New aspect of plant–rhizobia interaction: Alkaloid biosynthesis in Crotalaria depends on nodulation

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Infection of legume hosts by rhizobial bacteria results in the formation of a specialized organ, the nodule, in which atmospheric nitrogen is reduced to ammonia. Nodulation requires the reprogramming of the plant cell, allowing the microsymbiont to enter the plant tissue in a highly controlled manner. We have found that, in Crotalaria (Fabaceae), this reprogramming is associated with the biosynthesis of pyrrolizidine alkaloids (PAs). These compounds are part of the plant’s chemical defense against herbivores and cannot be regarded as being functionally involved in the symbiosis. PAs in Crotalaria are detectable only when the plants form nodules after infection with their rhizobial partner. The identification of a plant–derived sequence encoding homospermidine synthase (HSS), the first pathway-specific enzyme of PA biosynthesis, suggests that the plant and not the microsymbiont is the producer of PAs. Transcripts of HSS are detectable exclusively in the nodules, the tissue with the highest concentration of PAs, indicating that PA biosynthesis is restricted to the nodules and that the nodules are the source from which the alkaloids are transported to the above ground parts of the plant. The link between nodulation and the biosynthesis of nitrogen-containing alkaloids in Crotalaria highlights a further facet of the effect of symbiosis with rhizobia on the ecologically important trait of the plant’s chemical defense.

Significance

Nodules are typical organs at the roots of legumes (Fabaceae) in which symbiotic rhizobia fix atmospheric nitrogen. The fabaceous genus Crotalaria is known to produce toxic alkaloids as part of its chemical defense. We show that, in Crotalaria, nodulation triggers the biosynthesis of these alkaloids. The first specific enzyme of their biosynthesis is of plant origin and is expressed exclusively in the nodules.

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Data deposition: The sequences reported in this paper have been deposited in the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI) database, www.ebi.ac.uk (accession nos. LN810071 (C. spectabilis mRNA for UBQ), LN810072 (C. spectabilis mRNA for ACT), LN810069 (C. spectabilis mRNA for DHS), and LN810070 (C. spectabilis mRNA for HSS)).

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against herbivores, depicting a further mechanism by which nodulation should have a strong impact on plant fitness.

Results

**PA Biosynthesis Is Triggered by Nodulation.** To test the hypothesis that nodulation triggers PA biosynthesis in *Crotalaria*, we cultivated *C. spectabilis* under controlled conditions. Nodulation was induced by infection with a *Bradyrhizobium* strain isolated from *C. juncea* (18).

In the first experiment, we compared the PA content of *C. spectabilis* plants infected with *Bradyrhizobium* with uninfected controls. Of note, *C. spectabilis* is described as producing exclusively the PA monocrotaline (19), simplifying the detection and quantification of PAs in this project. Only plants that had developed nodules contained monocrotaline at concentrations of about 1.5 mg per individual. PA levels in the uninfected controls were below the detection limit (<0.01 mg per individual) and were most likely attributable to residual PAs transferred from the seeds from which the plants were grown (Fig. 1A). *Crotalaria* is known to degrade PAs accumulated in the seeds within the first few weeks after germination, most likely to mobilize the alkaloid-bound nitrogen for the seedling before nitrogen can be provided by the symbiosis (20, 21). Nongerminated seeds contained about 0.33 mg monocrotaline per seed.

To establish that nodulation triggers de novo PA biosynthesis also in other *Crotalaria* species and to exclude the possibility that seed-derived PAs might influence these analyses, individuals of *C. retusa* were studied in a second experiment. Of note, these individuals had been cultivated in vitro for several years from cuttings and did not contain any detectable amounts of PA. Ten weeks after infection with *Bradyrhizobium*, *C. retusa* plantlets contained 5.98 mg/g (dry weight) monocrotaline whereas the controls were still devoid of PAs.

