Biosynthesis

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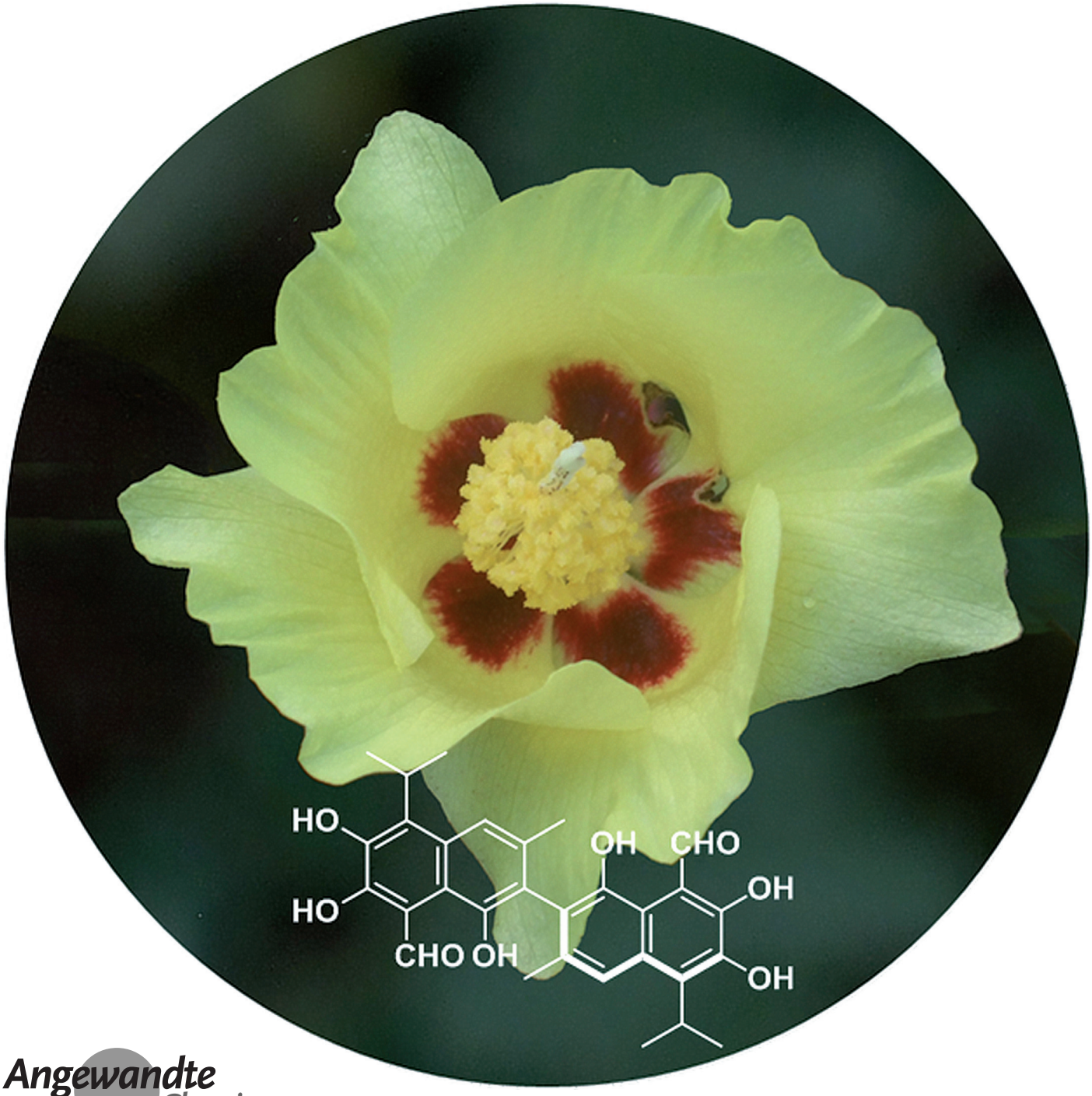
Dirigent Proteins from Cotton (*Gossypium* sp.) for the Atropselective Synthesis of Gossypol

Isabelle Effenberger, Bin Zhang, Ling Li, Qiang Wang, Yuxiu Liu, Iris Klaiber, Jens Pfannstiel, Qingmin Wang, and Andreas Schaller**

