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Tissue distribution, core biosynthesis and diversification of pyrrolizidine alkaloids of the lycopsamine type in three Boraginaceae species

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Abstract

Three species of the Boraginaceae were studied: greenhouse-grown plants of *Heliotropium indicum* and *Agrobacterium rhizogenes* transformed roots cultures (hairy roots) of *Cynoglossum officinale* and *Symphytum officinale*. The species-specific pyrrolizidine alkaloid (PA) profiles of the three systems were established by GC–MS. All PAs are genuinely present as *N*-oxides. In *H. indicum* the tissue-specific PA distribution revealed the presence of PAs in all tissues with the highest levels in the inflorescences which in a flowering plant may account for more than 70% of total plant alkaloid. The sites of PA biosynthesis vary among species. In *H. indicum* PAs are synthesized in the shoot but not roots whereas they are only made in shoots for *C. officinale* and in roots of *S. officinale*. Classical tracer studies with radioactively labelled precursor amines (e.g., putrescine, spermidine and homospermidine) and various necine bases (trachelanthamidine, supinidine, retronecine, heliotridine) and potential ester alkaloid intermediates (e.g., trachelanthamine, supinine) were performed to evaluate the biosynthetic sequences. It was relevant to perform these comparative studies since the key enzyme of the core pathway, homospermidine synthase, evolved independently in the Boraginaceae and, for instance, in the Asteraceae [Reimann, A., Nurhayati, N., Backenkohler, A., Ober, D., 2004. Repeated evolution of the pyrrolizidine alkaloid-mediated defense system in separate angiosperm lineages. Plant Cell 16, 2772–2784.]. These studies showed that the core pathway for the formation of trachelanthamidine from putrescine and spermidine via homospermidine is common to the pathway in *Senecio* ssp. (Asteraceae). In both pathways homospermidine is further processed by a β-hydroxyethylhydrazine sensitive diamine oxidase. Further steps of PA biosynthesis starting with trachelanthamidine as common precursor occur in two successive stages. Firstly, the necine bases are structurally modified and either before or after this modification are converted into their O^9-esters by esterification with one of the stereoisomers of 2,3-dihydroxy-2-isopropylbutyric acid, the unique necic acid of PAs of the lycopsamine type. Secondly, the necine O^9-esters may be further diversified by O^7- and/or $O^{3'}$-acylation.

Keywords: *Heliotropium indicum*, Boraginaceae; *Cynoglossum officinale*; *Symphytum officinale*; Hairy root cultures; Pyrrolizidine alkaloid biosynthesis; Alkaloid tissue distribution; Structural diversification

1. Introduction

Within the diverse class of pyrrolizidine alkaloids (PAs) the two major types are represented by macrocyclic diesters either with a 12-membered ring (senecionine type) or an 11-membered ring (monocrotaline type) and monoesters or open-chain diesters containing the unique branched aliphatic C_7 necic acid, 2,3-dihydroxy-2-isopropylbutyric acid. Macrocyclic PAs are found in the Asteraceae (tribe Senecioneae) and the Fabaceae (e.g., genus *Crotalaria*); PAs of the lycopsamine type are known from the families Asteraceae (tribe Eupatorieae) and many genera of the

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Boraginaceae and Apocynaceae (Hartmann and Witte, 1995; Hartmann and Ober, 2000).

Recently it was shown that the PA-specific biosynthetic pathways evolved polyphyletically within the angiosperms. The first pathway-specific enzyme of PA biosynthesis, homospermidine synthase (HSS, EC 2.5.1.45), was recruited at least four times independently: once within the Boraginaceae and within the Orchidaceae, respectively, and even twice within the Asteraceae, i.e. independently within the Senecioneae and the Eupatorieae (Reimann et al., 2004). Homospermidine synthase which till now has been only detected in PA-producing plant species, catalyzes the formation of the polyamine homospermidine, the unique precursor of the necine base moiety of all PAs representing 1-hydroxmethylpyrrolizidine derivatives. The enzyme has been isolated, purified, cloned and characterized from root cultures of *Senecio vulgaris* (Böttcher et al., 1993, 1994; Ober et al., 2000) and *S. vernalis* (Ober and Hartmann, 1999; Ober et al., 2003b). In *Senecio* senecionine *N*-oxide, the backbone structure of macrocyclic PAs of the senecionine type, is exclusively synthesized in the roots and translocated to shoots through the phloem (Hartmann et al., 1989). In the shoots senecionine *N*-oxide is biochemically modified to yield the species-specific PA patterns (Hartmann and Dierich, 1998; Pelser et al., 2005). In *Senecio* PAs preferentially accumulate in the inflorescences (Hartmann and Zimmer, 1986) and are stored within cellular vacuoles (Ehmke et al., 1988). The site of PA biosynthesis in roots is restricted to small groups of endodermal and adjacent cortex cells located opposite the phloem as demonstrated by immunolocalization of homospermidine synthase (Moll et al., 2002).