Further analyses of the infected PA-producing *C. spectabilis* plants showed that absolute amounts of PAs per plant individual were highest in the leaves, followed by nodules, roots, and stems (Fig. 1A). When the concentration of PAs was considered, it was highest in the nodules, with 1.97 mg/g dry weight, a value almost 10-fold higher than that found in the leaves (0.21 mg/g dry weight) (Fig. 1A). Flowers and fruits were not tested because they were not produced under the cultivation conditions used to grow the plants.

**Exogenously Applied Nitrate Has No Impact on PA Biosynthesis.** To test whether the supply of nitrogen as a limiting factor had an influence on PA biosynthesis, infected *C. spectabilis* plants and uninfected controls were grown on two different concentrations of nitrate (5 mM and 20 mM). Overall plant growth was comparable on both media, producing similar amounts of biomass, independent of infection (Fig. 1B). However, the only infected plants that showed nodules were those that were grown on medium containing 5 mM nitrate (Fig. 1B), suggesting that nodulation was inhibited at higher levels of nitrate by autoregulation, as described for soybean-*Bradyrhizobium* symbiosis (22). Furthermore, PA levels of about 2 mg per plant individual were found only in nodulated plants whereas all other plants contained only low amounts of PAs that were most likely attributable to remaining seed PAs. These results confirmed the previous observation that PA production was strictly dependent on nodulation and showed that exogenously applied nitrate had no impact on PA biosynthesis.

**PAs Are Synthesized by the Plant.** The literature gives several examples of alkaloids and other toxins that are produced by endophytes (23). To rule out that PAs in *Crotalaria* were produced by the microsymbiont, we repeated our search for a plant HSS, but this time using infected PA-producing *C. spectabilis* plants. If the microsymbiont produces PAs, we would expect to find no plantspecific enzyme of PA biosynthesis. The same would be true if only the first pathway-specific intermediate (i.e., homospermidine) was provided by the microsymbiont. Of note, homospermidine is the predominant polyamine in many α-proteobacteria, including *Bradyrhizobium* (24).

From previous studies, we knew that sequences encoding HSS were highly similar to that encoding its parologue DHS and that functional identification required heterologous expression and activity tests (14, 25). Therefore, we searched for HSS- and DHS-coding cDNA sequences in *C. spectabilis* and used 1-d-old seedlings (radicula 1–5 mm in length) and nodules of infected plants for reverse transcription and PCR (RT-PCR) with degenerate primers. Seedlings were used because, in tobacco, germinating seeds have previously been shown to possess increased levels of the *dhs* transcript (26). Nodules were chosen because the high concentration of PAs in this tissue suggested the nodules as a site of PA biosynthesis.

Two sequences with a high degree of identity to the DHS- and HSS-coding cDNAs of plant origin were identified (Table 1). For both sequences, an inverse PCR approach with genomic DNA was used to identify the 5′ ends of the ORF. In both cases, the resulting genomic sequences showed the characteristic intron pattern of plant DHS/HSS-homologs, supporting the interpretation that both sequences were of plant origin (Fig S1). The complete ORFs were heterologously expressed in *Escherichia coli*, and the resulting recombinant protein was affinity-purified as described previously (14). Activity tests confirmed that the sequence identified from germinating seeds encoded a DHS whereas that from nodules encoded an HSS (Table 2), with specific activities comparable with those described previously for DHS and HSS from various other PA-producing species (14, 27). A phylogenetic tree calculated using cDNA sequences available in the
public databases suggests an independent origin of HSS also for the Fabaceae (Fig. S2), to date, the sixth confirmed origin of HSS within the angiosperms.

**HSS Transcript Is Detectable only in Nodules.** Quantitative real-time PCR analyses using both ubiquitin and actin as standards showed that the transcript encoding HSS was detectable exclusively in nodules that started to develop no earlier than 10 d after infection (Fig. 2). In the sample taken 10 d after infection, the root of only one of the three infected individuals (plant 1) showed nodules and was the only tissue in which a transcript of HSS was detectable (Fig. 2, Inset). No transcript was detectable in any tissue of the noninfected plants or in the shoots of infected plants. In roots of infected plants, low levels of a transcript encoding HSS were detectable, most likely attributable to small developing nodules that were not separated completely from the root tissue.

