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REVIEW PAPER

Dirigent proteins in plants: modulating cell wall metabolism during abiotic and biotic stress exposure

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Abstract

Dirigent (DIR) proteins were found to mediate regio- and stereoselectivity of bimolecular phenoxy radical coupling during lignan biosynthesis. Here we summarize the current knowledge of the importance of DIR proteins in lignan and lignin biosynthesis and highlight their possible importance in plant development. We focus on the still rather enigmatic Arabidopsis DIR gene family, discussing the few members with known functional importance. We comment on recent discoveries describing the detailed structure of two DIR proteins with implications in the mechanism of DIR-mediated catalysis. Further, we summarize the ample evidence for stress-induced dirigent gene expression, suggesting the role of DIRs in adaptive responses. In the second part of our work, we present a preliminary bioinformatics-based characterization of the AtDIR family. The phylogenetic analysis of AtDIRs complemented by comparison with DIR proteins of mostly known function from other species allowed us to suggest possible roles for several members of this family and identify interesting AtDIR targets for further study. Finally, based on the available metadata and our *in silico* analysis of *AtDIR* promoters, we hypothesize about the existence of specific transcriptional controls for individual *AtDIR* genes and implicate them in various stress responses, hormonal regulations, and developmental processes.

Key words: Biotic and abiotic stress response, cell walls, dirigent protein, lignan, lignin, regioselectivity, stereoselectivity.

Introduction

Dirigent proteins: when the stereoselectivity matters

Pasteur (1860) demonstrated that many organic molecules form enantiomeric pairs of non-superimposable mirror-image molecular structures, characterized by their oppositely signed optical rotation. However, while *in vitro* synthesis produces racemic mixtures consisting of equal numbers of left- and right-handed molecules, biosynthesis often provides just one of the two enantiomers. Based on this, Pasteur concluded that biosynthesis involves a chiral force. That was further exemplified in the beginning of the 20th century by

Fischer (1890–1919), who demonstrated that functional biomolecules are composed specifically of the D-sugars and the L-amino acids (Mason, 1991). Since then, homochirality (i.e. using preferentially one of the possible enantiomers) has been proposed as a feature intrinsic to all living systems, and stereospecificity of biosynthetic reactions is considered as a necessary prerequisite for life's origin on the Earth (Weber and Pizzarello, 2006).

The regio- and stereoselectivity of bimolecular phenoxy radical coupling reactions takes place in various biosynthetic pathways, including the production of suberin, flavonolignans, and alkaloids in plants, fruiting body development in fungi, cuticle melanization and sclerotization in insects, and the formation of pigments in aphids and cell wall polymers in algae (reviewed by Davin *et al.*, 1997). In vascular plant development, the bimolecular phenoxy radical coupling has special importance in the biosynthesis of lignin (Nose *et al.*, 1995) and lignan (Paré *et al.*, 1994; Davin and Lewis, 2000).

It has been established that the oxidative coupling of coniferyl alcohol by peroxidases or laccases *in vitro* lacks regio- and stereoselectivity (Fig. 1), resulting in a mixture of (+/–) 8,8', (+/–) 8,5', and 8-*O*-4' linkages (Fig. 1). However, vascular plants are known to be capable of forming high proportions of regio- and stereoselectivity products of this reaction, including 8,8'-coupled (+)-pinoresinol, indicating the presence of a co-ordinating factor, which was initially named (+)-pinoresinol synthase (Paré *et al.*, 1994). The factors determining the stereoselective coupling of coniferyl alcohol (CA) radicals in the biosynthesis of (+)-pinoresinol were further investigated using crude cell wall extracts of *Forsythia suspensa* (Davin *et al.*, 1997). Non-specific (lacking both regio- and stereoselectivity) bimolecular radical coupling was obtained when a protein fraction containing a laccase was added to CA *in vitro,* resulting in the production of a racemic mixture of (+/–)-dehydrodiconiferyl alcohols, (+/–)-pinoresinols, and (+/–)-eryrhro/threo guaiacylglycerol 8-*O*-4' coniferyl alcohol ethers (Fig. 1). However, when the reaction mixture was combined with fraction containing an ~27 kDa protein, both regio- and stereoselectivity were restored, resulting in the production of essentially only (+)-pinoresinol. The ~27 kDa protein of (at that time) unknown nature was designated as a dirigent (DIR) protein (from Latin *dirigere*, to align, guide) and suggested to function by capturing CA radicals produced by an oxidizing compound to mediate stereoselective coupling of (+)-pinoresinol (Davin *et al.*, 1997).

In order to characterize DIR proteins further and to establish their involvement in lignan and lignin formation, Gang *et al.* (1999) cloned several genes encoding DIR proteins from different species. The recombinant proteins produced were able to confer strict regio- and stereoselectivity of the monolignol free radical coupling. The *DIR* expression in developing xylem and in other lignified tissues indicated the roles of DIRs in lignification (Burlat *et al.*, 2001).

Further studies identified DIR proteins in at least 104 species throughout the plant kingdom, suggesting that DIR families are probably present in all vascular plants (Davin *et al.*, 2008). In addition to DIR proteins involved in (+)-pinoresinol formation, the presence of (–)-pinoresinol-forming DIRs has been suggested in species such as Daphne (*Daphne tangutica*) and flax (*Linum usitatissimum*) (reviewed by Kim *et al.*, 2012). Direct evidence for the involvement of DIR proteins from *Arabidopsis thaliana* in the formation of (–)-pinoresinol has been provided in the case of AtDIR6, one of the few functionally characterized DIRs from Arabidopsis (Pickel *et al.*, 2010; Vassão *et al.*, 2010).

In the following text we provide an overview of the known and possible contributions of DIR proteins to lignan and lignin biosynthesis. We discuss recent structural insights into the hypothetical mechanism of the action of DIR proteins and recapitulate current evidence and implications for the role of DIR proteins in cell wall signaling as well as in biotic and abiotic stress responses. We provide a basic bioinformatics-based characterization of the *A. thaliana* DIR family (AtDIRs) and suggest possible approaches useful in our and others' efforts to decipher the role of the fascinating DIR proteins in plant development and stress responses.

Function of DIR proteins

Stereoselective radical–radical coupling in lignan biosynthesis

Lignins and lignans are derived from phenylpropanoid metabolism. This pathway leads to the production of monolignols (coniferyl, sinapyl, and *p*-coumaryl alcohols), which are precursors in both lignan and lignin biosynthesis. (Buchanan *et al.*, 2000) (see Supplementary Fig. S1 at *JXB* online). The term lignan specifies a class of dimeric phenylpropanoid (C_6C_3) metabolites linked by an 8–8' bond, while alternatively linked dimers are known as neolignans (Buchanan *et al.*, 2000). Twenty-three lignan and neolignan types across the plant kingdom have been described (Teponno *et al.*, 2016), indicating their wide range of usage. The lignan (and neolignan) biosynthetic pathway starts with the synthesis of phenylalanine, a precursor of coniferyl alcohol (Hao and Mohnen, 2014; Barros *et al.*, 2015). The dimerization/radical coupling of two coniferyl alcohol molecules leading to (+/–)-pinoresinol formation is mediated by oxidases, such as peroxidases or laccases, with the assistance of DIRs, which ensure the stereoselectivity of coniferyl alcohol dimerization (Davin *et al.*, 1997; Halls and Lewis, 2002; Halls *et al.*, 2004). This step is crucial because the optical activity is a property-determining feature in most of the lignans (Akiyama *et al.*, 2007*a*, 2009). Pinoresinol can be converted to other lignan types including piperitol, laciresinol, sesamin, secoisolaresinol, and their glucosides (Dinkova-Kostova *et al.*, 1996; Satake *et al.*, 2015).

