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Pinoresinol reductase 1 impacts lignin distribution during secondary cell wall biosynthesis in Arabidopsis

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ABSTRACT

Pinoresinol reductase (PrR) catalyzes the conversion of the lignan (−)-pinoresinol to (−)-lariciresinol in *Arabidopsis thaliana*, where it is encoded by two genes, *PrR1* and *PrR2*, that appear to act redundantly. *PrR1* is highly expressed in lignified inflorescence stem tissue, whereas *PrR2* expression is barely detectable in stems. Co-expression analysis has indicated that *PrR1* is co-expressed with many characterized genes involved in secondary cell wall biosynthesis, whereas *PrR2* expression clusters with a different set of genes. The promoter of the *PrR1* gene is regulated by the secondary cell wall related transcription factors SND1 and MYB46. The loss-of-function mutant of *PrR1* shows, in addition to elevated levels of pinoresinol, significantly decreased lignin content and a slightly altered lignin structure with lower abundance of cinnamyl alcohol end groups. Stimulated Raman scattering (SRS) microscopy analysis indicated that the lignin content of the *prr1-1* loss-of-function mutant is similar to that of wild-type plants in xylem cells, which exhibit a normal phenotype, but is reduced in the fiber cells. Together, these data suggest an association of the lignan biosynthetic enzyme encoded by *PrR1* with secondary cell wall biosynthesis in fiber cells.

1. Introduction

Lignans are dimers derived from the stereospecific oxidative coupling of hydroxycinnamyl alcohols. The initial step of lignan dimerization is mediated by an oxidase (e.g. laccase) acting in concert with a dirigent protein that confers enantio-selectivity to the free radical-derived coupling (Davin et al., 1997). Approximately 3000 different lignans are widely distributed in the plant kingdom (Schmidt et al., 2010). The numerous beneficial effects of lignans on human health, via estrogenic and anticancer activities, are well documented (Dixon, 2004; McCann et al., 2005). However, the exact roles of lignans *in planta* remain elusive although it has been hypothesized that they are involved in plant defense (Naoumkina et al., 2010).

The most characterized lignans are the group with 9(9′)-oxygen linkages (Umezawa, 2003). This class of lignans arises from the enantio-selective dimerization of two coniferyl alcohol units (**1**) to give rise to pinoresinol (**2**). Pinoresinol is then sequentially reduced to lariciresinol (**3**) and secoisolariciresinol (**4**) by a bi-functional pinoresinol/lariciresinol reductase (PLR) (Nakatsubo et al., 2008; Umezawa, 2003). Because of its stereo-selectivity, PLR is suggested to have enantiomeric control on the lignan biosynthetic pathway even though the dirigent protein is considered to be the asymmetric inducer (von Heimendahl et al., 2005). Conventional PLRs can utilize both pinoresinol and lariciresinol as substrates (i.e. are bifunctional) (Hano et al., 2006; von Heimendahl et al., 2005). However, in *Arabidopsis thaliana*, the two pinoresinol reductase enzymes show only weak or no activity toward lariciresinol, and are therefore named PrR1 and PrR2 rather than PLR (Nakatsubo et al., 2008).

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PLR genes have organ-specific expression patterns. In flax (*Linum usitatissimum*), genes encoding two PLRs with different enantiospecificity have been cloned. Both Lu*PLR1* and Lu*PLR2* are expressed in flax seed tissues, whereas only Lu*PLR2* is expressed in stem and leaf tissues (Hemmati et al., 2010). In Arabidopsis, both PrR1 and PrR2 catalyze pinoresinol reduction in a redundant manner in root tissue, where lignans are mostly accumulated, but only PrR1 is active in stem tissue (Nakatsubo et al., 2008).

The basic unit of pinoresinol, coniferyl alcohol, is also shared by the lignin biosynthesis pathway. Lignin is the second most abundant biopolymer on earth, and a major component of plant secondary cell walls. It is derived from three major subunit precursors; so-called H (*p*-coumaryl alcohol), G (coniferyl alcohol) and S (syringyl alcohol) monolignols (Bonawitz and Chapple, 2010; Zhao and Dixon, 2011). Even though coniferyl alcohol is the common unit of both lignin and lignans, there is no evidence that lignans *per se* are components of the plant wall structure. It is also not clear how plants allocate coniferyl alcohol for the biosynthesis of lignans versus lignin.

Recently, it has been shown in both flax and pine that the lignan-related gene *PLR* is up-regulated along with cell wall biosynthetic genes in highly lignifying stem tissues or during compression wood formation (Huis et al., 2012; Villalobos et al., 2012). Furthermore, a comparison of co-expressed gene networks with primary and secondary wall cellulose synthases in a variety of different plant species identified gene families that are consistently co-regulated with cellulose biosynthesis; among these genes was *PrR1* (Ruprecht et al., 2011). Because Arabidopsis PrR1 is only expressed highly in mature stem tissue, we have further investigated the potential role of this gene in cell wall biosynthesis. Our results confirm that *PrR1* is co-expressed with secondary cell wall biosynthetic genes, indicate directly that *PrR1* is regulated by regulators of secondary cell wall formation, and show that loss of function of *PrR1* results not only in changes in lignin levels, but also in alterations in lignin structure and tissue-specific lignin distribution.

