Enzyme Catalysis

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An Enantiocomplementary Dirigent Protein for the Enantioselective Laccase-Catalyzed Oxidative Coupling of Phenols**

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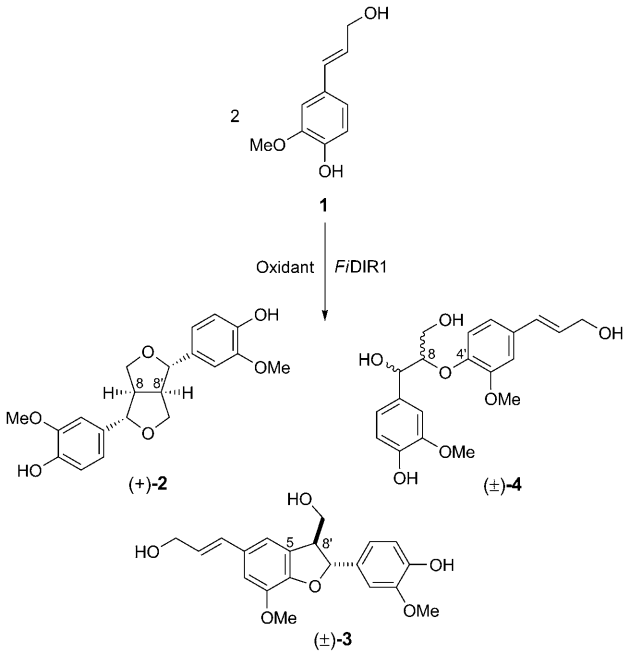
The oxidative coupling of phenols marks a key step in the biosynthesis of lignans, flavonolignans, and alkaloids and plays a central role in plant secondary metabolism.[1] The dimerization of propenylphenol derivatives to form lignans generally imparts high regio-, diastereo-, and enantioselectivity in vivo,[2] whereas in vitro enantioselectivity is negligible.[3,4] For example, after enzymatic oxidation of (*E*)-coniferyl alcohol (**1**) in vitro coupling results in the formation of only racemic pinoresinol ((±)-**2**).

Regio- and stereoselective phenol coupling is observed not only in plant secondary metabolism but also in bacteria, lichen, and fungi.[5] Examples include the regioselective formation of the isomers vioxanthin in *Penicillium citreoviride* and pigmentosin A in *Hypotrachyna immaculate*,[6] and the formation of the enantiomeric perylene quinones, hypocrellin and hypocrellin A, in *Hypocrella bambusae* and *Shiraia bambusicola*, respectively.[7] The rationale for the dramatically different routes taken during oxidative phenol coupling in vivo and in vitro remains an open question.

In 1997, Lewis et al. showed that in the presence of a dirigient protein (DP) from *Forsythia intermedia* (*Fi*DIR1), the oxidative coupling of **1** (Scheme 1) results in enantiomerically pure (+)-pinoresinol ((+)-**2**) as well as (±)-dehydrodiconiferyl alcohol ((±)-**3**) and *erythro*/*threo*-(±)-guaiacylglycerol 8-*O*-4′ coniferyl ether ((±)-**4**).[8,9] Additional DPs were subsequently found in *Thuja plicata*. Like *Fi*DIR1, the *T. plicata* DPs lack catalytic activity but mediate the enantioselective formation of (+)-**2** in the course of enzymatic oxidation of **1**.[2,10] There are also indications for a protein in *Linum usitatissimum* that allows the preferential formation of



Scheme 1. Oxidative coupling of **1** with *Fi*DIR1.[8]

(−)-**2**.[11] The atropselective coupling of hemigossypol to (+)-gossypol in *Gossypium hirsutum* further shows that enantioselective phenol coupling in plants is not restricted to the formation of **2**.[12]

Continuing studies of DPs and their effects on phenol coupling selectivity are required to achieve a better understanding of lignan biosynthesis and for the development of phenol coupling as a generally applicable tool in enantioselective synthesis. The latter aspect is of particular importance, since there is a high demand, but no general solution, for enantioselective phenol coupling in organic synthesis, even though some promising approaches exist.[13]

We present herein the cloning, expression, purification, and functional characterization of a DP from *Arabidopsis thaliana*, which mediates the laccase-catalyzed enantioselective oxidative phenol coupling of **1** to (−)-**2**. We further show that the enantioselectivity of the newly characterized *Arabidopsis* DP is opposed to that of the known *Forsythia* DP. In analogy to enantiocomplementary enzymes[14] we would like to propose the term enantiocomplementary dirigent protein (EDP) to describe such proteins.