Transcript levels of dhs, the paralogue to hss, were analyzed for comparison. dhs expression has been described as being correlated with cell proliferation (28) and was shown, in contrast to hss, to be transcribed uniformly in most plant tissues (26, 29, 30). Moreover, for *Crotalaria*, we found transcript levels of dhs to be constant in all analyzed tissues, but at an extremely low level (on average, 800-fold lower than that of hss, using actin as a reference) (Fig. S3).

**Discussion**

For *Crotalaria*, our results show that PA biosynthesis is coupled to nodulation. A central question in our study has been which partner of the symbiosis provides homospermidine for PA biosynthesis: the microsymbiont or the plant. In many bacterial phyla, homospermidine is found to replace spermidine, which is the prevalent triamine in other bacteria and in eukaryotes (24).

Indeed, homospermidine has been found repeatedly in nodules of fabaceous plants and in the associated rhizobia (31, 32). In homospermidine-producing bacteria, a bacterial HSS (EC 2.5.1.44) catalyzes the formation of homospermidine from two molecules of putrescine in an NAD⁺-dependent reaction (33, 34). Despite the similar reaction mechanism, bacterial HSS and plant-derived HSS are not related with respect to the degree of sequence identity (12, 35) and to their predicted 3D structure (24). Both the presence of introns at conserved positions in the genomic DNA encoding the HSS identified in *C. spectabilis*, and the ability of this enzyme to catalyze the formation of homospermidine from two molecules of putrescine and spermidine, establishes that the identified HSS is not of bacterial, but of plant, origin. Furthermore, the close spatiotemporal correlation between HSS transcript abundance and PA concentration strongly indicates that the nodules are the site of PA biosynthesis and the source from which the alkaloids are distributed throughout the plant.

PA biosynthesis is described as a constitutive pathway in various plant lineages, including the Senecioneae, Eupatorieae (both Asteraceae), and Boraginaceae (36). The coupling of PA biosynthesis to nodulation in *Crotalaria* offers a mechanism by which PA biosynthesis can be triggered by exogenous factors. This mechanism might ensure an adequate supply of nitrogen because alkaloid biosynthesis is nitrogen-intensive. For wild tobacco, 6% of the plant nitrogen is calculated to be bound in the alkaloid nicotine, not considering the amount of nitrogen bound by enzymes involved in alkaloid biosynthesis (37). Our results also show that elevated levels of nitrate in the soil inhibit nodulation and result in plants that do not produce PAs. This observation clearly demonstrates that the nodulation, and not the amount of bioavailable nitrogen within the plant, is the trigger for PA biosynthesis. We speculate that nitrogen levels in the natural habitat of *Crotalaria* are a limiting factor and that, thus, symbioses with rhizobia should be highly favored. Under these conditions, the symbiosis has an impact on the plant’s fitness, not only by providing bioavailable nitrogen from the atmosphere, but also by regulating the ecologically important trait of the plant’s chemical defense.

Because PA biosynthesis is linked with nodulation, the fascinating interactions between *Crotalaria* and specialized insects are also dependent on this symbiosis. *Utetheisa ornatrix*, an arctiid moth that has been intensively studied with respect to its interactions with PAs, uses *Crotalaria* as larval food plant. PAs are sequestered and retained through metamorphosis into the adult stage and used for the insect’s defense (38). Both parents provide PAs for egg protection, the male transferring the PAs to the female via the spermatophore. Males with a higher load of PAs have been shown to be more attractive for females, a quality that is signaled by PA-derived courtship pheromones. A PA-specific enzyme, a PA N-oxigenase, enables this class of insects to store PAs in a polar protocytic form (39, 40).