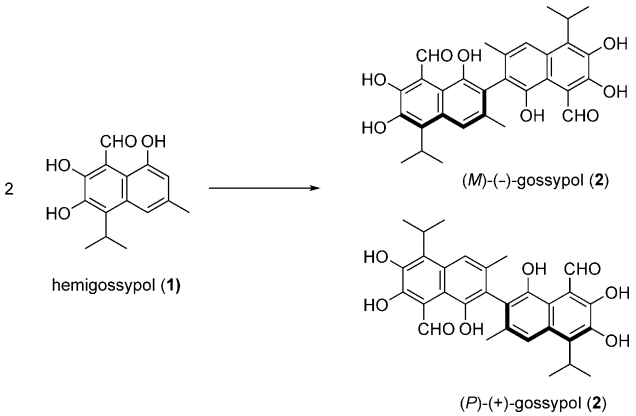


Abstract: *Gossypol is a defense compound in cotton plants for protection against pests and pathogens. Gossypol biosynthesis involves the oxidative coupling of hemigossypol and results in two atropisomers owing to hindered rotation around the central binaphthyl bond. (+)-Gossypol predominates in vivo, thus suggesting stereochemically controlled biosynthesis. The aim was to identify the factors mediating (+)-gossypol formation in cotton and to investigate their potential for asymmetric biaryl synthesis. A dirigent protein from Gossypium hirsutum (GhDIR4) was found to confer atropselectivity to the coupling of hemigossypol in presence of laccase and O_2 as an oxidizing agent. (+)-Gossypol was obtained in greater than 80% enantiomeric excess compared to racemic gossypol in the absence of GhDIR4. The identification of GhDIR4 highlights a broader role for DIRs in plant secondary metabolism and may eventually lead to the development of DIRs as tools for the synthesis of axially chiral binaphthyls.*

Axially chiral arene–arene linkages are frequent in biologically active natural products, and such compounds are rewarding targets for organic synthesis. Biosynthetic routes to biaryls typically involve the oxidative coupling of phenols or naphthols. While oxidative coupling is also the most direct approach towards biaryl formation in organic synthesis, it frequently suffers from poor regio- and enantioselectivity and unwanted side reactions. Therefore, novel strategies for the enantioselective formation of biaryl systems are urgently needed.[1] In this respect, the biosynthetic repertoire evolved by nature for the highly selective coupling of aromatic compounds provides a largely untapped reservoir for biotechnological approaches towards enantioselective biaryl synthesis.[2]

There are at least two mechanisms that confer regio- and enantioselectivity to oxidative coupling reactions in biological systems. First, the oxidizing enzymes, cytochrome P450-dependent enzymes or laccases, may provide the required selectivity. The intramolecular coupling of (*R*)-reticuline during morphine biosynthesis and of (*S*)-reticuline during magnoflorine biosynthesis, for example, and the intermolecular reaction for bisbenzylisoquinoline alkaloid formation are catalyzed by highly specific cytochrome P450-dependent oxidases in plants.[3] In *Aspergillus niger*, a P450 enzyme catalyzes the stereoselective coupling of dimethyl siderin as the penultimate step of (*P*)-(+)-kotanin biosynthesis,[4] and in streptomycetes, P450 enzymes are responsible for the regioselective formation of biarylic pre-anthraquinones.[5] In *Daldinia eschscholzii*, it is the conformational preference of laccase for specific naphthol dimer radicals that leads to the enantiomeric excess of (−)-dalesconols.[6] The second mechanism involves an unspecific oxidizing enzyme for radical formation and so-called dirigent proteins (DIRs) that confer regio- and enantioselectivity to the subsequent coupling reaction. The discovery of DIRs in 1997 led to a new model for phenoxy radical–radical coupling control in plant secondary metabolism.[7] However, the concept is still a matter of debate because DIRs are thus far only known for a single reaction during lignan biosynthesis, in which DIRs control the bimolecular coupling of coniferyl alcohol radicals to either (+)- or (−)-pinoresinol.[7,8] We show herein that DIRs are also responsible for the atropselective formation of gossypol, which supports a more general role for DIRs in plant secondary metabolism.

Gossypol (**2**, Scheme 1) is a natural product found in the flowers, seeds, roots and foliage of cotton plants, where it serves as a defense compound against insect pests and pathogens.[9] It has attracted a lot of interest for its multiple pharmacological activities and because of its toxicity to humans and non-ruminant animals, which limits the use of



Scheme 1. Oxidative coupling of **1** results in two atropisomers of **2**.

cottonseed oil and protein for food and feed.[10] The biosynthesis of **2** involves the formation of 8-hydroxy-(+)-δ-cadinene from farnesyl diphosphate by cadinene synthase and the P450 monooxygenase CYP706B1.[11] At least one more P450 enzyme is involved in the subsequent steps leading to hemigossypol (**1**), from which **2** is generated in a peroxidase-catalyzed bimolecular radical coupling reaction.[12] Two atropisomers, (+)- and (−)-**2**, arise because of hindered rotation around the central C–C bond of the tetra-*ortho*-substituted biaryl system. While organic synthesis typically yields racemic mixtures of the two isomers (with the notable exception of (+)-**2** synthesis by asymmetric Ullmann coupling of chiral oxazoline-activated naphthyls),[13] biosynthesis of **2** appears to be under stereochemical control. DIRs have been suggested to be involved in the process, and DIR activity for (+)-**2** formation has in fact been detected in protein extracts from cotton embryos and flower petals.[12c,14] However, the