Lignin initiation

Lignins are three-dimensional, amorphous heteropolymers with species-specific compositions. In angiosperms, lignin consists mainly of coniferyl and sinapyl alcohols, with *p*-coumaryl alcohol contributing only ~2% (Bonawitz *et al.*, 2014). Lignin polymerization can involve at least five different linkage sites to form *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, which differ in their degree of methoxylation

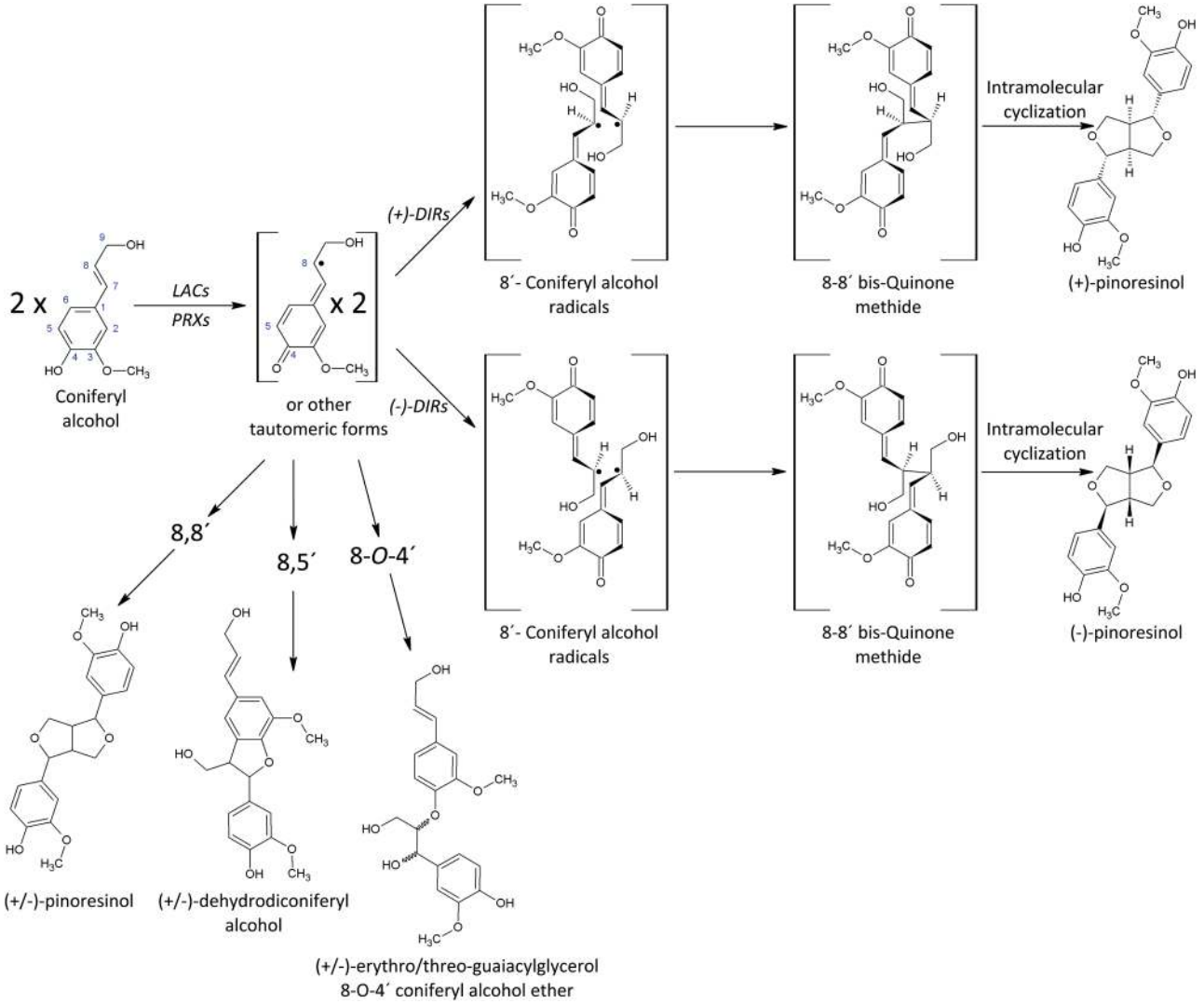

Fig. 1. Products of phenoxy radical coupling of E-coniferyl alcohol radicals. In the presence of peroxidases or laccases but in the absence of DIR proteins, the result is a racemic mixture of approximately equal amounts of (+/–) 8,8'-, (+/–) 8,5'-, and 8-*O*-4'-linked products. The presence of DIR proteins results in regio- and stereoselective coupling to give (–)-pinoresinol or (+)-pinoresinol depending on the specificity of DIR. LACs, laccases; PRXs, peroxidases; DIRs, dirigent proteins.

(Supplementary Fig. S1). The monolignol polymerization is initiated by the formation of lignin oligomers through an 'end-wise' reaction, which involves the 8-carbon site of one monomeric phenylpropanoid and a different acceptor site in the growing lignin oligomer and occurs via enzyme-mediated formation of phenylpropanoid radicals (reviewed by Behr *et al.*, 2015). This reaction differs from phenylpropanoid dimerization in lignan biosynthesis, where an 8–8' bond is preferentially formed, resulting in lignan precursors. In addition, lignin, in contrast to lignan, is an optically inactive complex polymer. However, even if the stereoselectivity is unnecessary, the regioselectivity of the coupling reaction is still needed to confer the observed predominance of 8-*O*-4' bonds in plant-isolated lignin (Davin and Lewis, 2005*b*, and references therein). Moreover, monolignols can be targeted to precise sites, called lignin initiation sites. For example, during tracheid formation, *p*-coumaryl alcohol is targeted toward the middle lamella, whereas coniferyl alcohol is initially deposited in the S1 sublayer and cell corners, where the lignification is initiated and extends back toward the plasma membrane (Davin and Lewis, 2000). Although the detailed molecular mechanism is still unclear, specific gene expression seems to be involved in the control over the lignin composition of each cell (Liu, 2012).

The evidence supporting the involvement of protein(s) harboring an array of DIR- (monolignol radical) binding sites in lignin biosynthesis was provided by a co-localizing signal generated by αDIR polyclonal antibodies with lignin initiation sites (i.e. in the S1 layer of the secondary cell walls of lignifying tracheary elements). It was suggested that a protein with similarity to DIR proteins (i.e. able to bind monolignol radicals) could be responsible for both directing the targeting of coniferyl alcohol to the lignification initiation sites and the regioselectivity resulting in the predominance of 8-*O*-4' bonds (Davin and Lewis, 2000). Davin *et al.* (2008) highlighted that such a protein and its dirigent sites have to be distinctly different from those involved in stereoselective radical–radical coupling leading to lignan biosynthesis, such as

to (+)-pinoresinol or (–)-pinoresinol. This prediction seems to be in line with recent findings implicating ENHANCED SUBERIN1 (ESB1/AtDIR10) in Casparian strip (CS) formation in Arabidopsis (Hosmani *et al.*, 2013); see below. Further, one of the major quantitative trait loci in soya encodes a DIR-like protein PDH1, involved in the regulation of soybean pod dehiscence. Based on the gene expression data, PDH1 seems to be also involved in the control of lignin deposition (Funatsuki *et al.*, 2014).

Other forms of stereoselective and regioselective coupling mediated by DIRs: in atropselective synthesis of gossipol

Besides the established roles of DIR proteins in lignan and lignin formation, an additional role has been demonstrated in the formation of the phenolic terpenoid (+)-gossypol in cotton (*Gossypium* sp.) (Liu *et al.*, 2008; Effenberger *et al.*, 2015). (+)-Gossypol is a phenolic aldehyde found in flowers, seeds, roots, and the foliage of cotton plants, and it has an important role in the response to pathogens (Gao *et al.*, 2013). DIR proteins from *Gossypium hirsutum* (GhDIR14) (Liu *et al.*, 2008), *G. barbadense* (GbDIR2), and *G. hirsutum* (GhDIR3) (Effenberger *et al.*, 2017) confer atropselectivity to this terpenoid, gossypol, formed from C–C coupling of two hemigossypol radicals. The possible rotation around the binaphthyl C–C bond allows the existence of two atropisomers with different properties and optical activities. When hemigossypol is incubated with peroxidase/H_2O_2, laccase/O_2, or ammonium persulfate, only a racemic mixture is obtained (Benedict *et al.*, 2006). However, when hemigossypol is incubated with crude extracts containing DIR activity, the (+)-gossypol form was preferentially formed (Liu *et al.*, 2008). Both + and – isomers are involved in plant defense. Nevertheless, only (–)-gossypol has antispermatogenic as well as antiviral activities, and only the (+) isomer is toxic to non-ruminant animals (Effenberger *et al.*, 2017). In this case, the control of atropselectivity could be of biotechnological relevance. For instance, the generation of cotton transgenic plants lacking the toxic (–)-gossypol but retaining (+)-gossypol (with a role in plant defense) might enable use of cotton seeds as a source of food protein (Effenberger *et al.*, 2015).

The importance of lignans and lignin

Lignans have an important role in plant defense against pathogens (Davin *et al.*, 2008; Davin and Lewis, 2005*b*), by inhibiting microbe-derived degradative enzymes such as cellulases, polygalacturonases, glucosidases, and laccases (MacRae and Towers, 1984). Additionally, it has been suggested that lignans can function as insect antifeedants by disrupting the insect endocrine system (Harmatha and Dinan, 2003). Importantly, lignans could also be used as drugs and chemopreventive agents in conventional medicine. Podophyllotoxin (from *Podophyllum peltatum*) has antiviral properties, and one of its derivatives (Etopophos®) has applications in cancer chemotherapy (reviewed by Davin *et al.*, 2008).

