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MINI-REVIEW

Dirigent proteins: molecular characteristics and potential biotechnological applications

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Abstract Dirigent proteins (DIRs) are thought to play important roles in plant secondary metabolism. They lack catalytic activity but direct the outcome of bimolecular coupling reactions toward regio- and stereospecific product formation. Functionally described DIRs confer specificity to the oxidative coupling of coniferyl alcohol resulting in the preferred production of either (+)- or (−)-pinoresinol, which are the first intermediates in the enantiocomplementary pathways for lignan biosynthesis. DIRs are extracellular glycoproteins with high β-strand content and have been found in all land plants investigated so far. Their ability to capture and orientate radicals represents a unique naturally evolved concept for the control of radical dimerization reactions. Although oxidative coupling is commonly used in biological systems, its wider application in chemical synthesis is often limited by insufficient selectivity. This minireview gives an overview of functionally described DIRs and their molecular characteristics and wants to inspire further research for their use in biotechnological applications.

Keywords Dirigent protein · Lignan · Phenoxy radical coupling · Stereospecificity · Biotechnology

Introduction

Chemical synthesis of natural products has been widely used to overcome their limited availability from natural sources as caused by their low abundance in source organisms or difficult isolation and purification schemes. Although a great variety of molecules are accessible by classical chemical or biomimetic approaches (Nicolaou et al. 2000, 2003; Gravel and Poupon 2008; Lindsley et al. 2011), the production procedures are often very complicated because of the complex structure and the defined absolute configuration of many natural substances (Finefield et al. 2012). Oxidative coupling is a prominent reaction type in the biosynthesis of many natural products, especially for phenolic compounds (Keseru and Nogradi 1998; Dewick 2009). Despite considerable progress in recent years in terms of selectivity (Kuhl et al. 2012; Giri et al. 2009), a wider application of this reaction type in chemical synthesis has still not caught on, since the control of regio- and stereospecificity remains challenging (Lessene and Feldman 2002; Whiting 1991; Iqbai et al. 1994; Chioccara et al. 1993; Lindsley et al. 2011). The application of oxidative coupling is nonetheless promising because of its fast reaction rate and efficiency under mild reaction conditions, alleviating the need for substrate activation or protection groups (Jasperse et al. 1991). While biotechnological approaches with enzymes as catalysts have successfully been used to solve the problem of selectivity in many cases (Koeller and Wong 2001; Bornscheuer et al. 2012), their potential in phenol coupling reactions remains largely unexplored.

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Stereo- and regiospecific control of phenoxy radical coupling

Radical coupling in general is a two-step process. Firstly, radicals are generated from the substrate by the action of an oxidizing agent. In the second step, the two radicals are quenched by the formation of a covalent C–C or C–O bond. In the presence of conjugated double bond systems, delocalization of the unpaired electrons can lead to the generation of multiple products. In natural product (bio-)synthesis, where stereo- and enantiospecific features of the product are often associated with bioactivity (Mori 2011; Leffingwell 2003), the

generation of side products or stereoisomers of incorrect absolute configuration can be considered as a waste of energy and resources. Therefore, the (bio-)synthesis of compounds generated by oxidative phenoxy coupling requires regio- and enantioselective control. While regioselectivity during radical coupling may be, in part at least, provided by thermodynamically favored mesomeric structures of the generated radicals, enantioselectivity is energetically indiscriminant. Nonetheless, optimization by natural selection resulted in the evolution of at least two mechanisms that confer regio- and enantiospecificity to phenoxy radical coupling. In the first one, the absolute configuration of the product is determined by the oxidizing enzyme. In *Aspergillus niger*, for example, the P450 enzyme KtnC was shown to control regio- and enantiospecificity of the oxidative coupling of demethyl siderin to *P*-(+)-orlandin, an intermediate in *P*-(+)-kotanin biosynthesis (Girol et al. 2012), and in *Daldinia eschscholzii*, a laccase catalyzes the formation of (−)-dalesconol with app. 67 % enantiomeric excess (*e.e.*) (Fang et al. 2012). In these examples, radical formation and subsequent coupling are achieved by the same enzyme, whereas the second mechanism requires two components: an oxidizing agent to generate radicals by one-electron abstraction and a second factor that forces the free radicals to undergo regio- and stereospecific coupling. This mechanism was discovered in 1997 by Lewis and coworkers, who identified a protein in the insoluble cell wall fraction of *Forsythia intermedia* that affected the free radical coupling of coniferyl alcohol in the presence of inorganic oxidants, resulting in the formation of (+)-pinoresinol with 100 % *e.e*. (Davin et al. 1997). The protein was named dirigent protein (DIR) from the Latin word "dirigere" (to guide or to align) (Davin et al. 1997), and its discovery led to a new concept for the control of intermolecular phenoxy radical coupling in plant secondary metabolism. In the following sections, the up-to-date knowledge of DIRs is summarized, focusing on functionally characterized DIRs and their molecular properties and evaluating their potential for biotechnological application.

Dirigent proteins in natural product biosynthesis

The few DIRs that have been characterized to date are all involved in lignan biosynthesis. Lignans are structurally defined as 8,8′-coupled phenylpropanoid dimers (Moss 2000). They are widespread in the plant kingdom (Umezawa 2003), and some of them exhibit strong antioxidative or bioactive properties (MacRae and Towers 1984; Saleem et al. 2005). The canonical pathway for lignan biosynthesis was first established in *Forsythia* (Lewis and Davin 1999; Suzuki and Umezawa 2007). *E*-coniferyl alcohol derived from the phenylpropanoid pathway serves as universal precursor for all lignans in angio- and gymnosperms (Lewis and Davin 1999). The only exception known to date is liridendrin which is synthesized from sinapyl alcohol (Fujimoto and Higuchi 1977; Katayama and Ogaki 2001) and was first described in *Liridendron tulipifera*, a deciduous tree from eastern North America (Dickey 1958). In the first step of the pathway, oxidation of *E*-coniferyl alcohol—putatively catalyzed by a laccase (Davin et al. 1997)—leads to the formation of radicals, which dimerize to yield pinoresinol (Fig. 1). The successive reduction of pinoresinol by pinoresinol lariciresinol reductases (PLRs) generates larici- and secoisolariciresinol, respectively (Dinkova-Kostova et al. 1996). Secoisolariciresinol is oxidized by secoisolariciresinol dehydrogenases (SIRD) concomitant with the formation of a lacton moiety to afford matairesinol (Xia et al. 2001; Moinuddin et al. 2006). Further modifications of matairesinol or upstream precursors account for the numerous lignan structures described (Umezawa 2003).