The transport of PAs from the site of synthesis in the belowground organs to the above-ground parts of the plant accords well with the optimal defense theory (41), suggesting that defense compounds are accumulated preferentially in tissues with the highest probability of herbivore attack. In *Senecio*, PAs are synthesized in the roots and are accumulated mainly in the reproductive tissues of the flower heads (42). Our finding that, in *Crotalaria*, about 60% of the total PAs occur in the above ground parts of the plant and only about 20% in the nodules (Fig. L4) suggests that PAs have also been selected in *Crotalaria* as chemical defense compounds. Whether PAs additionally have a direct effect on the microsymbiont remains open. In the tree legume *Leucaena leucocephala*, the plant-derived nonproteinogenic amino acid mimosine, which can be degraded by the rhizobial symbiont, is regarded as having a positive effect as a source of carbon and nitrogen (43). According to the literature, degradation of PAs is a rare event, with one prominent example: i.e., the germinating seeds of *Crotalaria*, in which PAs have been suggested to serve, not only as chemical defense, but also as nitrogen source for the developing embryo (21). This early phase of life, in which the symbiosis with rhizobia is not yet established, is characterized by a special need for nitrogen mobilization within the germinating seed. To date, no reports exist for PA degradation in *Crotalaria* roots infected by rhizobia. Because *Crotalaria* is reported to export nitrogen from indeterminate nodules as amides, predominantly as asparagine (44, 45), and because PAs do not possess a pronounced antimicrobial activity

### Table 1. Amino acid sequences identified in seedlings and nodules from *C. spectabilis* show a high degree of identity with plant DHS

|                | DHS of *C. scassellatii*, % | HSS of *B. elkanii* USDA76, % |
|----------------|-----------------------------|-------------------------------|
| DHS from seedling | 95.7                         | 23.7                          |
| HSS from nodules  | 82.4                         | 22.8                          |

Amino acid sequences were compared with deoxyhypusine synthase (DHS) of *C. scassellatii* and homospermidine synthase (HSS) of Bradyrhizobium elkanii USDA76.

### Table 2. Specific activities of recombinant DHS and HSS of *C. spectabilis*

|                | Specific activity, pkat/mg |
|----------------|---------------------------|
| **C. spectabilis** |                            |
| DHS assay       | 133                        |
| HSS assay       | 4955*                      |
| **DHS, deoxyhypusine synthase; HSS, homospermidine synthase. cDNA sequences identified from germinating seeds or nodules were expressed in E. coli and were assayed after metal-chelate affinity purification. Values noted with an asterisk are interpreted as main activity.** |
that would allow the bacterial symbiont to be kept in check (16). PAs have presumably not been selected for a specific function within the symbiosis. The accumulation of proteins as seed-storage compounds is widespread within the Fabaceae and is certainly attributable to the capacity of these plants to fix atmospheric nitrogen. However, seeds filled with proteins and carbohydrates are also an attractive meal for herbivores (46). Therefore, many species of the Fabaceae produce and accumulate secondary metabolites as a chemical defense, of which nitrogen-containing compounds such as alkaloids or amines are more frequent than in other non–nitrogen-fixing plants (47). In addition to the PAs in the seeds of Crotalaria (48, 49), the physostigmin of the calabar bean (Phyostigma venenosum) and quinolizidine alkaloids of the lupins are prominent examples of alkaloidal defense compounds. Therefore, an intriguing question for the future will be to test whether Crotalaria is the only clade within the Fabaceae in which alkaloid biosynthesis is coupled to nodulation.

Materials and Methods

Plant Material, Culture, and Infection with Bradyrhizobium. Seeds of C. spectabilis were obtained from Wolf Seeds (www.wolfseeds.com). Plants of C. retusa are part of the in vitro plant collection of the Botanical Institute (Kiel University).

The Bradyrhizobium strain used in this study was originally isolated from nodules of C. juncea from the Philippines (18) and was obtained from the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) Gene Bank (Shimadzu). The injector temperature was set at 250 °C, and the temperature program was as follows: 150 °C (3 min), 150 °C–300 °C (3 min)–6 °C·min (−1) ·min, and 300 °C (3 min). The injection volume was 1 μL. As a carrier gas, He was used with a flow rate of 4.2 mL/min. Heliotrin (Latoxan) and monocrotaline (Sigma-Aldrich) were used as internal and external standards, respectively.

