

***In planta* studies on the biosynthesis
of pyrrolizidine alkaloids and the
involvement of copper-containing
amine oxidases**

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I. Abstract

Many plants especially from the families of Asteraceae, Boraginaceae, Heliotropiaceae, Fabaceae and Orchidaceae produce pyrrolizidine alkaloids (PAs) that are often toxic to fend off herbivores. Till this work only the first step of PA biosynthesis was elucidated. The entry-step of the PA biosynthesis is performed by an enzyme called homospermidinesynthase (HSS) which transfers an aminobutyl moiety of spermidine onto putrescine and creates the symmetric triamine, homospermidine. This enzyme, resulting from a duplication of the gene encoding the enzyme deoxyhypusine synthase (DHS) was shown to have developed several times independently.

Using common comfrey (*Symphytum officinale*, Boraginaceae) as a model, different aspects of PA biosynthesis have been studied. It was shown that PA biosynthesis, though constitutive, is dynamic with respect to its regulation. A second site of PA biosynthesis was identified *in planta* within leaves subtending the reproductive parts (inflorescence) of the plants.

Furthermore the work showed that this variability is also found on the intraspecific and intraindividual level by analyzing PA distribution and amounts in common comfrey. This result was validated also in another model system the Indian heliotrope (*Heliotropium indicum*, Heliotropiaceae) in respect to the variability of PA levels. This variability of PA levels was mirrored by the expression levels of HSS, an observation that was also valid for a copper-containing amine oxidase (CuAO). This CuAO was regarded as a candidate for a further step in PA biosynthesis, i.e. the oxidation of homospermidine.

The sequence of the CuAO candidate was used to design virus-induced gene silencing (VIGS) experiments to knock down the candidate CuAO. Successful knock-downs showed reduced PA levels confirming the hypothesis of this CuAO is involved in PA biosynthesis. Chemical elucidation of the homospermidine transformation by the candidate CuAO resulted in the identification of a double oxidized and already double cyclized PA precursor. This result was further validated by tracer-feeding experiments to further support the involvement of the candidate CuAO, now named homospermidine oxidase in PA biosynthesis.

II. Zusammenfassung

Viele Pflanzen vor allem aus den Familien der Asteraceae, Boraginaceae, Heliotropiaceae, Fabaceae und Orchidaceae produzieren oft giftige Pyrrolizidinalkaloide (PAs), um sich vor Fraßfeinden zu schützen. Bis zu dieser Arbeit war nur der erste Schritt der PA-Biosynthese aufgeklärt. Dieser wird von dem Enzym Homospermidinsynthase (HSS) katalysiert, das eine Aminobutyl-Einheit von Spermidin auf Putrescin transferiert und dadurch das symmetrische Triamin Homospermidin produziert. Dieses Enzym, welches aus einer Genduplikation des Primärstoffwechsellenzym Desoxyhypusinsynthase entstanden ist, entwickelte sich mehrfach unabhängig.

Anhand des Arzneibeinwells (*Symphytum officinale*, Boraginaceae) als Modellpflanze wurden verschiedene Aspekte der PA-Biosynthese untersucht. Es zeigte sich, dass die PA-Biosynthese, obwohl sie konstitutiv ist, einen dynamischen Aspekt der Regulation besitzt. Ein zweiter Ort der PA-Biosynthese wurde *in planta* in jenen Blättern identifiziert, welche sich unterhalb den reproduktiven Organen (Blütenstand) der Pflanze befinden.

Desweiteren macht die Arbeit deutlich, dass die PAs auch eine deutliche intraspezifische und intraindividuelle Variabilität ihrer Mengen ihrer Verteilung zeigen. Diese Beobachtung konnte in einer weiteren Modellpflanze, der Indischen Sonnenwende (*Heliotropium indicum*, Heliotropiaceae), in Bezug auf die Menge der PAs bestätigt werden. In dieser Pflanze wurde zudem beobachtet, dass sich die Variabilität der PA-Level in der Transkriptmenge der HSS widerspiegeln. Gleiches wurde für eine kupferhaltige Aminoxydase (CuAO) beobachtet, von der vermutet wurde, dass sie an der PA-Biosynthese, der Oxidation des Homospermidins, beteiligt sein könnte. Der Hinweis auf eine mögliche Koregulation von HSS und der CuAO wurde genutzt, um virus-induced gene silencing (VIGS) Experimente durchzuführen. Infolge einer erfolgreichen Reduktion der CuAO-Transkriptmengen wurden auch reduzierte Mengen an PAs beobachtet, was die Hypothese einer Beteiligung der CuAO an der PA-Biosynthese unterstützt. Die biochemische Charakterisierung der CuAO zeigte zudem, dass dieses Enzym in der Lage ist, aus Homospermidin die Bildung eines Pyrrolizidingrundkörpers zu katalysieren. Dass dieser ein mögliches Intermediat der

Biosynthese sein kann, wurde durch Fütterungsexperimente mit markierten Vorstufen belegt..

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