Lignan biosynthesis is under stereochemical control. While plant-derived dibenzylbutyrolignans are optically pure in most cases, furofuran-, furan-, and dibenzyllignans exhibit at least an enantiomeric excess (Umezawa 2003). The enantiomeric configuration of lignans is determined during the first step of biosynthesis, as two chiral centers are introduced during the formation of a covalent bond between C-8 and C-8′. This once established absolute configuration is maintained throughout the downstream biosynthetic pathway (Fig. 1).

In contrast, when *E*-coniferyl alcohol is oxidized in vitro by organic or inorganic oxidizing agents, or enzymatically through the action of non-specific laccases or peroxidases (Sterjiades et al. 1992; Frías et al. 1991; Davin et al. 1997; Bao et al. 1993; Chioccara et al. 1993), bimolecular coupling of the resulting radicals leads to racemic mixtures of 55.6 % (±)-dehydrodiconiferyl alcohols, 27.7 % (±)-pinoresinols, and (under the addition of water) 16.7 % *erythro*/*threo*-(±)-guaiacylglycerol coniferyl alcohol ethers (Halls et al. 2004) (Fig. 1). While the stereoselectivity of subsequent enzymes in the biosynthetic pathway could potentially explain the accumulation of selected enantiomerically pure end-products in vivo (von Heimendahl et al. 2005), such a pathway would be highly inefficient, as only a small fraction of the initial coupling products would actually end up in lignan biosynthesis. The employment of DIRs in combination with an oxidizing agent to control the coupling outcome in regio- and stereospecific terms provides a more efficient alternative.

The hallmarks of DIR-mediated couplings include (1) the dependence on an oxidizing agent, (2) altered product ratio as compared to the free coupling reaction, and (3) the loss of selectivity when DIRs are denatured, as first described for FiDIR1 from *Forsythia* (Davin et al. 1997). In agreement with the proposed biosynthetic pathway for lignans, coniferyl alcohol radicals are the only substrates of FiDIR1, and other monolignol coupling reactions are not affected (Davin et al. 1997). FiDIR1 directs the coupling of coniferyl alcohol radicals toward the formation of (+)-pinoresinol (Davin et al. 1997). Lignans derived from (+)-pinoresinol are widespread in phylogenetically diverse taxa of the plant kingdom including