Starting point in our search for EDPs was the recent report on a pinoresinol reductase from *Arabidopsis thaliana* specifically converting (−)-**2** into (−)-lariciresinol.[15] This finding suggested the existence of an EDP responsible for the

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formation of (−)-**2**. Data base searches identified two *Arabidopsis* genes, At1g64160 and At4g23690, coding for proteins highly similar to known DPs (Figure 1). Adopting the nomenclature proposed by Ralph et al., we will refer to

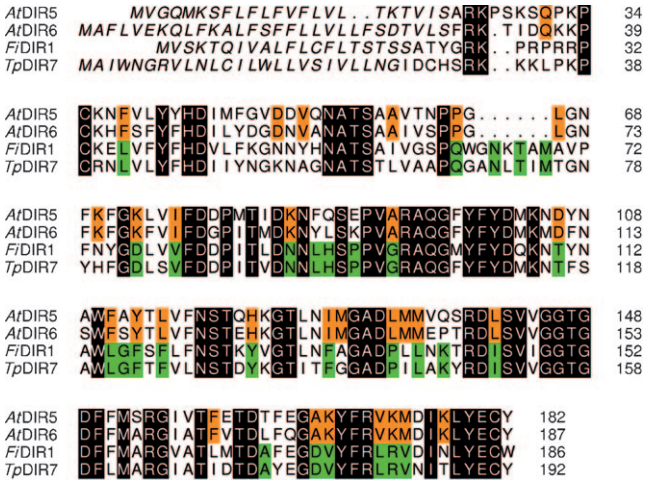
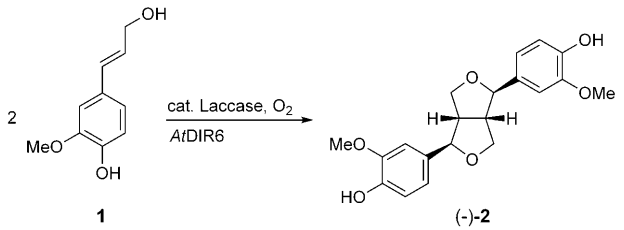


Figure 1. Sequence comparison between DPs and EDPs from *F. intermedia*, *T. plicata*, and *A. thaliana*. Residues conserved in all sequences are indicated in black. Sequence conservation between *Thuja* and *Forsythia* is highlighted in green and conservation within *Arabidopsis* sequences in yellow. Predicted N-terminal signal peptides (www.cbs.dtu.dk) are shown in italics.

the two proteins as *At*DIR5 (At1g64160) and *At*DIR6 (At4g23690), respectively.[16] With an amino acid sequence conservation of 52% with *Fi*DIR1, the two proteins are good candidates for the enantioselective coupling of **1** to (−)-**2** in *Arabidopsis* (Scheme 2).



Scheme 2. Oxidative coupling of **1** with *At*DIR6.

The open reading frame of *At*DIR6 was cloned into an expression vector, transformed into cultivated plant (*Solanum peruvianum*) cells, and a suspension cell culture was established from the cell line exhibiting the highest *At*DIR6 expression level. The secreted *At*DIR6 protein was purified to apparent homogeneity by fractionated ammonium sulfate precipitation and conventional chromatographic techniques. It was obtained in a yield of 0.2 mg L^{-1} as five isoforms differing in the degree of glycosylation (Figure 2). The five isoforms with masses of 20.4, 20.9, 21.4, 21.9, and 22.4 kDa were deglycosylated using CF_3SO_3H resulting in a single protein species of 18.6 kDa. The N-terminus of this protein

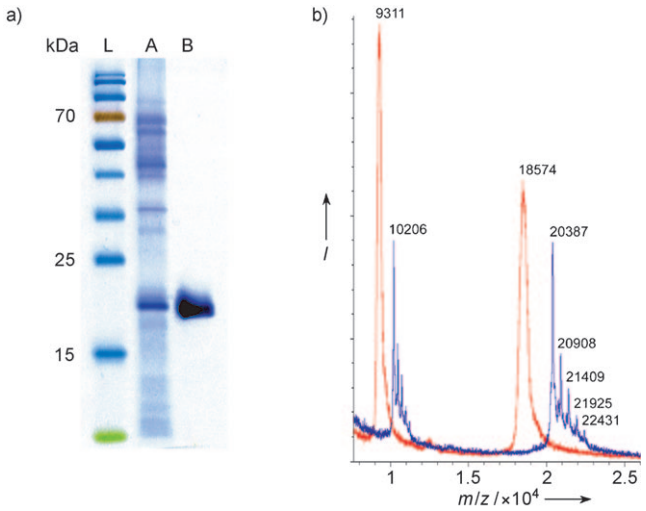


Figure 2. Purification of *At*DIR6. a) Stripe domain structure polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the crude extract (A), purified *At*DIR6 (B), and a size marker (L). b) MALDI-TOF-MS analysis of the purified protein prior to (blue) and after (red) deglycosylation using CF_3SO_3H.

was identified by mass spectrometry (MS) confirming the predicted processing site of the signal peptide (Figure 1). The mass calculated for mature *At*DIR6 is consistent with 18.6 kDa determined for the deglycosylated protein.

To investigate a potential "dirigent" (directing) activity of *At*DIR6 we first analyzed the product spectrum generated during the oxidative coupling of **1** catalyzed by laccase from *Trametes versicolor* alone, with O_2 as the oxidizing agent. In addition to (±)-pinoresinol ((±)-**2**) we isolated and identified (±)-**3** and (±)-**4**. Using chiral HPLC (Chiralpak IB) the pinoresinol formed in absence of DPs was shown to be a racemic mixture of (+)- and (−)-**2** (Figure 3a).