Identification and Heterologous Expression of cDNAs Coding for HSS and DHS. For the identification of cDNA sequences encoding HSS and DHS of C. spectabilis, degenerate primers were designed according to an alignment of cDNA sequences encoding DHs from five Fabaceae species: i.e., Glycine max (NM_001250823), Medicago truncatula (exon 1 and 2 from contig 13725 and exons 3–7 from contig 60537; www.phytozone.net) and, from three Crotalaria species as described previously, C. juncea (AJ968376, AJ968377), C. retusa (AJ704838), and C. scassellatii (AJ968375) (13, 17). Total RNA was extracted with TRIzol (Life Technologies) from 1-d-old seedlings and from nodules harvested from plants cocultivated for 5 wk in vitro with Bradyrhizobium. RT (with primer P1) and PCR with a pair of degenerate primers (P2/P3) was performed as described previously (17) and resulted in fragments of 419 bp in length (for primer sequences, see Table S1). The 5′ ends were identified by using the inverse PCR strategy (56). For this purpose, genomic DNA isolated with the Wizard Genomic DNA Purification Kit (Promega) was digested with HincII (for DHS) and SacI (for HSS), diluted and self-ligated, before being

![Graph](image.png)

**Fig. 2.** Transcript encoding HSS is detectable only in nodules of C. spectabilis. Tissue-specific analysis of relative transcript levels of HSS in infected and noninfected plants. Transcript levels of ubiquitin served as a reference and were set at 1. The values of the individuals are given (Inset) for the root at 10 d after infection. Plant 1 was the only individual that had developed nodules at this time point.
used as a template with primer pairs P6/P7 (for DHS) and P8/P9 (for HSS) and AccuTaq LA DNA Polymerase (Sigma-Aldrich) at an annealing temperature of 64 °C. In the resulting fragments of 6.5 kbp and 4.6 kbp, the start codons for the ORF of DHS and HSS, respectively, were identified. Primer pairs P10/P11 (DHS) and P12/P13 (HSS) were designed to amplify the complete ORFs with proofreading Platinum px DNA Polymerase (Life Technologies), introducing NdeI/XhoI restriction sites that allowed cloning in the NdeI/XhoI-linearized pET22b (Novagen). Resulting constructs were sequenced and used for heterologous expression in E. coli BL21(DE3) cells according to Ober and Hartmann (12). Recombinant proteins were purified by using nickel-nitritrocacetic metal-chelate affinity chromatography and biochemically characterized by their enzymatic activity as described previously (14). Proteins were quantified according to the method of Bradford (57). Amino acid sequence identities were calculated by using the alignment tool of Geneious software (V. 7.1.3; Biomaters) with the Bloom52 matrix and gap-open and gap-extension penalties of 6 and 1, respectively. The HSS-encoding sequence of R. elkanii USDA76 was extracted from the whole genome shotgun sequence (ARAG00000005.1, 1,021.001-1,023.000).

Identification of Reference Genes of C. spectabilis and Primer Design for Quantitative Real-Time PCR. The 3′ ends of cDNA sequences encoding ubiquitin (UBQ) and actin (ACT) were identified with degenerate primers (P14 for UBQ and P15 for ACT) in combination with an oligo(dT) primer (P1). These sequences and the 3′-untranslated regions of the cDNA encoding DHS and HSS of C. spectabilis were used to design specific primers for RT-qPCR. Primer specificity was confirmed by using plasmas containing the 3′ regions of the ORF and the 3′-untranslated region of the genes as interest as template. Primers were regarded as specific if a PCR product was detectable only with the respective template but not with the cDNA of the other genes under study.

Quantitative Real-Time PCR. All samples were collected from three individuals grown under identical conditions as biological replicates and stored at −80 °C until used. Total RNA was extracted with the Direct-zol RNA MiniPrep kit (Zymo Research) in combination with an oligo(dT) primer (P1). The HSS-encoding sequence of R. elkanii USDA76 was extracted from the whole genome shotgun sequence (ARAG00000005.1, 1,021.001-1,023.000).

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