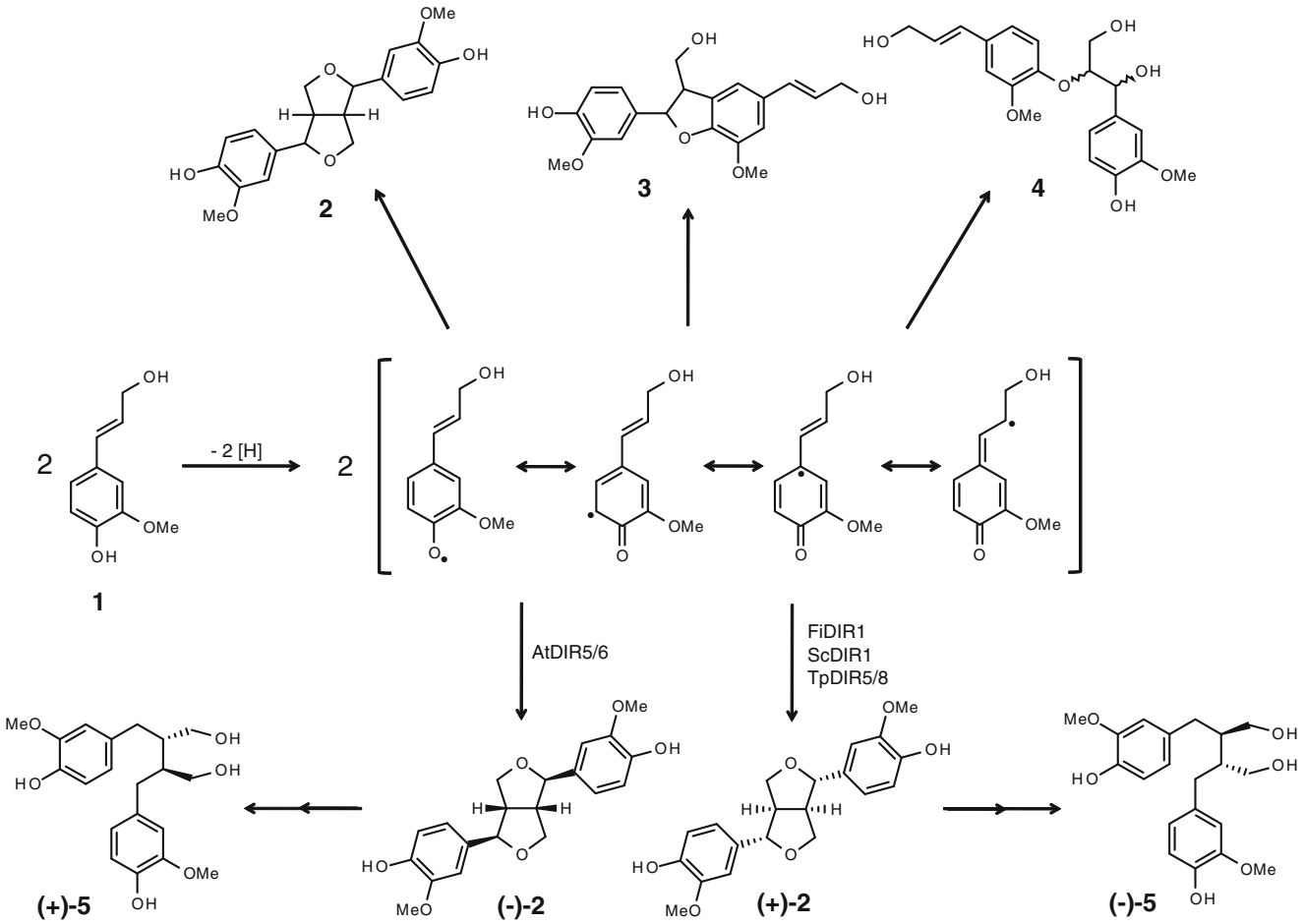

Fig. 1 Overview of the coniferyl alcohol radical coupling reaction and its link to lignan biosynthesis. Coniferyl alcohol (**1**) is oxidized by one-electron oxidation. The resulting radicals are stabilized by resonance and couple in open solution to (±)-pinoresinol (**2**), (±)-dehydrodiconiferyl alcohol (**3**), and *erythro/threo* -(±)-guaiacylglycerol coniferyl alcohol ethers (**4**). In the presence of AtDIR5/6 **(−)-2** and in the presence of FiDIR1, ScDIR1 or TpDIR5/8 **(+)-2** are formed, which are converted by PLRs and SIRDs to (+)- or (−)-secoisolariciresinol (**5**) in the subsequent steps of the lignan biosynthetic pathway

gymno- and angiosperms (Austrobaileyales and Lamiales). The presence of (+)-pinoresinol-forming DIRs related to FiDIR1 might thus be expected in all these taxa. This has been confirmed for several species including western red cedar (*Thuja plicata*) and *Schisandra chinensis* (Table 1). The heartwood of western red cedar contains large amounts of 8–8′ linked lignans, mainly plicatic acid and derivatives thereof (Yasuda et al. 1989). The absolute configuration of plicatic acid is *2R*, *3S*, *4R* ((−)-plicatic acid) (Gardener et al. 1966; Swan et al. 1967), which is in agreement with (+)-pinoresinol being its precursor (Kim et al. 2002a). Nine DIRs were identified in *T. plicata*, and as expected, those that were characterized functionally (TpDIR5 and TpDIR8) were found to direct the coupling of *E*-coniferyl alcohol radicals toward (+)-pinoresinol (Kim et al. 2002b). *S. chinensis*, a medicinal plant native to northern China, produces dibenzocyclooctadiene lignans with different enantiomeric configurations, including schizandrins and gomisins (reviewed in Lu and Chen 2009). While the biosynthetic pathway of *Schisandra* lignans has not been fully established, DIRs are likely to be involved for enhanced efficiency. Recently, such a DIR has been characterized (ScDIR1), and similar to previously characterized DIRs from *Thuja* and *Forsythia*, it was found to mediate (+)-pinoresinol formation in vitro (Kim et al. 2012). One of the pharmaceutically and economically most important (+)-pinoresinol-derived lignans is podophyllotoxin. Its derivatives etopside and teniposide are widely used in the treatment of various cancers (reviewed in Gordaliza et al. 2004). Podophyllotoxin and derivatives have been found in several *Podophyllum* (mayapple) (Bedows and Hatfield 1982; Jackson and Dewick 1983) and *Linum* (flax) species (Berlin et al. 1986; Broomhead and Dewick 1990; Fuss 2003). Its biosynthesis occurs via the general lignan pathway including matairesinol (Xia et al. 2000; Seidel et al. 2002). With an absolute configuration of (−)-podophyllotoxin (Petcher et al. 1973), (+)-pinoresinol would be the appropriate precursor. Two DIRs have been identified in *Podophyllum peltatum*, but their expected specificity for (+)-pinoresinol formation has not been confirmed yet (Xia et al. 2000).

Table 1 Molecular characteristics of DIR precursors and processed DIRs from *F. intermedia* (Fi) (Davin et al. 1997), *T. plicata* (Tp) (Kim et al. 2002b), *A. thaliana* (At) (Pickel et al. 2010; Kim et al. 2012), *S. chinensis* (Sc) (Kim et al. 2012), and *Gossypium* (Gh and Gb) (Zhu et al. 2007)

Protein	Accession number	Activity	Precursor				Processed			
			AA	MW (kDa)	p*I*	SP	AA	MW (kDa)	pp*I*	NGS
FiDIR1	AAF25357	**(+)-Pinoresinol**	186	21.0	8.4	**TSS^{20}-A^{21}T** (0.81)	166	18.8	8.0	4
FiDIR2	AAF25358	N.I.	185	20.8	6.8	VYG^{23}-H^{24}K (0.88)	162	18.4	6.3	4
TpDIR2	AAF25360	N.I.	192	21.6	8.6	ADC^{28}-H^{29}R (0.86)	164	18.4	7.9	5
TpDIR5	AAF25363	**(+)-Pinoresinol**	190	21.1	7.7	ADC^{26}-H^{27}S (0.85)	164	18.3	6.6	4
TpDIR8	AAF25366	**(+)-Pinoresinol**	192	21.3	6.9	**LNG^{25}-I^{26}D** (0.85)	167	18.5	6.5	4
ScDIR1	ADR30610	**(+)-Pinoresinol**	195	21.9	8.6	AFG^{28}-R^{29}K (0.55)	167	18.8	8.0	3
AtDIR5	NP_176598	**(−)-Pinoresinol**	182	20.7	8.4	VIS^{23}-A^{24}R (0.90)	159	18.1	7.0	2
AtDIR6	NP_194100	**(−)-Pinoresinol**	187	21.4	8.4	**VLS^{29}-F^{30}R** (0.50)	158	**18.1**	8.5	**2**
AtDIR10	NP_180435	**N.D.**	447	41.5	4.7	AAA^{21}-A^{22}R (0.91)	426	39.4	4.6	1
AtDIR13	NP_192858	**N.D.**	184	20.9	6.3	VLS^{25}-F^{26}R (0.74)	159	18.1	6.3	2
GhDIR1	ACU55135	N.I.	190	21.0	9.4	ALA^{24}-E^{25}H (0.68)	166	18.4	9.4	4
GhDIR2	ACU55136	N.I.	197	21.9	9.8	TRG^{29}-E^{30}N (0.66)	168	18.7	9.3	5
GbDIR1	AAS73001	N.I.	176	18.9	6.1	VQS^{22}-Q^{23}Y (0.91)	154	16.5	5.6	7
GbDIR2	AAY44415	N.I.	174	18.6	5.8	VRS^{22}-Q^{23}Y (0.85)	152	16.2	5.1	7