The spectrum of products was the same when laccase-catalyzed oxidation of **1** with O_2 as oxidizing agent was carried out in presence of *At*DIR6, however, the yield of **2** increased with a concomitant decrease in **3** and **4**. More importantly, in the presence of *At*DIR6, the oxidative coupling of **1** resulted in the preferential formation of (−)-**2** (Figure 3b). The enantiomeric excess of (−)-**2** was (26.8 ± 3.1)% *ee* when *At*DIR6 was present at a concentration of 1.9 μM. The *ee* value increased to (49.2 ± 2.1)% when the concentration of **1** was reduced and that of *At*DIR6 kept constant (Figure 4b). With increasing concentrations of *At*DIR6, the enantiomeric excess could be improved to (78 ± 3.6)% *ee* at 11.7 μM *At*DIR6, and the maximum was not reached (Figure 4a).

The data clearly show that *At*DIR6 is a genuine DP with a novel dirigent activity. Directing the formation of **1** to (−)-**2**, *At*DIR6 is enantiocomplementary to known DPs. To further corroborate this finding, we also cloned and purified *Fi*DIR1, the dirigent protein characterized by Lewis and co-workers.[8] *At*DIR6 and *Fi*DIR1 were then compared with respect to their dirigent activities. During laccase-catalyzed coupling of **1** to **2** we observed opposite enantioselectivities for *Fi*DIR1 and *At*DIR6, that is, the preferential formation of (+)-**2** by *Fi*DIR1 and (−)-**2** by *At*DIR6 (Figure 3b,c). With *At*DIR6

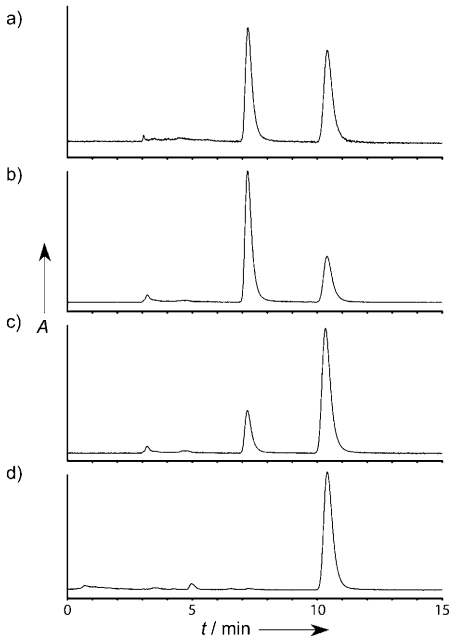


Figure 3. Demonstration of dirigent activity. The enantiomeric composition of **2** was analyzed by HPLC (*A* at 280 nm) after the reaction of **1** with a) laccase, b) laccase and *At*DIR6, c) laccase and *Fi*DIR1. d) (+)-**2** standard.

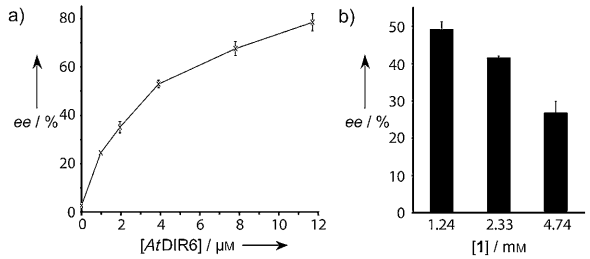


Figure 4. Enantiomeric excess of (−)-**2** as a result of varying concentrations of a) *At*DIR6 and b) **1**. In (a) the concentration of **1** was kept constant at 1.7 mM and in (b) the concentration of *At*DIR6 was kept constant at 1.9 μM.

and *Fi*DIR1, two DPs with opposite enantioselectivity are now available in recombinant form enabling the enantioselective synthesis of both (+)-**2** and (−)-**2**.

During the formation of (−)-**2** by *At*DIR6 and (+)-**2** by *Fi*DIR1 high (E)DP concentrations are required to achieve high *ee*-values. It is assumed that high (E)DP concentrations are necessary because unselective coupling of free radicals competes with the directed coupling of (E)DP-bound radicals.[2,9]

The mechanism of action of DPs and EDPs and, particularly, the molecular basis of their opposite enantioselectivity will be the subject of future investigations. However, first indications can be obtained from a sequence comparison between DPs and EDPs (Figure 1). A number of amino acid residues stand out that are conserved between *Fi*DIR1 and the *T. plicata* DP, but differ from the *Arabidopsis* sequences. Since *Forsythia* and *Arabidopsis* are phylogenetically much closer, the sequence differences cannot be explained by phylogenetic distance but may rather reflect functional differences of the proteins. The respective amino acids may thus be relevant for the observed enantiocomplementarity of DPs and EDPs. The analysis and comparison of DP and EDP structures will ultimately resolve this question. On basis of structural data it may become possible to devise minimal DPs suited for the development of a universal enantioselective phenol coupling process.

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