Moreover, lignans could serve as a storage pool of monolignols for lignification. Expression of lignan synthesis genes was up-regulated during xylogenesis in lignified tissues of maritime pine and flax (Huis *et al.*, 2012; Villalobos *et al.*, 2012). Immunolabeling experiments revealed the presence of lignans in the secondary cell walls of flax (Attoumbré *et al.*, 2010). Interestingly, functional disruption of PINORESINOL REDUCTASE 1 (PrR1), which catalyzes the conversion of pinoresinol to lacinoresinol, leads to decreased lignin content and altered distribution in the inflorescence stem of Arabidopsis (Ruprecht *et al.*, 2011; Zhao *et al.*, 2015). The promoter of *PrR1* was also shown to be under the transcriptional control of secondary cell wall-specific (and thus lignification-inducing) transcription factors, NAC secondary wall thickening-promoting factor (NST3), and MYB46 (Zhao *et al.*, 2015).

Lignin confers stability and hydrophobicity to the plant vascular system; it forms a barrier against microbial pathogens to limit the spread of pathogen-derived toxins and enzymes into the host by altering compressibility and porosity of the cell wall (Bonello *et al.*, 2003; Miedes *et al.*, 2014). Moreover, lignin plays an important role in the spatial delimitation of silique shattering for seed release, as well as in seed protection (reviewed in Barros *et al.*, 2015). The ability of lignin to control the permeability of cell wall is of especial importance in the case of CS formation, where lignification occurs before secondary cell wall formation and is thought to form a diffusion barrier regulating vascular solute transport (see later).

Dirigent proteins in Arabidopsis

AtDIRs in lignan biosynthesis

Arabidopsis DIR and DIR-like (AtDIR) proteins constitute a protein family with 26 members whose specific functions are still barely understood (Ralph *et al.*, 2006; Kim *et al.*, 2012). Kim *et al.* (2012) investigated 16 out of 26 AtDIRs. In this study, several *AtDIR* genes were cloned and their recombinant proteins biochemically analyzed. In particular, AtDIR5 and AtDIR6 (but not AtDIR10/ESB1 or AtDIR13) were found to be able to generate (–)-pinoresinol *in vitro* when incubated together with a laccase. Accordingly, increasing *AtDIR6* expression levels enhanced the abundance of (–)-pinoresinol and (–)-lariciresinol (a derivative of (–)-pinoresiol), indicating that *AtDIR6* was involved in preferential coupling leading to (–)-pinoresinol *in vivo*.

The analysis of promoter::GUS (β-glucuronidase) reporter constructs suggested that *AtDIR6*, *AtDIR10*/*ESB1*, and *AtDIR13* are strongly but not exclusively expressed in the root (Kim *et al.*, 2012). *AtDIR10*/*ESB1* was also expressed in the lignifying leaf vasculature and hydathodes, and activity of *AtDIR13* was detectable in cotyledons (Vassão *et al.*, 2010; Kim *et al.*, 2012). In contrast, the expression of A*tDIR12*/*DP1* seems to be specific for the outer seed coat at 7 d post-anthesis (Esfandiari *et al.*, 2013). Accordingly, knocking-out of *AtDIR12*/*DP1* resulted in a lack of seed-specific neolignans such as 3-{4-[2-hydroxy-2-(4-hexosyloxy-3-methoxyphenyl)-1-hydroxymethylethoxy]-3,5

dimethoxyphenyl} acryloylcholine (Böttcher *et al.*, 2008; Matsuda *et al.*, 2010), suggesting a role for *AtDIR12*/*DP1* in neolignan biosynthesis.

AtDIRs in Casparian strip formation

The CS is mainly composed of lignin and forms a longitudinally oriented belt passing through both transversal and anticlinal cell walls in the root endodermis (Naseer *et al.*, 2012). The CS extends across cell junctions and integrates the middle lamella of adjacent epidermal cells to form a continuous ring of lignin. Consequently, the CS constitutes a physical and chemical barrier (analogous to tight junctions in animals), tightly controlling water and nutrient transport while providing protection against soil-borne pathogens (Geldner, 2013).

The lignification of the CS occurs in a tightly controlled manner indicative of a precise targeting mechanism with subcellular resolution. Recent studies demonstrated that the localization of CS domain proteins (CASPs) to specific cell membrane domains is indicative of the site of CS formation. Here, CASPs are thought to form a protein scaffold directing transport across the plasma membrane and recruiting proteins required for CS formation and lignification (Roppolo *et al.*, 2011). The DIR domain-containing protein AtDIR10/ESB1 was shown to be targeted to the CS in a CASP-dependent manner. The *esb1-1* mutant showed that ESB1 is required in both the early deposition of lignin patches and their fusion in generating the mature CS. Loss of *AtDIR10*/*ESB1* resulted in the disruption of CASP1 localization, suggesting a reciprocal requirement for both AtDIR10/ESB1 and CASPs in the spatial control of CS lignification. The lack of AtDIR10/ESB1 also provoked an ectopic deposition of suberin, suggesting a possible cross-talk between CS and suberin biosynthesis (Hosmani *et al.*, 2013). Alternatively, the phenotype could be caused by the activation of the receptor-like cytoplasmic kinase [SCHENGENES (SGN)1–SGN3] pathway by the CASPARIAN STRIP INTEGRITY (CIF) peptides lost from the stele due to the defect in the CS barrier in the *esb1-1* mutant (Doblas *et al.*, 2017).

Recently, MYB36 has been suggested as the transcriptional regulator controlling the expression of genes required for CS formation. Analysis of microarray data of *myb36* knockout mutants predicted 23 endodermal-expressed genes to be regulated by MYB36. Among these are genes encoding the DIR proteins ESB1–ESB5 and ESB-like DIRs (AtDIR9, 10, 16, 18, 19, 24), CASPs, PEROXIDASE 64 (PER64), and a leucine-rich repeat receptor-like kinase (LRR-RLK). Additionally, ChIP-qPCR using the MYB36 genome–GFP/myb36-1 showed a direct association with CASP1, PER64, and ESB1 promoters (Kamiya *et al.*, 2015). These findings suggest that other members of the AtDIR family could be important for CS and/or secondary cell wall formation. That, however, remains to be shown.

Structure and mode of action of pinoresinol-forming AtDIRs

One of the first published attempts to decipher the structural features of DIR proteins was the homology-based model of AtDIR6 (Pickel *et al.*, 2012). The model predicted the monomer of AtDIR6 as an eight-stranded antiparallel β-barrel with a central hydrophobic cavity for substrate binding, structurally resembling allene oxide cyclase (Hofmann *et al.*, 2006; Pickel *et al.*, 2012). Based on earlier biochemical and kinetic data (Halls and Lewis, 2002; Halls *et al.*, 2004; Davin and Lewis, 2005*a*), it was suggested that DIR proteins form dimers, where each monomer binds a single CA radical (produced by laccase or oxidase) in a way that favors 8–8' coupling (Halls *et al.*, 2004). However, the crystal structure obtained for the (+)-pinoresinol-forming DISEASE RESISTANCE RESPONSE 206 (PsDRR206) from pea indicated a tightly packed homotrimer with the hydrophobic binding pockets placed on the outer surface of each monomer and too distant from each other to allow inter-pocket CA side chain interactions. Based on this, it was proposed that each binding site enables stereoselective coupling (using either two CA radicals or a radical and a monolignol) (Kim *et al.*, 2015). The trimeric nature of DIRs and the docking of two CA molecules per DIR monomer was recently supported from structural studies of AtDIR6, a (–)-pinoresinol-forming DIR (Kim *et al.*, 2012; Gasper *et al.*, 2016).

In AtDIR6 (Fig. 2), the binding cavity of each monomer was seen to consist of two lobes (pockets A and B), with each lined with a set of hydrophilic and potentially catalytic residues (Fig. 3). These residues are conserved between (+)- and (–)-pinoresinol-forming DIRs and are required for DIR activity (Kim *et al.*, 2012; Gasper *et al.*, 2016).