Accession numbers and activity are indicated. Number of AA, MW, and p*I* were calculated using ProtParam (http://web.expasy.org/protparam/). Cleavage site of signal peptides (SP with score) and N-glycosylation sites were predicted with SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. Experimentally verified data are highlighted in bold letters

N.I. no information available, *N.D.* not detected for coniferyl alcohol radicals, *AA* amino acids, *MW* molecular weight, *pI* isoelectric point, *NGS* *N*-glycosylation sites

Lignans with opposite enantiomeric configuration have been isolated from various plants like (+)-secoisolariciresinol from *Wikstroemia sikokiana* (Okunishi et al. 2000), (−)-lariciresinol from *Arabidopsis thaliana* (Nakatsubo et al. 2008), (+)-matairesinol from *Daphne odora* and *Daphne genkwa* (Okunishi et al. 2001), as well as (+)-secoisolariciresinol-*O*-β-D-diglucopyranoside and (−)-pinoresinol-*O*-β-D-diglucopyranoside from flax (*Linum usitatissimum*) seeds (Qiu et al. 1999). Those lignans are derived from (−)-pinoresinol and, therefore, have to be synthesized by an enantiocomplementary biosynthetic pathway (Suzuki and Umezawa 2007). The existence of parallel pathways for the synthesis of lignans with opposite enantiomeric configuration is supported by the identification of PLRs that show substrate specificity for either (+)- or (−)-pinoresinol. PLR from *Linum album*, for example, reduces (+)-pinoresinol, while *L. usitatissimum* PLR acts upon the (−)-enantiomer (von Heimendahl et al. 2005). Furthermore, two PLRs with opposing enantioselectivity have been characterized in western red cedar, suggesting that enantiocomplementary pathways for lignan biosynthesis may exist even within a single species (Fujita et al. 1999). Consistent with this notion, *Arctium lappa* produces lignans with opposite absolute configurations in seeds and petioles, respectively (Suzuki et al. 2002).

The presence of enantiocomplementary pathways for lignan biosynthesis implies the existence of two types of DIRs, the well-characterized type for the production of (+)-pinoresinol, and a second type mediating (−)-pinoresinol formation. First indications for the existence of an enantiocomplementary pair of DIRs were obtained in *A. lappa*. Using enzyme preparations from petioles in phenoxy radical coupling assays, the preferred formation of (+)-pinoresinol (33 % *e.e.*) was observed, while (−)-pinoresinol was preferentially formed (22 % *e.e.*) by protein extracts from ripening seeds (Suzuki et al. 2002). However, the corresponding DIRs have not been identified yet. Indirect evidence for the existence of a (−)-pinoresinol forming DIR was obtained also in *A. thaliana* roots, which accumulate (−)-lariciresinol at 88 % *e.e.* (Nakatsubo et al. 2008). Further investigations of the lignan biosynthetic pathway in *A. thaliana* revealed that lariciresinol is formed by two pinoresinol reductases (AtPrR1 and AtPrR2). In double mutants lacking functional *atprr1* and *atprr2* genes, lariciresinol was completely absent, rather pinoresinol accumulated with 74 % *e.e.* in favor of the (−)-enantiomer, indicating that pinoresinol formation is under stereospecific control (Nakatsubo et al. 2008). Considering these genetic data, *A. thaliana* provided an ideal model system for the identification of the enantiocomplementary DIR responsible for (−)-pinoresinol formation. Among 24 homologs identified by sequence comparison, AtDIR6 and AtDIR5 showed the highest sequence similarity with the known (+)-pinoresinol-forming DIRs (Pickel et al. 2010). AtDIR6 was cloned and expressed in a plant cell culture system. The purified recombinant protein exhibited the long-sought enantiocomplementary activity, mediating the formation of (−)-pinoresinol in vitro (Pickel et al. 2010). The (−)-pinoresinol-forming activity was

later confirmed for AtDIR6 and also found for AtDIR5 (Kim et al. 2012).

In addition to their role in lignan biosynthesis, DIRs are likely to be involved in other phenoxy radical coupling processes as well. This includes the biosynthesis of gossypol in cotton (*Gossypium hirsutum*). Gossypol, a highly bioactive sesquiterpenoid dimer (Wang et al. 2008; Jiang et al. 2012), is formed by phenoxy radical coupling of two molecules of hemigossypol (Veech et al. 1976; Benedict et al. 2006) (Fig. 2a). Thereby a chiral axis is introduced leading to two possible atropisomers, (+)-*S*- and (−)-*R*-gossypol, with the (−)-atropisomer exhibiting greater biological activity (Band et al. 1989; Matlin et al. 1985). The enantiomeric composition of gossypol varies among *Gossypium* species and even among different varieties. Most cotton species contain (−)- and (+)-gossypol at a ratio of app. 2:3, while, e.g., *G. hirsutum* var. *marie-galante* (moco cotton) accumulates (+)-gossypol at 90 % *e.e.* (Cass et al. 1991). The highest levels of (−)-gossypol were detected in *Gossypium barbadense* (Stipanovic et al. 2009) and some wild species (Stipanovic et al. 2005), but never exceeded 68 % *e.e.* Strong evidence for the involvement of DIRs in the final step of gossypol biosynthesis was obtained in *G. hirsutum* var. *marie-galante*. Protein extracts from embryos and flower petals promoted the formation of (+)-gossypol with 46 to 59 % *e.e*. in vitro, while control reactions yielded racemic product (Benedict et al. 2006; Liu et al. 2008). Two putative DIR-like proteins have been cloned from *G. barbadense* (Zhu et al. 2007), and two sequences from *G. hirsutum* are available in GenBank (cf. Table 1), but an involvement in gossypol formation has not been confirmed.

In addition to the systems described, the widespread occurrence of radical coupling steps in biosynthetic pathways leading to enantiomerically pure natural products and the considerable size of DIR gene families in higher plants (Ralph et al. 2006, 2007) provide a great potential for a more general involvement of DIRs in stereochemical control of secondary metabolism. The concept of DIR-mediated coupling control may also account for the specificity observed in the biosynthesis of distilbenoids (Rivière et al. 2012), biflavanoids (Iwashina 2000), disesquiterpenoids (Zhan et al. 2011), tannins (Ascacio-Valdés et al. 2011; Khanbabaee and van Ree 2001; Bors and Michel 2002), and other natural products.