[*] I. Effenberger, Prof. Dr. A. Schaller
Institut für Physiologie und Biotechnologie der Pflanzen
Universität Hohenheim (260), 70593 Stuttgart (Germany)
E-mail: andreas.schaller@uni-hohenheim.de
I. Klaiber, Dr. J. Pfannstiel
Serviceeinheit Massenspektrometrie
Universität Hohenheim (690), 70593 Stuttgart (Germany)
Dr. B. Zhang, Dr. L. Li, Q. Wang, Prof. Dr. Y. Liu, Prof. Dr. Q. Wang
State Key Laboratory of Elemento-Organic Chemistry, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin)
Nankai University, Tianjin 300071 (P.R. China)
E-mail: wangqm@nankai.edu.cn
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proteins responsible for atropselective biosynthesis of **2** have not been identified yet, and this was the goal of the present study.

In a candidate gene approach, two DIR-related cDNAs reported by Zhu et al. from sea island cotton (*G. barbadense*; GbDIR1 and GbDIR2) were considered to be involved in gossypol biosynthesis because they are pathogen-inducible and gossypol formation is known to be upregulated in cotton plants as part of the pathogen defense response.[9,15] The closest homologue of GbDIR1 and 2, GhDIR4, was cloned from *G. hirsutum* var. marie-galante, a variety of upland cotton containing (+)-**2** in greater than 90% enantiomeric excess (*ee*). The open reading frame of GhDIR4 was extended with a hexa-histidine tag and expressed in a transgenic plant cell culture system. Recombinant GhDIR4 was purified to homogeneity from cell-wall extracts (Figure S18a in the Supporting Information). The N terminus of the purified protein was identified by mass spectrometry, which indicated that the signal peptide is cleaved between Ser22 and Gln23 (Figure S19). The apparent molecular weight of GhDIR4 differed under denaturing and native conditions (31 kDa for SDS-PAGE, 125 kDa for gel filtration; Figure S18a,b), thus suggesting a homotetramer as the native state of the protein. However, considering the recently solved crystal structure of a trimeric (+)-pinoresinol-forming DIR from pea plants, the possibility of a homotrimer should not be dismissed.[16]

To investigate potential "dirigent" activity, the product spectrum generated during oxidative coupling of **1** was compared in the presence and absence of GhDIR4. One-electron oxidation of **1** was catalyzed by laccase from *Trametes versicolor* with O_2 as the oxidizing agent, which results in (*rac*)-**2** when no DIR is present. Preferential formation of (+)-**2** was observed upon addition of GhDIR4 (Figure 1a). The enantiomeric excess of (+)-**2** increased to more than 80% with increasing concentrations of GhDIR4 (Figure 1b). Only (*rac*)-**2** was observed when GhDIR4 was heat-inactivated or substituted with DIRs known to be involved in lignan formation (Figure S20b). GhDIR4, on the other hand, was inactive with coniferylalcohol as the substrate (Figure S20c). The data indicate that GhDIR4 is a novel dirigent protein that confers atropselectivity to the oxidative coupling of **1**. Its specificity for (+)-**2** is consistent with (+)-**2** being the predominant isomer in *G. hirsutum* var. marie-galante.

An enantiocomplementary (−)-**2**-forming DIR might be expected in plants that contain an excess of this particular isomer. However, most cotton plants produce (+)- and (−)-**2** at a 3:2 ratio. *G. barbadense* is a rare exception, with a 30–35% excess of (−)-**2** in at least some of its organs, thus suggesting the presence of a (−)-**2**-forming DIR in this species.[17] We thus cloned the pathogen-inducible GbDIR1 from *G. barbadense*, along with GaDIR1 from *G. arboreum*, a species with the more typical 3:2 excess of (+)-**2**. The two novel DIRs were expressed (Figure 2a) and their activities were compared with that of GhDIR4 (Figure 2b). GaDIR1 also directed (+)-**2** formation, which is consistent with (+)-**2** being the predominant isomer in *G. arboreum* (Figure 2b). No activity was detected for GbDIR1 during the coupling of **1**, thus suggesting that this protein does not contribute to

