indicate beginning secondary growth are labeled by asterisks. Cross section (E) and longitudinal section (F) of young roots of *J. vulgaris*. Sections have been labeled with antibody against HSS of *C. officinale* (A-D) and against HSS of *J. vulgaris* (E and F) before detection with AlexaFluor488 secondary antibody. Exodermis and xylem elements show yellow/orange autofluorescence attributable to incrustation of lignin in the cell walls. Size bars = 20 µm. CO, cortex parenchyma; EN, endodermis; EX, exodermis; LR, lateral root; PC, procambium; PE, pericycle; PH, phloem; XY, xylem.

Figure 6. Tissue-specific expression of HSS in *J vulgaris*. Immunoblot of proteins extracted from various tissues (A) and of roots by three different extraction procedures (B) with antibody specific for HSS of *J. vulgaris*. Roots of a tissue culture have been extracted according to the standard protocol (I), in the presence of soil material taken from the field without (II) and with (III) the addition of proteinase inhibitor.

Figure 7. Localization of HSS in various PA-producing plant species. CO, cortex parenchyma; EN, endodermis; EP, epidermis; EX, exodermis; GC, guard cell; PC, procambium; PE, pericycle; PH, phloem; PP, palisade parenchyma; RC, root cap; RH, rhizodermis; SM, spongy mesophyll; T, trichome; XY, xylem.
Supplemental Material:

Supplemental Table 1. Nucleotide sequence of primers. Restriction sites used for cloning are underlined. H.i., *H. indicum*; S.o., *S. officinale*; C.O., *C. officinale*; J.v., *J. vulgaris*; for, forward; rev, reverse.

Supplemental Figure 1. Cross sections of the lower epidermis of young leaves of *Heliotropium indicum* labeled with HSS-specific antibody according to the standard protocol (A), and after preincubation of the antibody with BSA (B), HSS (C and D), and DHS (E and F) at a molar ratio of antibody to added protein of 10:1 (C and E) and 1:3 (B, D, and F). Size bar = 10 µm.

Supplemental Figure 2. Cross sections of the stem epidermis of *Heliotropium indicum* labeled with HSS-specific antibody according to the standard protocol (A), and after preincubation of the antibody with BSA (B), HSS (C and D), and DHS (E and F) at a molar ratio of antibody to added protein of 10:1 (C and E) and 1:3 (B, D, and F). Size bar = 10 µm.

Supplemental Figure 3: Cross sections of primary roots of *Symphytum officinale* labeled with HSS-specific antibody according to the standard protocol (A), and after preincubation of the antibody with BSA (B), HSS (C and D), and DHS (E and F) at a molar ratio of antibody to added protein of 10:1 (C and E) and 1:3 (B, D, and F). Size bar = 10 µm.

Supplemental Figure 4. Cross sections of primary roots of *Cynoglossum officinale* labeled with HSS-specific antibody according to the standard protocol (A), and after preincubation of the antibody with BSA (B), HSS (C and D), and DHS (E and F) at a molar ratio of antibody to added protein of 10:1 (C and E) and 1:3 (B, D, and F). Size bar = 10 µm.

Supplemental Figure 5. Cross sections of primary roots of *Jacobaea vulgaris* (Syn. *Senecio jacobaea*) labeled with HSS-specific antibody according to the standard protocol (A), and after preincubation of the antibody with BSA (B), HSS (C and D), and DHS (E and F) at a molar ratio of antibody to added protein of 10:1 (C and E) and 1:3 (B, D, and F). Size bar = 10 µm.
Heliotropium indicum

(A) RNA, HSS, and DHS gel images for different samples:
- root, young stem, old stem, leaf, young flower bud, open flower, fruit
- negative control (water), positive control (HSS/DHS-coding plasmid)
- 100 bp ladder

(B) RNA, HSS, and DHS gel images for different samples:
- axis of inflorescence, flower, fruit
- negative control (HSS/DHS-coding plasmid)
- 100 bp ladder

(C) Protein gel images for different samples:
- root, young stem, old stem, leaf, young leaf, flower bud, fruit
- open flower
- + control (50 ng HSS protein)
- protein ladder
- leaf, 1 cm in length, leaf, 2 cm in length, leaf, 3 cm in length, leaf, 4 cm in length, leaf, 5 cm in length, leaf, >6 cm in length
- control (buffer)
- stem epidermis
**Jacobiaca vulgaris**

**A**
- root, old
- root, young
- stem
- leaf, old
- leaf, young
- flower bud
- open flower
- + control (100 ng HSS protein)

**B**
- protein ladder

([Image of gel electrophoresis results])

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**Senecio vernalis**
*Jacobaea vulgaris*  
(Senecioneae, Asteraceae)
- expression in cells of endodermis and cortex that are opposite of the phloem  
- expression exclusively in primary roots

**Eupatorium cannabinum**  
(Eupatorieae, Asteraceae)
- expression in cells of the cortex parenchyma, not in the endodermis  
- expression only in the young, growing plant  
- no expression in front of an emerging lateral root

**Phalaenopsis spec.**  
(Orchidaceae)
- expression in the mitotically active cells of the apical meristem of aerial roots  
- additional expression in young, developing flower buds

**Heliotropium indicum**  
(Heliotropiaceae)
- expression in cells of the lower leaf epidermis, but not in trichomes or guard cells  
- expression in the shoot epidermis

**Symphytum officinale**  
(Boraginaceae)
- expression in cells the endodermis  
- additional expression in young leaves underneath flower buds  
- no expression in front of an emerging lateral root

**Cynoglossum officinale**  
(Boraginaceae)
- expression in cells the endodermis and later also in the pericycle  
- no expression in front of an emerging lateral root