For example, resveratrol and derivatives thereof can be oxidized by fungal laccases (Pezet et al. 1991; Nicotra et al.

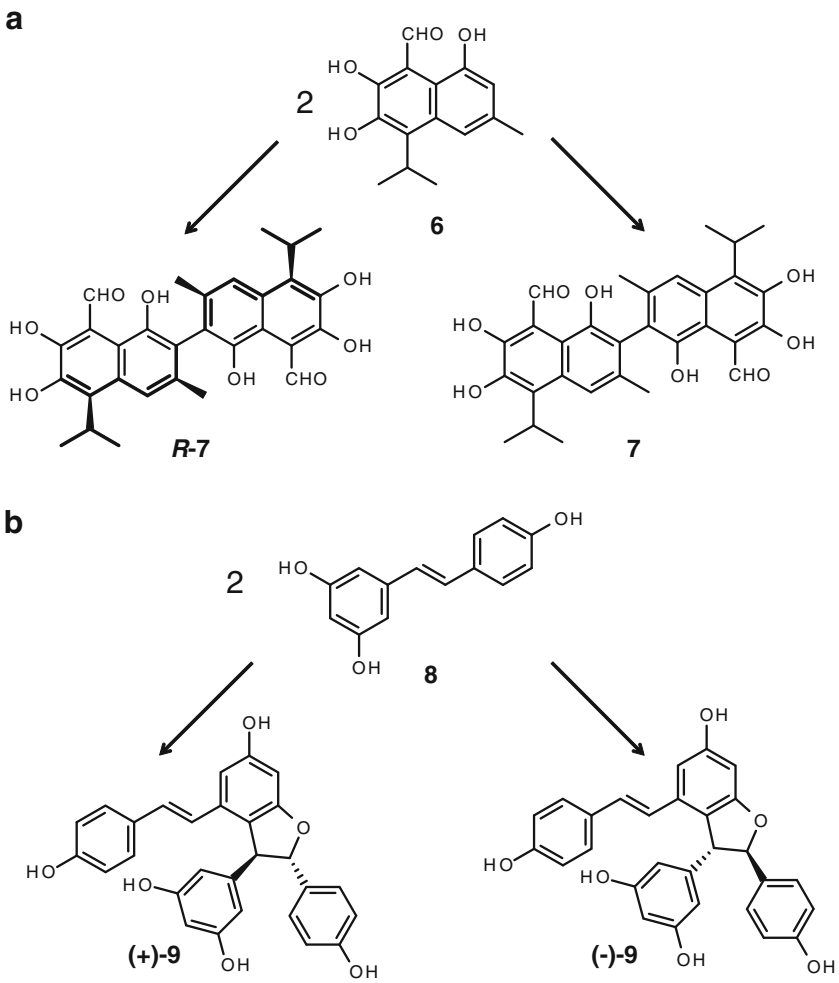

Fig. 2 Reaction scheme of the radical coupling of hemigossypol (**6**) to racemic gossypol (**7**) or (−)-*R*-gossypol (***R*-7**) under the influence of putative DIRs and the enantioselective generation of ε-viniferin (**9**) from resveratrol (**8**) with (+)-ε-viniferin (**(+)-9**) accumulating in Vitaceae and (−)-ε-viniferin (**(−)-9**) in other taxa

2004; Ponzoni et al. 2007) or horseradish peroxidase (Langcake and Pryce 1977). Dimerization or oligomerization of the resulting radicals yields di- or oligostilbenoids, respectively. Using inorganic oxidants, the distilbene (±)-ε-viniferin was obtained as a racemate (Takaya et al. 2005). Also the laccase-mediated oxidation of 3,5-dimethoxy-4′-hydroxystilbene yielded a racemic *trans*-resveratrol dehydrodimer as the main product (Ponzoni et al. 2007). These studies show clearly that there is no thermodynamic preference for the production of one enantiomer over the other. In planta, however, ε-viniferin biosynthesis is stereochemically controlled (Fig. 2b). The formation of (+)-ε-viniferin in Vitaceaeous plants (He et al. 2008; Rivière et al. 2012) and of (−)-ε-viniferin in Dipterocarpaceae (Ito et al. 2009) and Cyperaceae (Kurihari et al. 1990) points toward the involvement of corresponding enantiocomplementary DIR activities.

In general, the concept of DIR-mediated coupling control may apply in all cases, where opposite enantiomers of a given metabolite accumulate in vivo, maybe in different species or in different tissues, while uncontrolled coupling in vitro results in diverse or racemic products. The action of DIRs is also not necessarily limited to bimolecular radical coupling.

From a functional point of view, the term "dirigent protein" should be extended to include all proteins that guide the transformation of reactive molecules, like radicals, epoxides, and others, toward one of multiple possible products, by excluding all but one of the possible reaction channels. In that broader sense, also allene oxide cyclases (AOCs), which mediate the stereospecific cyclization of an unstable epoxide (Ziegler et al. 2000), show dirigent activity.

Molecular characteristics of dirigent proteins

Primary structure

In a detailed phylogenetic analysis, app. 150 DIR sequences have been grouped into six subfamilies (Ralph et al. 2007). The functionally characterized pinoresinol-forming DIRs cluster in subfamily a. As no functional data are available for any subfamily other than subfamily a, the members of the other subfamilies are referred to as DIR-like proteins (Ralph et al. 2006). This also includes DIR-like proteins from *Gossypium* that are located within subfamily b and putatively involved in gossypol formation. Although sequence similarity can fall into the arbitrary range between or within subfamilies, six conserved motives have been identified that are characteristically present in all DIRs and DIR-like proteins (Ralph et al. 2006). While some DIR-like proteins contain additional domains of varying size (Kittur et al. 2007; Ralph et al. 2006), the functionally characterized DIRs are relatively small proteins of app. 180 amino acids, with molecular weights ranging from 18 to 21 kDa (Table 1), and they are encoded by intronless genes (Kim et al. 2002a; Ralph et al. 2006, 2007).