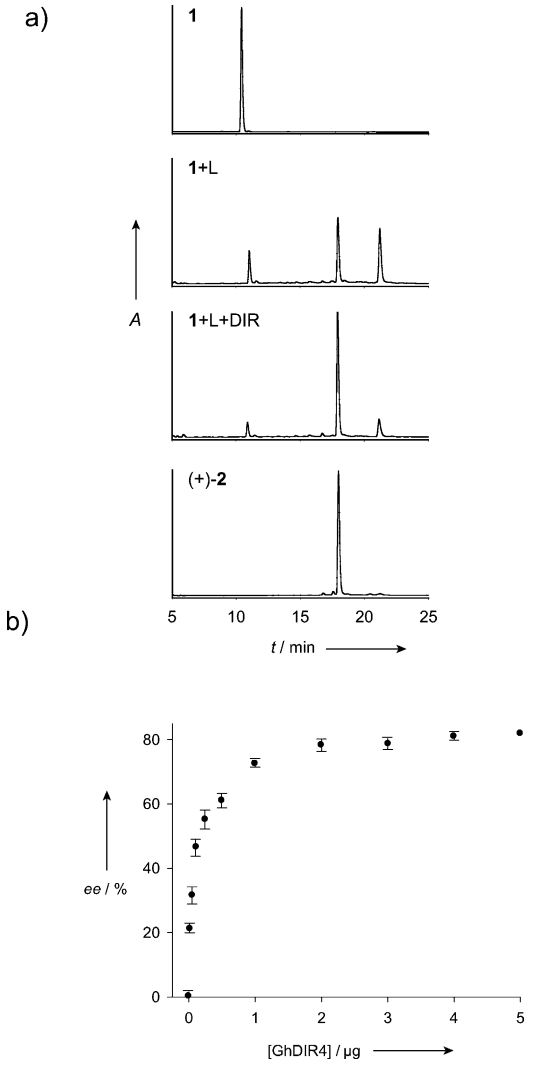

Figure 1. Demonstration of dirigent activity. The enantiomeric composition of **2** resulting from oxidative coupling of **1** was analyzed by derivatization with a chiral amine and separation of the Schiff-base adducts by reversed-phase HPLC. a) HPLC profiles are shown for the substrate (**1**), the reaction with laccase/O_2 (**1** + L), the reaction with laccase/O_2 in presence of GhDIR4 (**1** + L + DIR), and a (+)-**2** standard. b) Enantiomeric excess of (+)-**2** with increasing amounts of GhDIR4.

gossypol biosynthesis and may act on different substrates. The (−)-**2**-forming DIR thus remains elusive. Plants showing a more pronounced *ee* of (−)-**2**, *Thespesia danis*, for example, a tree from eastern Africa,[18] ought to be considered as sources for such activity.

We present herein the cloning and characterization of cotton DIRs that mediate the laccase-catalyzed atropselective coupling of **1** to (+)-**2**. The results highlight the importance of DIRs for stereochemical control in plant secondary metabolism. The road from the discovery of (+)-**2**-forming DIRs towards the development of DIRs as tools for the synthesis of axially chiral binaphthols will certainly be long. Important first steps have been made with the development of efficient expression systems for DIR production,[19] and the application of targeted mutagenesis for the engineering of DIR selectivity.[20] With respect to the use of cotton seeds as a source of

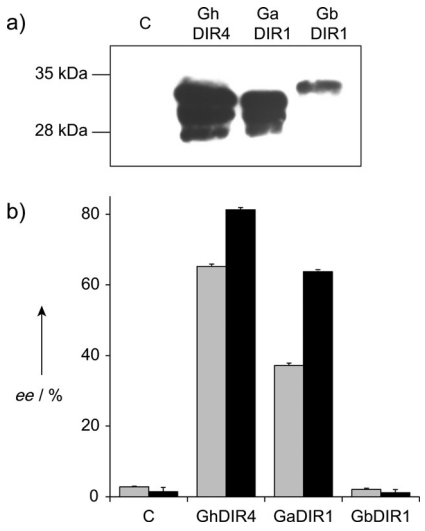

Figure 2. The activity of GaDIR1 and GbDIR1 compared to that of GhDIR4. a) Western Blot of total leaf protein (1 μg) of agro-infiltrated *N. benthamiana* plants expressing the indicated DIR proteins compared to the mock-infiltrated control (C). Multiple bands represent differentially glycosylated DIR isoforms. b) Dirigent activity in leaf extracts (gray and black bars represent 10 and 30 μg total leaf protein, respectively) as shown by the *ee* of (+)-**2**.

food protein, the identification of (+)-**2**-forming DIR genes offers the possibility to engineer cotton plants that are devoid of the toxic (−)-**2** isomer while retaining high levels of (+)-**2** for plant defense against pests and pathogens.

Acknowledgements

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Keywords: biaryls · biosynthesis · C–C coupling · dirigent protein · enantioselectivity

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