2. Results

2.1. Differential expression of PrR1 and PrR2 in Arabidopsis

The different gene expression patterns of *PrR1* and *PrR2* were confirmed by analysis of microarray expression data from the Arabidopsis eFP Browser; *PrR1* transcripts are expressed in most tissues, with highest level in the lignified second internodes (Fig. S1), whereas *PrR2* transcript levels are high in root tissues, where most lignan accumulates, with almost no transcripts detectable in the 2nd internode (Fig. S2).

To investigate genes which are co-expressed with *PrR1* and *PrR2*, we examined the recently built cell wall co-expression database (http://csbl.bmb.uga.edu/publications/materials/shanwang/CWRPdb/index.html; Wang et al., 2012). A bi-clustering analysis of Arabidopsis microarray data with a focus on cell wall-related genes was applied to build co-expressed gene modules. In each module graph, the node is shown as a yellow diamond (known/annotated cell wall-related genes), an aquamarine square (known/annotated transcription factor genes) or a red circle (other genes); an edge connecting two nodes means that these two genes are co-expressed (Fig. 1). Similar co-expression graphs are also available in the ATTED-II database (http://atted.jp/data/locus/At4g13660.shtml), which uses different methods for clustering microarray data and calling co-expression modules.

Based on this analysis, *PrR1* is co-expressed with many characterized cell wall-related genes represented by yellow diamonds and aquamarine squares (Fig. 1). For instance, *LAC17* is a laccase that has been directly implicated in lignin biosynthesis (Berthet et al., 2011); cellulose synthase-like A9 (*CSLA9*) is a beta-mannan

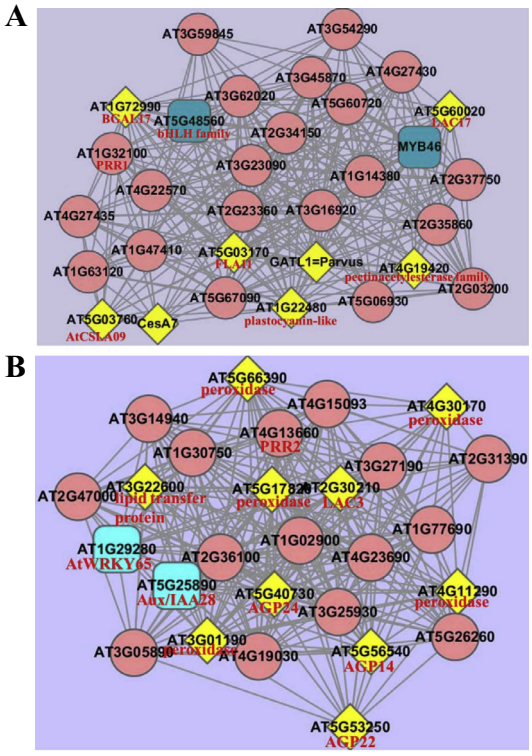

Fig. 1. Gene co-expression networks for Arabidopsis PrR1 and PrR2. (A) PrR1 is co-expressed with many known secondary cell wall biosynthetic genes. (B) PrR2 is co-expressed with an entirely different set of genes.

synthase involved in hemicellulose biosynthesis (Davis et al., 2010); cellulose synthase A7(*CesA7*) is involved in cellulose synthesis and *MYB46* is a transcriptional master switch of secondary cell wall formation (Zhong et al., 2007). These data confirm the co-expression of *PrR1* with *CesA* genes as previously reported (Ruprecht et al., 2011). In contrast, *PrR2* clusters with a totally different set of genes, including a laccase that has not been implicated in lignin polymerization, one peroxidase that has been implicated in lignin biosynthesis (Fig. 1; Table S1) and a protein associated with casparian strip formation, a process which involves peroxidase-mediated lignification in specific cell types in the root (Lee et al., 2013).

2.2. PrR1 is regulated by the secondary cell wall transcription factors SND1 and MYB46

To investigate whether *PrR1* is under the control of secondary wall transcription factors, we used an Arabidopsis leaf protoplast-based promoter *trans*-activation system. Protoplasts were transfected with a construct in which firefly luciferase (reporter) is driven by the promoter consisting of a 1 kb DNA fragment upstream of the start codon of *PrR1*. SND1 and MYB46 were chosen as potential trans-activators for this assay since both are known to be master switches of the entire secondary cell wall biosynthetic program (Zhong et al., 2006, 2007). Co-expression of cauliflower mosaic virus (CaMV) 35S promoter driven SND1 or MYB46 activated the expression of the firefly luciferase reporter gene driven by the *PrR1* promoter by around 30-fold (Fig. 2).

2.3. Loss of function of PrR1 affects both lignan and lignin biosynthesis

The *prr1-1* knock-out mutant SALK_058467 was obtained from the ABRC at Ohio State University (http://abrc.osu.edu/) (Nakatsubo et al., 2008). This line contains a T-DNA insertion