Post-translational modifications

Characteristic for pinoresinol-forming DIRs is an N-terminal signal peptide, which targets the protein for secretion into the apoplastic space (Table 1). Cleavage of the signal peptide has been validated experimentally for FiDIR1 (Gang et al. 1999), TpDIR8 (Kim et al. 2002b), and AtDIR6 (Pickel et al. 2010) (Table 1). Depending on the expression system, small differences were observed between the predicted and the actual sites of processing. Secretion was confirmed for FiDIR1 by in situ localization within the cell wall of *F. intermedia* stems (Burlat et al. 2001; Gang et al. 1999; Davin and Lewis 2000). Also AtDIR6 was found to be secreted when expressed in a plant cell culture (Pickel et al. 2010). However, only a small fraction was present in the culture supernatant; the majority of AtDIR6 remained non-covalently bound to the cell wall by ionic interactions.

The experimentally determined molecular weight of purified DIRs differs considerably from that predicted on basis of the amino acid sequences (Gang et al. 1999; Kim et al. 2002a; Pickel et al. 2010). The mass difference could be attributed to extensive glycosylation, since several isoforms were observed for heterologously expressed FiDIR1 and AtDIR6 that were converted into a single smaller species by chemical or enzymatic deglycosylation (Pickel et al. 2010; Gang et al. 1999). The sequences of the pinoresinol-forming DIRs comprise two to five *N*-glycosylation consensus motives (NxS/T) (Marshall 1972; Gavel and Heijne 1990), whereas four to seven potential *N*-glycosylation sites are predicted for DIRs from *Gossypium* (Table 1; Fig. 3). For AtDIR6, glycosylation at the predicted sites (N^{59} and N^{123}) was confirmed by mass spectrometry (Pickel et al. 2010). The glycan chain was found to be of the paucimannosidic-type (Pickel et al. 2012), which is common in plant proteins and has also been shown for the subtilisin-like proteinases P69B (Bykova et al. 2006) and SBT3 from *Solanum lycopersicum* (Cedzich et al. 2009). Considering that paucimnannosidic glycans are derived from bigger complex N-type glycans by the successive removal of two Lewis^a epitopes (0.5 kDa) (Lerouge et al. 1998), the occurrence of the five different AtDIR6 isoforms with mass differences of app. 0.5 kDa can be explained by incomplete processing during secretion.

Glycosylation of secreted proteins may assist in the correct folding of proteins during passage through the secretory pathway; it may be important for the regulation of enzyme activity, enhance protein solubility, or contribute to its stability (Shental-Bechor and Levy 2008, 2009; Hanson et al. 2009). Also for AtDIR6, glycosylation was found to by functionally important. Deglycosylation of AtDIR6 over time correlated with a gradual loss of soluble protein and

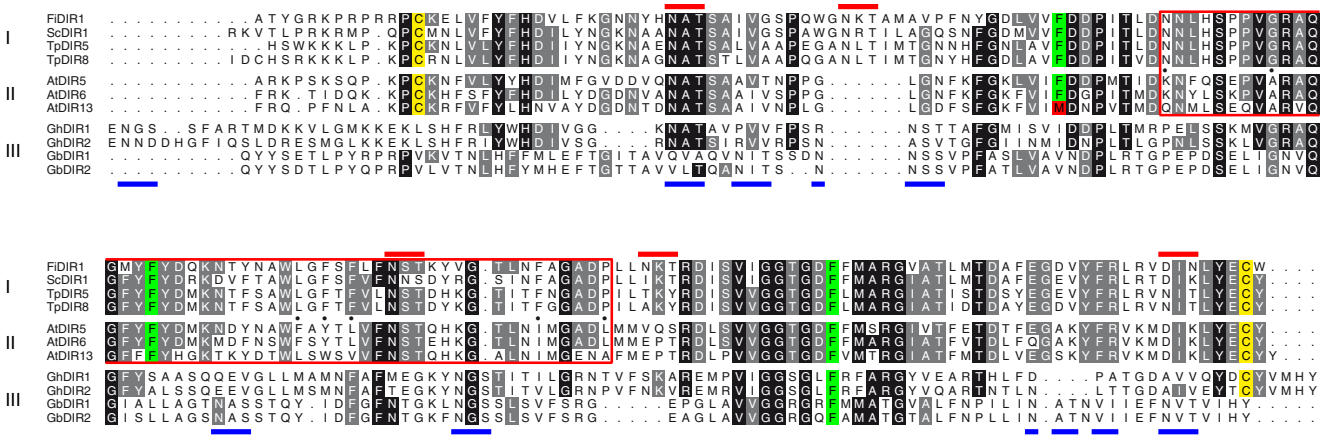

Fig. 3 Sequence alignment of functionally characterized DIRs involved in the formation of (+)-pinoresinol (I, FiDIR1, ScDIR1, TpDIR5/8), (−)-pinoresinol (II, AtDIR5/6) and without detected functionality towards coniferyl alcohol radicals (II, AtDIR13) as well as *Gossypium* DIRs putative involved in the coupling of hemigossypol (III, GhDIR1/2 and GbDIR1/2). Predicted glycosylation sites of pinoresinol-forming DIRs are marked by a *red bar*, those of potentially gossypol-forming DIRs by a *blue bar*. Cysteine residues participating in disulfide formation as shown for AtDIR6 (Pickel et al. 2012) are highlighted in *yellow*. Functionally relevant phenylalanines of ScDIR1 are indicated in *green* (Kim et al. 2012), the corresponding residue exchanged for Met in the inactive AtDIR13 is shown in *red*. The region implicated in coupling control is framed in *red* (Kim et al. 2012); amino acids that are differentially conserved between (+)- and (−)-pinoresinol forming DIRs in this region are marked by *dots*

dirigent activity (Kazenwadel et al. 2012). The formation of inclusion bodies upon expression in *Escherichia coli* (personal observation) provides further indirect evidence for the necessity of glycosylation and would be consistent with a requirement for glycosylation during protein folding, for protein stability or solubility. The inability to obtain soluble protein in *E. coli* may also be due to the reducing conditions in the bacterial cytoplasm that do not support disulfide bridge formation. Two cysteine residues are conserved at the N- and C-termini of (+)- and (−)-forming DIRs (Figs. 3 and 4a). Their engagement in the formation of a disulfide bond was experimentally confirmed for AtDIR6 (Pickel et al. 2012). The disulfide bridge may contribute to protein stability by stabilizing the tertiary structure of DIRs.