Although the biochemistry and physiology of PAs in *Senecio* is quite well understood only little information is available about PAs in other plant taxa. The polyphyletic origin of homospermidine synthase within the angiosperms provokes intriguing questions regarding evolutionary and mechanistic aspects about the origin of the biosynthetic pathways in PA containing species of distant angiosperm families. In this study we compare the core pathway of PA biosynthesis in three species of the Boraginaceae and the species-specific strategies of structural diversification. By means of classical tracer techniques the alkaloid distribution between major plant organs, identification of the sites of PA biosynthesis and the sequence of structure diversification in *Heliotropium indicum* (major object) are studied. For comparison PA producing hairy root-cultures of *Cynoglossum officinale* and *Symphytum officinale* are included.

2. Results and discussion

2.1. Tissue-specific alkaloid levels in H. indicum

H. indicum is a phytochemically well studied species, containing PA profiles that greatly vary between populations (Hartmann and Witte, 1995). The alkaloid profile of the *H. indicum* population used in our experiments includes six PAs, i.e. indicine, and its 3′-acetylesters as major alkaloids. These are accompanied by minor quantities of lycopsamine and the three indicine aralkyl esters, 3′-benzoyl-, 3′-cinnamoyl- and 3′-dihydrocinnamoyl-indicine that are previously not known from *H. indicum*. PAs are detectable in all plant organs. Indicine is the dominating PA in the inflorescences (>75% of total PAs) whereas the vegetative plant tissues contain about equal proportions of indicine and its 3′-acetylester. The minor alkaloids generally account for less than 5–10% of total PAs. The inflorescences with all developmental flower stages possess 71% of total plant PAs (Table 1), whereas the vegetative shoot including leaves and all stem parts display only 10%; roots account for the remaining 19%. Consequently inflorescences, especially the flowers and flower buds hold the highest PA tissue concentrations, reaching up to 1.5% of dry weight. This PA tissue distribution roughly corroborates the results of earlier studies (Catalfamo et al., 1982). Preferential accumulation of PAs in the reproductive tissues is also known from species of the Asteraceae, e.g. *Senecio vulgaris* and *S. vernalis* (Hartmann and Zimmer, 1986) and Orchidaceae, e.g. *Phalaenopsis* hybrids (Frölich et al., 2006).

2.2. Biogenetic tracer feeding experiments with H. indicum

Since the PA distribution all over the plant does not indicate a preferential site of synthesis, detached plant organs were incubated with ^{14}C-labelled putrescine as described earlier for *Senecio vulgaris* (Hartmann et al., 1989). Abscised inflorescences, leaves and stems were allowed to take up the tracer via the transpiration stream.

Table 1
Contents and concentrations of total PAs in the different organs of a single representative four-months-old specimen of *Heliotropium indicum*

Plant organ analyzed	Total PAs		
	Content (mg)	Concentration (mg/g dry wt)	Rel. abundance (%)
Inflorescence			
Flower buds	7.1	11.3	71
Open flowers	47.1	15.2	
Withered flowers	13.0	5.2	
Fruits	7.3	3.5	
Stems			
Upper part	2.5	1.4	3
Lower part	0.6	0.1	
Leaves			
Young	1.4	2.1	7
Medium	1.6	1.0	
Old	4.3	0.4	
Roots			
Young	8.8	4.9	19
Old	11.7	4.7	
Whole plant	105.4	3.2	100