Comparing the structures of PsDRR206 and AtDIR6 (Supplementary Fig. S2) highlights several important differences. First, there is an important difference in the architecture of the β1–β2 loop of both proteins. In AtDIR6, there is one more short β sheet (β1') in the region corresponding to the β1–β2 loop of PsDRR206. Further, the β1–β2 loop of PsDRR206 traverses the interaction interface between the neighboring monomers. This places the N-terminal part of the β2 sheet of one monomer of PsDRR206 next to the β2 of the interacting monomer (Supplementary Fig. S2). These two β2 strands contribute to the interaction interface by seven hydrogen bonds between their backbone chains (Kim *et al.*, 2015). No such domain swapping could be seen in AtDIR6 (Gasper *et al.*, 2016). Secondly, there are striking differences in the architecture of the active sites of both proteins. In PsDRR206, the loops surrounding the active center are more flexible and therefore not well resolved in the structure. Notably, in contrast to AtDIR6, where the loops are bent inward, the loops of AtDRR206 are bent outwards from the active center, making it more open (Supplementary Fig. S2B–D) (Gasper *et al.*, 2016). This has an important consequence for the positioning of the key residues of pocket A, which are well (ideally for catalysis) positioned in the case of AtDIR6 (Asp137, Arg144). However, this is not the case for corresponding residues of PsDRR206 (Asp134, Arg141), which are protruding out into the solvent and forming the hydrogen bond with β5, respectively (Supplementary Fig. S2B). Based on that, Apo-DRR206 was proposed to exist in a non-catalytic state, requiring the binding of the two CA radicals for the rearrangement of the active residues and DIR

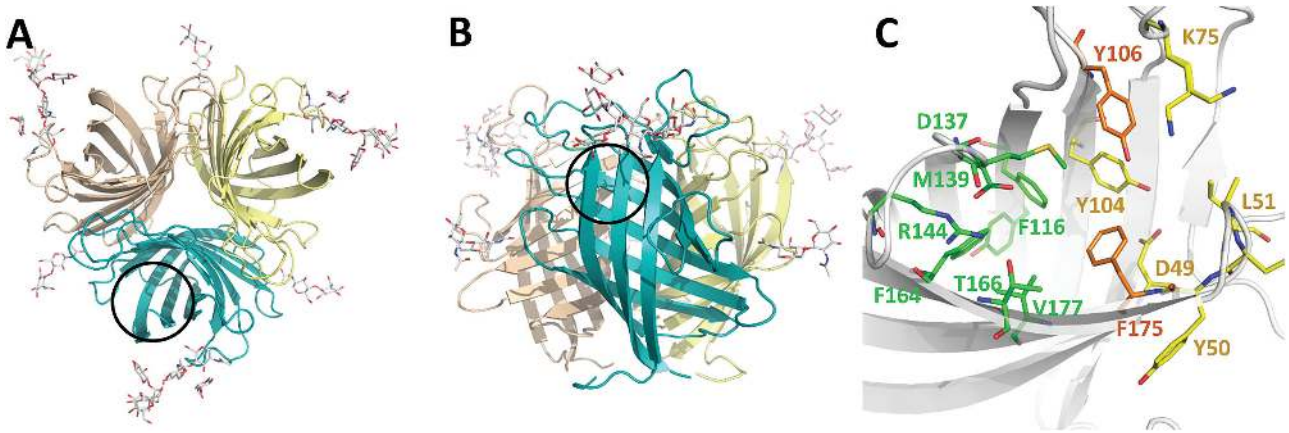

Fig. 2. Structure of AtDIR6. AtDIR6 trimer (A) top and (B) side view. Each monomer is shown in a different color. Glycosylation is shown as sticks. The location of the active site is highlighted by a black circle in one of the monomers. (C) AtDIR6 active site. Residues forming pocket A are shown in green, residues forming pocket B shown in yellow, and residues in between pockets are shown in orange. Figures were generated with PyMOL 4, using the PDB code 5LAL.

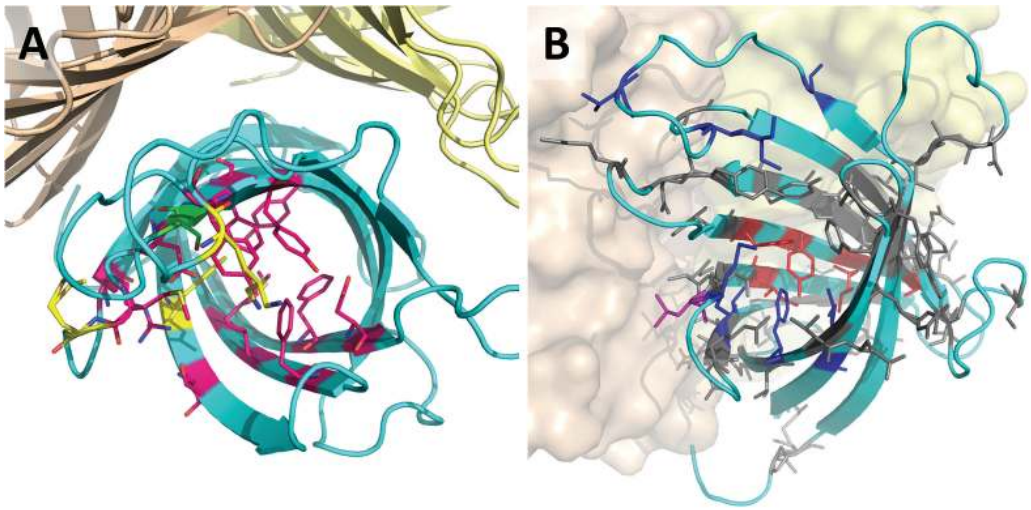

Fig. 3. Localization of functional and conserved residues of AtDIR6. (A) AtDIR6 residues important for protein activity. Based on Gasper *et al.* (2016). Mutation of green residues results in an increase of activity, mutation of yellow residues in a slight decrease in activity, and mutation of magenta residues in a strong decrease in activity. (B) DIR6 monomer with residues conserved within DIR proteins highlighted. Color code: cyan, non-conserved; gray, fully conserved across (+) and (–) DIRs; blue, (–) DIR conserved; magenta, (+) DIR conserved; red, differentially (+) and (–) conserved residues.

activation. In comparison, free AtDIR6 is probably in a pre-catalytic conformation with the active site residues lined up for catalysis (Gasper *et al.*, 2016).

Both AtDIR6 and PsDRR206 contain an omega loop (part of the β2–β3 loop, Supplementary Fig. S2) that comprises a cluster of highly conserved amino acid residues (His39, Thr84, and Ser91 in PsDRR206). The omega loop is tightly adjacent to the active center and might have a role in proper substrate positioning (Kim *et al.*, 2015).

Based on the AtDIR6 structure and extensive mutational analysis, an updated model describing how AtDIR6 facilitates lignan synthesis was proposed (Gasper *et al.*, 2016). Each subunit of the DIR homotrimer binds two CA radicals. The binding to pockets A and B accommodates both radicals in their extended, planar *all-trans* conformation, in which they are precisely positioned to enable 8–8' coupling ata the *re–re* face. This determines the regioselectivity of 8–8' pinoresinol coupling over other coupling options. Also the enantioselectivity of DIRs seems to be a direct consequence of the precise substrate positioning allowing regioselective 8–8' coupling. Gasper *et al.* (2016) suggested that if coupling occurs at the *re–re* face, the *S*,*S*-configured *bis*-quinone methide (bisQM) intermediate (see also below) is formed as the precursor of (–)-pinoresinol. However, *si–si* face coupling yields the *R*,*R*-bisQM and, consequently, (+)-pinoresinol. Interestingly, larger portions of the DIR protein must be changed (not simply individual amino acids) to change its stereoselectivity (Kim *et al.*, 2012; Gasper *et al.*, 2016).

Originally it was hypothesized that DIRs actually lack enzymatic activity and are in reality responsible mostly for the correct positioning of linked CA radicals. Surprisingly, the solved structure suggests a direct involvement of AtDIR6 in catalyzing the cyclization of a bisQM intermediate that is electron deficient at C7 and thus susceptible to nucleophilic attack (Freudenberg, 1959; Ralph *et al.*, 2009; Gasper *et al.*, 2016). In the process of pinoresinol formation, the terminal

OH groups of the propionyl side chains can act as nucleophiles. 1,6-Addition to the *p*-quinone methides allows the cyclization of the furan rings and re-aromatization of the cyclohexadienones. In the new mode of action proposed by Gasper *et al.* (2016), AtDIR6 contributes to the catalysis of pinoresinol formation by donating a proton to the hexadienone ring carbonyl of the bisQM (Fig. 4). The authors suggested that this reaction could be mediated via hydrogen bond formation or acid catalysis. Conserved amino acids Asp137, Arg144, and Thr166 in pocket A, and Asp49, Tyr104, and Tyr106 in pocket B are critical for this DIR function. Based on the physico-chemical properties and positioning of those conserved amino acid residues, the most probable candidates are Asp137, Arg144, and Asp49.

The role of DIR proteins in stress responses

DIRs in a response to biotic stress

The involvement of lignin, and phenylpropanoid biosynthesis in general, in plant defense against pathogens is well known (Caño-Delgado *et al.*, 2003; Miedes *et al.*, 2014). Several publications have described increased concentrations of lignin after pathogen infection (Mohr and Cahill, 2007; Quentin *et al.*, 2009), or highlighted the importance of soluble phenylpropanoids in Arabidopsis pathogen defense (König *et al.*, 2014). An important contribution of lignin to defense against pathogens was demonstrated in *Medicago sativa*, where selective down-regulation of lignin biosynthesis resulted in the constitutive expression of defense response genes. It was suggested that lignin deficiency triggers the defense response via the enhanced release of bioactive cell wall fragments as a result of impaired cell wall integrity (Gallego-Giraldo *et al.*, 2011) (see also below). Conversely, changes in flax resistance to pathogens putatively correlate better with the antioxidant potential of seed lignans, whereas the structural barriers provided by lignin and cellulose appear to be not as important (Zeitoun *et al.*, 2014).