Tertiary structure

As there is no crystal structure available, the tertiary structure of DIRs is unknown. However, structural modeling allowed a first insight into the three-dimensional topology of DIRs (Pickel et al. 2012). Even though there is little sequence conservation and no obvious homology with any other known protein family (Gang et al. 1999), structural and functional similarity was observed with allene oxide cyclases (Schaller and Stintzi 2009) and a structural homology search identified allene oxide cyclase 2 from *A. thaliana* (AtAOC2) as a likely homolog and good potential template for the modeling of AtDIR6 (Pickel et al. 2012). AOCs are involved in the stereospecific generation of *cis*-(+)-oxophytodienoic acid from 12,13-(*S*)-epoxy-octadecatrienoic acid (12,13-EOT) in the octadecanoid pathway for jasmonic acid biosynthesis (Ziegler et al. 2000; Hofmann and Pollmann 2008; Schaller and Stintzi 2009). The structural model of AtAOC2 resembles an eight-stranded antiparallel β-barrel with a central hydrophobic cavity for substrate binding (Hofmann et al. 2006). As compared to the structure of AtAOC2, the AtDIR6 model features an additional, ninth β-strand that extends the upper rim of the barrel and a short 3_{10}-helix (Pickel et al. 2012) (Fig. 4). Both features are supported by CD-spectroscopic data and secondary structure predictions (Pickel et al. 2012; Halls and Lewis 2002). The general validity of the model is confirmed by the two glycosylation sites that are located at the solvent-exposed outer surface of the barrel and by

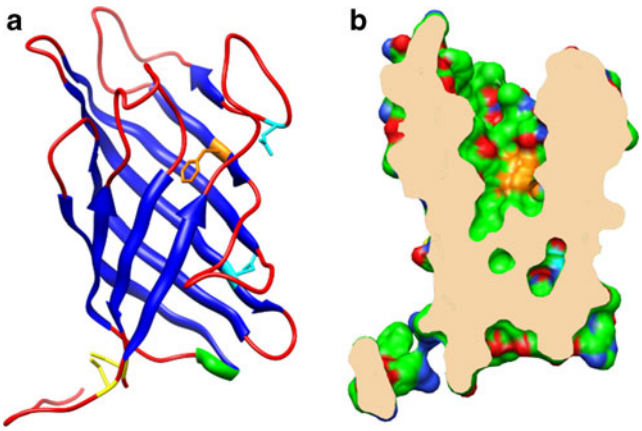

Fig. 4 Structural model of AtDIR6. The model shows nine antiparallel β-strands (*blue*) and a short 3_{10}-helix (*green*) separated by loop regions (*red*, **a**), that form a calyx-like structure with a bottom-closed hydrophobic cavity (**b**: section of **a** with capped surface shown in *beige*). Surface renderings indicate positive (*blue*) and negative charges (*red*). The glycan attachment sites (N^{59} and N^{123}, *cyan*), disulfide-forming cysteines (C^{40} and C^{186}, *yellow*), and the functionally important F^{82} (*orange*) are *highlighted* (the figure was generated with UCSF Chimera 1.6.2)

the position of the two cysteines engaged in the experimentally confirmed disulfide bridge, which is located at the bottom end of the barrel linking the N- and the C-terminus of the protein (Pickel et al. 2012) (Fig. 4a).

Hypothesized reaction mechanism of DIR-mediated coupling

Kinetic analysis of DIRs clearly support a two-step model of oxidative phenol coupling control, involving an oxidizing agent to generate the substrate radicals and DIRs to direct the coupling process in a regio- and stereospecific manner. As the presence of DIRs does not affect the overall conversion rate (Halls et al. 2004) and a high DIR to radical ratio is positively correlated to the amount and *e.e.* of pinoresinol that is generated (Pickel et al. 2010; Halls et al. 2004), DIR-mediated and free radical coupling have been recognized as competitive processes. While oxidation of coniferyl alcohol after binding to DIRs remains a possibility, this notion is consistent with the mode of action proposed by Halls et al. (2004), according to which the coniferyl alcohol radical is the substrate of DIRs. In the proposed model (Fig. 5), the radicals are bound by DIRs in a stoichiometry of about two per DIR homodimer, with the first radical binding reversibly followed by irreversible binding of the second, thereby initiating the formation of a C–C bond (Halls et al. 2004). Such a mode of action is also supported by the large difference in binding affinity for the alcohol and the radical. A K_D of 370 μM was determined for the binding of coniferyl alcohol to FiDIR1. For the coniferyl alcohol radical, on the other hand, a K_M of 10 nM was estimated from the saturation behavior of (+)-pinoresinol formation with respect to the apparent steady-state concentration of coniferyl alcohol radicals (Halls et al. 2004). The DIR homodimer provides a topology, which forces the unpaired electrons of the bound radicals to form a covalent bond in a regio- and enantiospecific manner. The resulting quinonmethide intermediate undergoes intramolecular cyclization reactions and is released. The coupling process and the release of pinoresinol were determined as the rate limiting steps (Halls et al. 2004).

The proposed reaction mechanism according to which the DIR homodimer captures two coniferyl alcohol radicals and orients them in a way to favor stereospecific 8-8′ coupling is consistent with cross-linking experiments and calibrated size exclusion chromatography that confirm native FiDIR1 and AtDIR6 as homodimers (Davin et al. 1997; Halls and Lewis 2002; Pickel et al. 2012), whereas a homotetrameric structure was determined for AtDIR6 heterologously expressed in *Pichia pastoris* (Kazenwadel et al. 2012).

The hydrophobic binding cavity of DIRs was probed by site-directed mutagenesis. Substitution of F^{90}, F^{113}, and F^{163} to A or Y abolished the dirigent activity of ScDIR1, revealing functional importance for these conserved residues, possibly in the stabilization of coniferyl alcohol radicals by π–π interactions (Kim et al. 2012). This finding may explain the apparent lack of activity for AtDIR13 (Kim et al. 2012). In AtDIR13, the residue corresponding to F^{90} in ScDIR1 is replaced by methionine (M^{78}; Fig. 2). The functional importance of F^{90} (or F^{82} in AtDIR6) is further supported by its conservation in AtAOC2 and pinoresinol-forming DIRs (Fig. 3) and by its location in the hydrophobic pocket of both classes of proteins (Pickel et al. 2012) (Fig. 4).

With the characterization of AtDIR6 (Pickel et al. 2010) and AtDIR5 (Kim et al. 2012) as being (−)-pinoresinol-forming and enantiocomplementary to previously characterized DIRs, protein regions and individual residues potentially involved in controlling the coupling mode could be identified by sequence comparisons (Pickel et al. 2010; Kim et al. 2012). Domain-swapping between ScDIR1 (N^{98} to P^{146}) and AtDIR6 (K^{90} to L^{138}) resulted in a conversion of enantiospecificity (Kim et al. 2012). Further studies and detailed site-directed mutagenesis will be required to resolve which of the residues that are differentially conserved in this region are responsible for the control of stereospecificity.

Evolution of DIRs

Structural homology search and subsequent modeling of AtDIR6 provided insight into the evolutionary ancestry of DIRs and revealed a distant relationship with lipocalins in the calycin superfamily (Pickel et al. 2012). Characteristic for lipocalins are their β-barrel structure with a central hydrophobic cavity for binding of small hydrophobic molecules and their function in transport processes (Flower et al. 2000). The

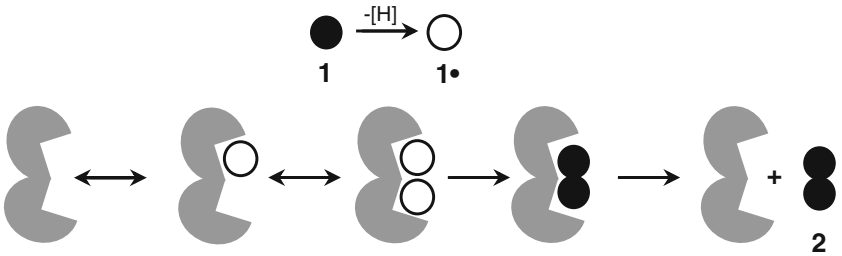

Fig. 5 Proposed reaction mechanism of DIR-mediated phenoxy radical coupling (Halls et al. 2004). Firstly, coniferyl alcohol (**1**, *black circle*) is oxidized to the corresponding radical (**1•**, *white circle*). A DIR homodimer (*gray structure*) successively binds two coniferyl alcohol radicals mediating regio- and enantiospecific coupling to either (+)- or (−)-pinoresinol (**2**, *black double circle*). Finally, the product is released

suggested evolution of DIRs from hydrophobic ligand-binding proteins is consistent with their proposed reaction mechanism: If the bound ligand is unstable, it is only a small step for the respective binding protein to become a dirigent protein, since the chiral protein environment will impart its stereochemical influence on the reaction. Similar to the mode of action proposed for DIRs, product formation in AOCs is also directed by steric restrictions imposed by the protein environment on the enzyme-bound substrate, an unstable allylic epoxide (12,13-EOT; Hofmann et al. 2006). As members of the calycin superfamily, ancestral AOCs primarily may have been binding proteins for 12,13-EOT that were optimized as catalysts in the course of evolution (Pickel et al. 2012). Similarly, chalcone isomerase evolved as a highly efficient and stereospecific enzyme from non-enzymatic fatty acid-binding proteins (Ngaki et al. 2012). Considering the reactivity of phenoxy radicals, optimization as catalysts to lower the activation energy barrier may not be necessary in case of DIRs. We would therefore expect that proteins with dirigent activity (as opposed to proteins with enzymatic activity) are frequent among lipocalins and that the dirigent concept will be more generally applicable to binding proteins for unstable ligands (Pickel et al. 2012).

Biotechnological applications of DIRs

The functional characterization of FiDIR1 revealed a novel way of how to direct the coupling of coniferyl alcohol radicals qualitatively and quantitatively toward (+)-pinoresinol under appropriate reaction conditions (Davin et al. 1997). Although a full control of the coupling outcome has not been achieved for other DIRs under the conditions employed (Pickel et al. 2010; Kazenwadel et al. 2012; Kim et al. 2002b, 2012), further optimization of oxidative capacity-to-DIR ratio and the chemical environment may eventually allow for complete repression of side product formation. Another approach to reduce the competing undirected coupling of free radicals in favor of the directed coupling of DIR-bound radicals is to decrease the spatial distance between oxidant and DIR by cross-linking or employment of nanostructures (Ren et al. 2011; Roessl et al. 2010).

For a more general application of DIRs as tools for stereospecific radical coupling in organic synthesis, their narrow substrate and product specificity is the most serious limitation. Although various DIRs for the formation of (+)- and (−)-pinoresinol have been described, there is still no firm evidence for the involvement of DIRs in other radical coupling processes. Nevertheless, the large number of DIRs and DIR-like proteins with unknown function in plants (Ralph et al. 2007), as well as the high sequence diversity within the DIR family and the prevalence of enantiopure natural products in the plant kingdom, provides the potential for the discovery of novel DIR-mediated coupling reactions. Alternatively, the substrate and product spectra of DIRs and their scope for organic synthesis may be increased by engineering of artificial DIRs. As demonstrated by the successful reversal of the coupling mode of pinoresinol-forming DIRs (Kim et al. 2012), the engineering of dirigent activities is possible. It will be thrilling to identify the minimal DIR structure and to generate minimal DIRs tailored to direct specific coupling outcomes in the future.

A second bottleneck for the utilization of DIRs in organic synthesis is their limited availability. As purification from native sources is generally not feasible in economical terms and expression in *E. coli* does not yield functional DIRs due to the lack of appropriate glycosylation and secretion systems, eukaryotic expression systems are the method of choice. Heterologous expression of AtDIR6 in *P. pastoris*, which is routinely used as protein expression host, has recently been achieved at a level of 47 mg/L (Kazenwadel et al. 2012). The purified protein showed 90 % activity compared to the one obtained from plant cells as expression host (Pickel et al. 2010).

The application of phenoxy radical coupling in organic synthesis is often prohibited in economic and ecological terms because of limited specificity and the requirement of high concentrations of sometimes toxic oxidants. The combined use of DIRs with an appropriate oxidant offers an attractive answer to these problems. The development of appropriate DIRs may provide a biotechnological solution for the regio- and stereospecific control of phenoxy radical coupling reactions, thereby providing synthetic access to a plethora of pharmaceutically interesting compounds.

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