Direct evidence connecting AtDIRs with pathogen defense in Arabidopsis is still lacking. Nevertheless, numerous examples clearly demonstrate the involvement of DIRs in the pathogen response in various species. For example, the infection of the moss *Physcomitrella patens* with *Colletotrichum gleosporioides* results in cell wall modifications including increased incorporation of phenolic compounds, which was correlated with the induction of a DIR-like protein-encoding gene (Reboledo *et al.*, 2015). Recently, it has been reported that *P. patens* also exhibits cell wall reinforcements upon treatment with *Pectobacterium carotovorum*-derived elicitors. Expression analysis showed that four genes encoding putative DIR-like proteins (*DIR-1*, *DIR-2*, *DIR-3*, and *DIR-4*) were induced during the treatment with different dynamics (Alvarez *et al.*, 2016). *DIR-4* seems also to be induced by *B. cinerea* and *C. aloeosporioides* infection (Ponce De Leon *et al.*, 2012; Reboledo *et al.*, 2015). In Sitka spruce (*Picea sitchensis*), transcript profiling and microarray-based gene expression analysis suggested that several DIR genes were induced upon attack by stem-boring weevils or after mechanical wounding. Similarly, *BrDIR12* and several *DIR-like* genes were differentially expressed in cabbage (*Brassica oleracea*) infected by *Fusarium oxysporum* (Arasan *et al.*, 2013).

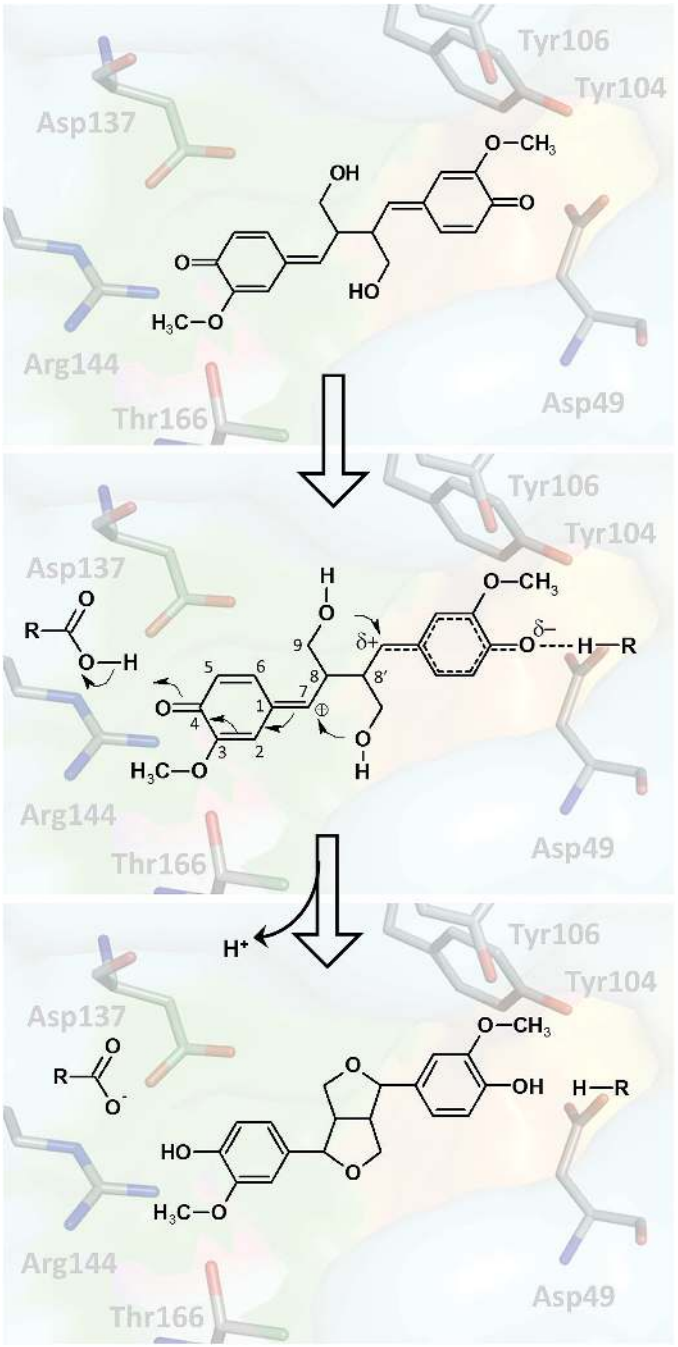

Fig. 4. Schematic representation of the bisQM intermediate cyclization catalyzed by AtDIR6. The cartoon combines the side view on the active site as shown in Fig. 2 with the key functional residues highlighted (sticks). The mechanism of bisQM cyclization proposed by Gasper *et al.* (2016) via acid catalysis (left) or hydrogen bond formation (right) is schematically shown. Both mechanisms seem to result in a partial or full positive charge on C7 that facilitates the nucleophilic attack during bisQM cyclization.

There is also evidence that DIRs and DIR-like proteins are spatially targeted during the response to pathogen infection (Ma, 2014). The rice mannose-binding jacalin-related lectin gene (OsJAC1) was shown to consist of a DIR domain and

an N-terminal jacalin-related lectin domain (JRL), which is predicted to bind mannose-containing oligosaccharides prevalent in fungi. The overexpression of *OsJAC1* in rice resulted in broad-spectrum resistance against major rice pathogens and seems to require the re-localization of OsJAC1 to infection sites. Similarly, overexpression of *OsJAC1* or the wheat ortholog (*TaJA1*) in barley results in enhanced resistance to powdery mildew fungus. Interestingly, both the DIR and JRL domains are required to generate resistance, as indicated by transient expression experiments (Weidenbach *et al.*, 2016).

The large number of different *DIR* genes expressed during wounding and pathogen infection supports the idea that DIR proteins participate in pathogen defense. This can be by generating defense compounds and/or participation in dynamic reorganization of the cell wall since both require radical coupling during the formation of pinoresinol and other lignans as well as their stereoisomers. This hypothesis is further supported by the large number of antibacterial activities observed for various lignan stereoisomers (Akiyama *et al.*, 2007*a*, 2009). However, plant pathogen defense via lignan production is not delimited to antibacterial effects. Lignans isolated from *Piperaceae* also have toxic effects on phytophagous insects (Bernard *et al.*, 1995). Other lignans were shown to act as antifungal agents (Carpinella *et al.*, 2005; Akiyama *et al.*, 2007*b*) and contribute to wound responses (Harju *et al.*, 2009). Interestingly, the highest transcriptional change observed upon fungal infection in *Vitis vinifera* was the up-regulation of a putative *DIR* gene (180-fold) (Borges *et al.*, 2013). The authors hypothesized that low *DIR* expression during the early stages of fungal infection favors lignin biosynthesis via peroxidase-mediated production of all three possible isomers from coniferyl alcohol: pinoresinol, dehydrodiconiferyl alcohol. and guaiacylglycerol 8-*O*-4'-coniferyl ether, since the latter two are precursors of lignin biosynthesis. In comparison, the up-regulation of *DIR* genes during later stages of infection increases antifungal activities through activation of lignan biosynthesis (Borges *et al.*, 2013).

DIRs during the response to abiotic stress

DIRs and peroxidases have frequently been implicated in modulation of lignification levels upon exposure to abiotic stress. The expression of several of the *DIR-like* genes was responsive to water, abscisic acid (ABA), and cold stress. More importantly, in the case of water stress, the expression of the most responsive *DIR* genes could be correlated with increased lignification (Arasan *et al.*, 2013).

In soybean roots, Mn toxicity is known to enhance peroxidase activity and wall lignification (Morita *et al.*, 2006). A recent proteomic study demonstrated that Mn toxicity also induced elevated accumulation of H_2O_2 in roots, which coincided with up-regulation of PEROXIDASE5- and DIR2-like protein levels, whereas levels of another DIR protein were reduced (Chen *et al.*, 2016). Similarly, in *M. sativa*, application of cold stress resulted in the transcriptional down-regulation of two peroxidases and a *DIR* gene, while another *DIR* gene was up-regulated by heat stress (Behr *et al.*, 2015). A *DIR* gene from *Boea hygrometrica* (*BhDIR1*) was implicated in the response to water and temperature stresses (Wu *et al.*, 2009). A *Saccharum* spp. dirigent gene (*ScDIR*) exhibiting stem-specific expression has been reported to respond to drought, salt, and oxidative stresses (Jin-Long *et al.*, 2012). Additionally, CaiQiu *et al.* (2010) reported enhanced expression levels of *TaDIR* in *Tamarix androssowii* after exposure to salinity–alkalinity stress.

The above examples imply that the mobilization of specific peroxidases and DIRs in response to particular stress conditions is possibly ensuring the specificity of the plant response. The DIR-related molecular mechanisms underpinning the ability of plants to cope with the given abiotic stress type, however, remain to be identified.

DIRs and maintenance of plant cell wall integrity

As we discussed above, the spatial control of lignin deposition is important in defense responses to biotic or abiotic stress. Several groups have implicated DIR proteins in the response to pathogens causing physical damage to the cell wall during infection and abiotic stress (osmotic and drought) in different plant species (Ralph *et al.*, 2006; Jin-Long *et al.*, 2012; Arasan *et al.*, 2013). Bearing in mind the current research on CS formation, it is conceivable that DIR proteins could mediate the spatial control of lignin deposition during development and stress responses. This would make them an essential element controlling cell wall modification/reinforcement during cell wall integrity maintenance.

During exposure to both biotic and abiotic stress, the conformation/structure of the differing plant cell walls needs to be adaptively altered to maintain stress-compromised functional characteristics essential for development and defense. The maintenance of wall integrity can often also neutralize the effects of targeted genetic manipulation through adaptive (compensatory) changes in other processes impacting cell wall composition and structure (Doblin *et al.*, 2014). It also implies that a mechanism exists to monitor cell wall status and to initiate specific compensatory responses. It is reasonable to assume that such responses probably require a tight spatial control at the cellular and subcellular level to target compromised wall matrix sites of specific architecture to ensure that novel cell wall components required for the restoration of function are integrated correctly.

A dedicated cell wall integrity maintenance mechanism has been well documented in *Saccharomyces cerevisiae* (Levin, 2011). It involves osmo, mechano, and cell wall damage perception, signal translation via Rho guanine nucleotide exchange factor (GEFs) and mitogen-activated protein (MAP) kinase cascades, as well as adaptive changes in cytoskeleton organization and cell wall metabolic processes. Interestingly it has been shown that certain *A. thaliana* genes can rescue yeast strains deficient in components of the yeast maintenance mechanism (Reiser *et al.*, 2003; Nakagawa *et al.*, 2007). Several groups have shown in parallel that in Arabidopsis a similar mechanism exists (Ellis *et al.*, 2002; Caño-Delgado *et al.*, 2003; Hématy *et al.*, 2007; Wolf and Höfte, 2014). It involves at least a plasma membrane-localized receptor-like kinase (THESEUS1) as well as a putative stretch-activated calcium

channel (MID1-COMPLEMENTING ACTIVITY1), which seem to perceive plant cell wall integrity impairment. Homologs of these genes have been found in a large number of both mono- and dicotyledonous plant species including crops such as rice and strawberry, as well as *P. patens* (N. Gigli-Bisceglia and J. Antsiferova, personal communication), suggesting that the mechanism they are involved in exists throughout the plant kingdom (Kurusu *et al.*, 2012; Nguyen *et al.*, 2015; Zhang *et al.*, 2016).

Jasmonic acid and reactive oxygen species are required for signal translation in cell wall integrity maintenance, while cell wall modifications can involve the production of pectic polysaccharides as well as targeted deposition of lignin in cell types that normally do not exhibit lignin deposition (Caño-Delgado *et al.*, 2003; Denness *et al.*, 2011). To meet the specific functional requirements of the compensatory process, both the individual biosynthetic processes and the locations where they take place have to be tightly controlled. While the need for this tight control is obvious, our knowledge of the molecular machinery responsible for it is still very limited. However, as mentioned in the above text, DIR proteins might represent one of the potent effectors acting downstream of the cell wall integrity signaling cascade. That, however, remain to be demonstrated.

Bioinformatic analysis of Arabidopsis DIR family

Phylogenetic relationships of AtDIR proteins

In order to identify potential functional relationships in the AtDIR family, a phylogenetic tree was created based on the amino acid alignment of DIR domains from individual AtDIR sequences. As AtDIR17 contains just a partial DIR domain, it was excluded from the phylogenetic analysis.

As shown in the Fig. 5, AtDIRs might be divided into several distinct subclades. Some of them partially correspond to DIR (DIR-a) and DIR-like (DIR-b–DIR-e) subfamilies previously distinguished by Ralph *et al.* (2006) by comparing 72 DIRs and DIR-like proteins from several species including AtDIRs. In the tree based exclusively on DIR domains of all 26 members of the AtDIR family, we were able to identify three main subclades reliably. The subclade consisting of AtDIR5, 6, 12, 13, and 14 corresponds to the Ralph DIR-a subfamily and contains both of the known (–)-pinoresinol-forming Arabidopsis AtDIRs, AtDIR5 and AtDIR6. Further reliably distinguishable subclades include AtDIR1, 2, 11, and 21, which seems to correspond to DIR-like subfamily DIR-d, in Ralph *et al.* (2006) represented by AtDIR11 and AtDIR2. Finally, the last well-specified subclade consisting of AtDIR9, 10, 16, 18, 24, and 25 largely overlaps with DIR-like subfamily DIR-e (Ralph *et al.*, 2006). Further classification of AtDIR proteins would be rather approximate due to low bootstrap support of deep nodes (Fig. 5).

AtDIR 10, 16, 18, and 25 together with isoleucyl-tRNA synthetase (OVA2), a rather atypical member of the AtDIR family, comprise a group of multidomain DIRs. *OVA2* genes represent a special case among *AtDIR* genes, which is also reflected by its clustering outside the DIR-e subclade. One of three possible *OVA2* isoforms extends to the upstream transcription start site, thereby including the coding sequence of the *AtDIR3* gene. The stop codon of *AtDIR3* is spliced out, resulting in a predicted OVA2 (At5g49030.3) protein with an N-terminal DIR3 domain. Interestingly, AtDIR10, 16, 18, and 25 contain both a complete DIR domain and a partial N-terminal DIR domain.

AtDIR25 also contains a part of a JACALIN protein (represented in green in Fig. 5), reminiscent of the recently described OsJAC1 protein of rice (Weidenbach *et al.*, 2016). However, it is questionable whether AtDIR25 and OsJAC1 have similar functions since the JACALIN domain of AtDIR25 is not complete. The clustering of all but one multidomain AtDIRs in the DIR-e subclade suggests that the presence of additional domains in the AtDIR family is 'encoded' in the sequence of the DIR domain. The conservation of this mechanism is supported by the inclusion of DIR domain sequences from DIR proteins with extended N-termini from species other than Arabidopsis [*Gossypium hirsutum* (Gh), *Theobroma cacao* (Tc), *Corchorus capsularis* (Cc), and *Nicotiana sylvestris* (Ns)]. All these sequences clustered together in the DIR-e (multidomain) subclade (Supplementary Fig. S5).

One defining characteristic of this subclade in Arabidopsis could be the lack of the N-terminal portion of the aligned DIR domains (Supplementary Fig. S3). Whether there is any functional link between an absence of the N-terminal amino acid stretches (corresponding to a part of the β1 sheet) and functional properties of the members of the DIR-e subfamily remains to be investigated. Interestingly, another common feature of the subfamily is the absence of one of the key functional residues, corresponding to Arg144 in AtDIR6. The residue is located at the N-terminus of β6 and lines the pocket A, where it is thought to act as one of the potential proton donors facilitating cyclization of bisQM by AtDIR6 (Figs 2, 4). In all AtDIRs, the residue corresponding to Arg144 in AtDIR10/ESB1 is conserved, but it is replaced by serine in all members of the multidomain DIR-e subfamily (Supplementary Fig. S3).

Interestingly, AtDIR22 is the only AtDIR protein predicted not to contain a signal peptide for extracellular secretion, suggesting that this protein could be involved in the intracellular monolignol coupling and/or another role. Remarkably, a metabolic pathway involving intracellular coupling of the monolignol radical has been described recently (Dima *et al.*, 2015).

A comparison of the chromosome map (Supplementary Fig. S4) with the phylogenetic tree shows that some *AtDIR* genes are adjacent to each other on their respective chromosomes and also very similar at the sequence level (e.g. *AtDIR12*, *13*, and *14*; *AtDIR2*, *AtDIR3*, and *OVA2*). However, some of the *AtDIR* genes locating to the same region on the chromosome are rather distantly related based on the phylogenetic tree (e.g. *AtDIR4* and *AtDIR23*; *AtDIR7* and *AtDIR22*). On the other hand, some of the almost identical *AtDIR* genes are positioned far from each other on the chromosome map (e.g. *AtDIR16* and *AtDIR18*; *AtDIR9* and *AtDIR24*; *AtDIR10*

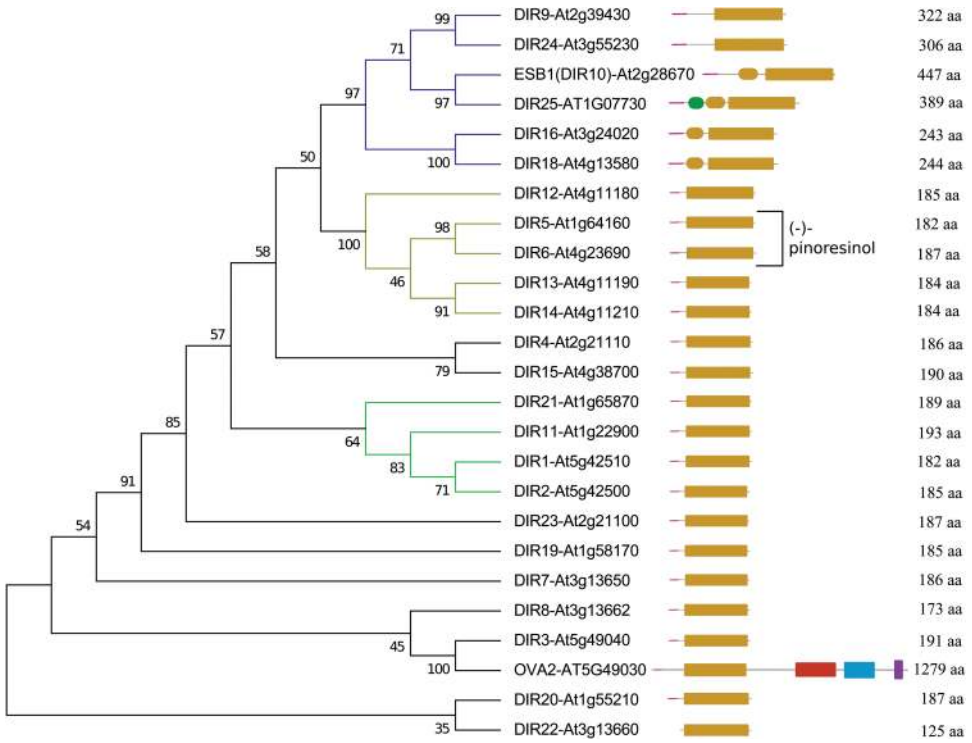

Fig. 5. Phylogenetic analysis of AtDIRs. The cluster analysis is based on the alignment of amino acid sequences of DIR domains of all 26 members of the AtDIR family. Three subfamilies identified with high reliability are highlighted (DIR-a, yellow; DIR-d, green; DIR-e, blue). DIR domains were extracted from the cdd database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The alignment of amino acid sequences was conducted in MEGA5 with the MUSCLE algorithm and visualized with the UGENE toolkit (Okonechnikov *et al.*, 2012). The evolutionary relationships were inferred using the Maximum Parsimony (MP) method. The percentages of replicate trees in which the associated sequences clustered together in the bootstrap test (percentage of 500 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was created using the Tree-Bisection-Regrafting (TBR) algorithm (Nei and Kumar, 2000). Phylogenetic analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

and *AtDIR25*). This implies the existence of several independent gene duplication events during the *AtDIR* family evolution and should be studied in more detail.

Identification of (+/–)-pinoresinol-forming AtDIRs

In order to identify AtDIRs potentially involved in stereoselective lignan biosynthesis, the DIR domains from DIR proteins known to mediate stereoselective pinoresinol formation from other species were added to the phylogenetic analysis. These include (+)-pinoresinol-forming FiDIR1 from *F. intermedia* (Davin *et al.*, 1997), TpDIR2 and TpDIR7 from *Thuja plicata* (Kim *et al.*, 2002), ScDIR1 from *Schizandra chinensis* (Kim *et al.*, 2012), PsDRr206 from *Pisum sativum* (Seneviratne *et al.*, 2015), and LuDIR1 from *Linum usitatissimum* (Dalisay *et al.*, 2015). The (–)-pinoresinol-forming DIR proteins LuDIR5 and LuDIR6 from *L. usitatissimum* (Dalisay *et al.*, 2015) were also included.

The resulting phylogenetic tree (Supplementary Fig. S5) clearly splits into four major subclades. Subclade I comprises the (+)-pinoresinol-forming DIR proteins FiDIR1, TpDIR2, TpDIR7, ScDIR1, and PsDRr206. Subclade II comprises the (–)-pinoresinol-forming DIR proteins LuDIR5, LuDIR6, AtDIR5, and AtDIR6, and additionally AtDIRs 12, 13, and 14, whose role in stereoselective pinoresinol formation is uncertain (see further in the text). Subclade III consists of the multidomian DIRs, whereas the remaining sequences belong to subclade IV.

Several amino acids are differentially conserved in (+)- and (–)-pinoresinol-forming DIR proteins from different species, indicating the functional importance of those amino acid residues for stereoselective pinoresinol formation (Kim *et al.*, 2015; Gasper *et al.*, 2016). Using this information, we found that only AtDIR5 and AtDIR6 contain all residues conserved in (–)-pinoresinol-forming DIRs (Supplementary Fig. S3). Remarkably, none of the conserved amino acid residues associated with (+)-pinoresinol formation activity is present in any AtDIR protein.

In conclusion, these data indicate that AtDIR5 and AtDIR6 might be the only AtDIRs involved in (–)-pinoresinol formation, while the (+)-pinoresinol-forming DIRs seem to be absent in Arabidopsis. These findings are in agreement with published experimental evidence showing that both AtDIR5 and AtDIR6 were able to produce (–)-pinoresinol, both in vitro and in vivo. In contrast, AtDIR13, in spite of being a member of the same subclade (DIR-a subfamily, Fig. 5), was not able to catalyze (–)-pinoresinol formation (Kim *et al.*, 2012), further highlighting the functional importance of conserved residues (Kim *et al.*, 2012; Gasper *et al.*, 2016).

Transcriptional regulation of AtDIRs

In order to analyze the transcriptional regulation of *AtDIR* genes in different tissues and in response to hormones and stress conditions, Genevestigator (Hruz *et al.*, 2008) and eFP browser (Winter *et al.*, 2007) were used to analyze the

available transcriptome microarray and RNAseq data sets (summarized in Supplementary Table S1 and Fig. 6). Most of the *AtDIR* genes reveal the highest expression levels in the roots (*AtDIR1*, *2*, *5*, *6*, *7*, *9*, *10*, *13*, *14*, *16*, *18*, *19*, *20*, *23*, *24*, and *25*), whereas only a few show the highest expression levels in other organs (*AtDIR3* and *AtDIR12* in the seed, *AtDIR7* in the hypocotyl, *AtDIR20* in the inflorescence and flower, *AtDIR8* and *AtDIR20* in the flower or pollen, and *AtDIR22* in the anther and stamen). *AtDIR4*, *AtDIR11*, and *AtDIR15* exhibited rather low expression levels in all organs. A more detailed comparison of the *AtDIR* expression levels in different root cell types is shown in Supplementary Fig. S6.

To investigate the transcriptional responses of *AtDIR* genes to hormone treatments and different types of stress, microarray-based expression profiling data sets from AtGenExpress were analyzed again using Genevestigator (fold-change >2, *P*-value <0.05). Certain *AtDIR* genes responded to ABA (*AtDIR19* and *23*), methyl jasmonate (*AtDIR5* and *13*), and *t*-zeatin (*AtDIR13*). Different *AtDIR* genes responded to biotic stress-related treatments (*AtDIR6*, *7*, *11*, *20*, and *21*), namely following treatments with the organisms *Pseudomonas syringae* (*AtDIR6*, *7*, *11*, *20*, and *21*), *Pseudomonas infestans* (*AtDIR20*, and *21*), *Golovinomyces orontii* treatment (*AtDIR20*), or the elicitors GST-necrosis-inducing phytophthora protein 1 (*AtDIR7*, *11*, and *20*), flagellin fragment 22 (*AtDIR 7*), and hairpin (hrpZ) (*AtDIR 11* and *20*). In parallel, the data showed that *AtDIR* genes also change their expression levels in response to osmotic stress (*AtDIR1*, *2*, *5*, and *21*), salt stress (*AtDIR5*, *7*, and *9*), drought (*AtDIR5*), wounding (*AtDIR5* and *7*), oxidative stress (*AtDIR5*), and heat stress (*AtDIR7* and *19*).

To expand our knowledge regarding the molecular mechanisms responsible for transcriptional regulation of individual *AtDIR* family members, we performed an *in silico* transcription factor binding assay. A total of 345 DNA-binding motif matrices of transcription factors from the TRANSFAC database were used for a calculation of binding affinities to each of the 25 *AtDIR* gene promoters (see Roider *et al.*, 2007; Manke *et al.*, 2008). Out of 345 motifs analyzed, 281 (81%) were found to have a significant affinity (*P*-value <0.05) for at least one of the *AtDIR* promoters. The results of this analysis (clustered according to the transcription factor binding profiles of individual *AtDIR* genes) are summarized in the heat map provided in Supplementary Fig. S7. All detected significant affinities are provided in Supplementary Table 2.

Since the composition of the TRANSFAC database used for this analysis might be biased due to over-representation of transcription factors associated with certain biological functions, or transcription factor families comprising transcription factors with very similar DNA-binding motif matrices, the results have to be considered critically. This is exemplified by ETHYLENE RESPONSE FACTORS (ERFs), which show a high degree of conservation with respect to their recognized binding sites. This leads to a possibly artificially enhanced number of transcription factors being implicated with certain *AtDIR* promoters carrying a particular binding site (data not shown). Bearing in mind these considerations, a comparative analysis of transcriptome data (Supplementary Table S1) with predicted transcription factor–promoter interactions (Supplementary Fig. S7; Supplementary Table S2) yields the following observations.

AtDIR3 is expressed in the seed

The expression of the *AtDIR3* gene seems to be seed specific and the motif matrices of the transcription factors ABI3 (At3g24650) and FUS3 (At3g26790) have significant affinities for the *AtDIR3* promoter. Interestingly these transcription

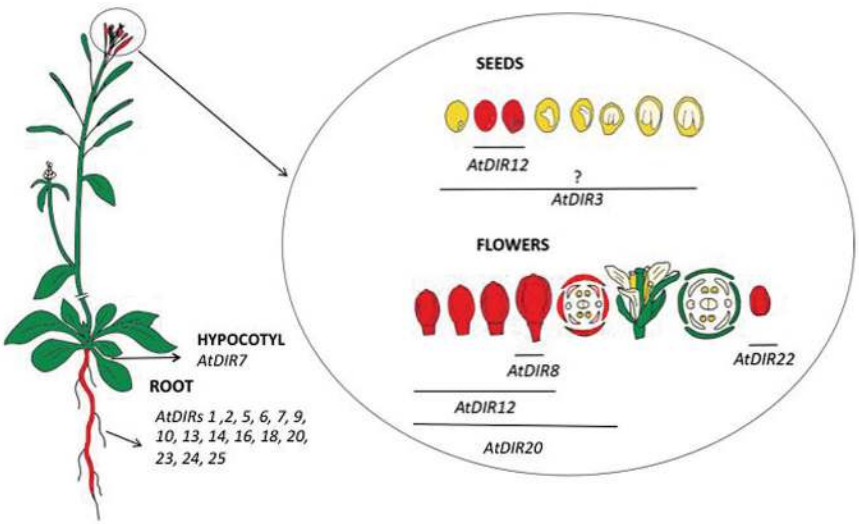

Fig. 6. Expression pattern of *AtDIR* genes. Schematic representation of the highest expression values of individual AtDIRs as determined by the eFP browser tool (Winter *et al.*, 2007) and Genevestigator (Hruz *et al.* 2008). Most of the *AtDIR* genes show the highest expression levels in the roots. However, a few *AtDIR* genes show the highest expression levels in flowers (floral organs) or seeds. The picture does not reflect the developmental stage at which the expression occurs (especially in the case of the root), but is used only for visualization purposes. For the cell-specific expression in the root, see Supplementary Fig. S6. For those *AtDIR* genes which are not represented on Affymetrix microarray chips, RNAseq data from the Genevestigator database were analyzed. According to these data, *AtDIR3* shows the highest expression levels in seeds, but the exact time point of expression during seed development is unclear, as indicated by the question mark.

factors have been shown independently to regulate seed-specific gene expression (Mönke *et al.*, 2004).

AtDIR5 responds to stress and methyl jasmonate

AtDIR5 gene expression might respond to methyl jasmonate and a wide range of stresses such as drought, salt stress, and wounding. Motif matrices of the transcription factors MYC2, MYC3, and MYC4 exhibit significant affinities for the *AtDIR5* promoter. These transcription factors have been demonstrated previously to mediate methyl jasmonate responses and are induced by drought, salt stress, and wounding (reviewed in Kazan and Manners, 2013).

AtDIR6 associates with secondary cell wall formation

AtDIR6 has been predicted to be co-expressed with *PrR1*, the pinoresinol reductase-encoding gene (Kim *et al.*, 2012). Previous work showed that *PrR1* expression is under transcriptional control of NST3 and MYB46, the master regulators of secondary cell wall formation (Zhao *et al.*, 2015). We found that the motif matrices of MYB46 and additionally MYB52 exhibit significant affinities for the *AtDIR6* promoter, and both transcription factors are involved in regulation of secondary cell wall formation (Zhong *et al.*, 2008).

AtDIR13 responds to cytokinins

Expression of the *AtDIR13* gene changes in response to zeatin treatment (Taniguchi *et al.*, 2007). Interestingly, the motif matrices of ARR10 (our analysis) and ARR1 (Taniguchi *et al.*, 2007), type-B ARRs, and members of the cytokinin signaling pathway (Hwang *et al.*, 2012) exhibit significant affinities for the *AtDIR13* promoter.

Concluding remarks and future outlook

Originally identified as responsible for the regio- and stereoselectivity of phenoxy radical coupling reactions, DIR proteins could be important regulators of plant development and plant stress response. Today, the AtDIR protein family is still uncharacterized. Clearly, AtDIR5 and AtDIR6 are involved in the stereoselective radical–radical coupling leading to (–)-pinoresinol. Unfortunately, the role of these genes in Arabidopsis development is not known. In contrast to that, the role of AtDIR10/ESB1 in CS formation is indisputable. The inability of AtDIR10/ESB1 to mediate regio- and stereoselectivity of coniferyl alcohol coupling mediated by its close homologs AtDIR5 and AtDIR6 suggests that very small changes in amino acid sequence may cause a loss of stereoselectivity. Alternatively, AtDIR10/ESB1 could have the ability to bind phenoxy monolignol radicals, which might be necessary for the role of AtDIR10/EBS1 in the initiation of lignification. The role of AtDIR10/ESB1 in targeting CASPs for precise positioning of the entire CS-forming machinery implies its ability to recognize specific plasma membrane domains pre-determined to guide lignin impregnations of the adjacent cell wall. Whether the additional N-terminal sequence of AtDIR10/ESB1 contains sequence motifs responsible for this and what the nature of the molecular targets of the interaction is remains to be determined. Our preliminary bioinformatics analysis implies that the other members of the Arabidopsis DIR-e subclade reveal (i) similarity at the level of the DIR domain (missing the β1 sheet); (ii) replacement of the conserved arginine by serine at the N-terminus of β6; and (iii) most of them contain an additional N-terminally located, incomplete DIR domain. Whether these protein structural characteristics are essential for the role in the lignification is still to be determined.

Based on the published evidence and our *in silico* transcription factor binding assay, it seems that the Gene Ontology (GO) term best characterizing AtDIRs is the stress response. However, transcription factor binding based on the TRANSFAC database (with over-representation of transcription factors related to development) is only a basic bioinformatics approach, and further analyses should be done. Nonetheless, the ability of lignans to mediate pathogen resistance and stress-induced expression of numerous *DIR* genes in various plant species seems to be in line with that hypothesis. The fact that several of the *AtDIR* genes are proposed to be responsive to plant hormones is in agreement with an important role for hormonal regulation in the mediation of adaptation to stress. The evidence that *DIR* genes could be a target of cell wall integrity signaling (largely under hormonal control) also fits the concept. However, experimental approaches have to be put into action to confirm these speculations and deepen our knowledge of the possible role of hormonal regulation of DIR expression.

The high homology in protein sequence of individual AtDIRs and the similar expression patterns imply possible functional redundancy in the frame of the family, potentially hampering functional characterization. Thus, taking advantage of using up to date molecular biology approaches, such as amiRNA-based knock-down of multiple AtDIRs, might represent a suitable strategy.

We hope we have convinced the readers that AtDIRs constitute an essential and fascinating protein family, enabling plants to adapt to dynamically changing environmental conditions. Based on the available evidence, it seems that the primary role of DIRs occurs at the level of control over cell wall metabolism and/or production of antibacterial compounds. That determines DIRs as a potent target in a number of biotechnological applications as powerful tools in improving plant stress resistance or production of pharmaceutically interesting compounds.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Monolignol production via phenylpropanoid pathway.

Fig. S2. Comparison of AtDIR6 and PsDRR206.

Fig. S3. Multiple alignment of amino acid residues of AtDIR proteins.

Fig. S4. Chromosome map showing the positions of *AtDIR* genes.

Fig. S5. Identifying stereospecific pinoresinol-forming *AtDIR* genes.

Fig. S6. Root cell type-specific expression of *AtDIR* genes.

Fig. S7. Heatmap of *P*-values of transcription factor binding affinities.

Table S1. Transcriptional regulation and proposed roles of *AtDIR* genes from *Arabidopsis thaliana*.

Table S2. TRANSFAC analysis of DNA binding motif affinities.

Acknowledgements

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