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NOTE

Flavonoids and lignins: pathway elucidation and modification for improved biomass properties

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Introduction

Flavonoids and lignins are two major classes of plant secondary metabolites widespread across plant kingdom. They are not only essential for plant growth and physiology, but also important for human activities. Understanding the occurrences, structures and biosynthetic mechanisms of flavonoids and lignins therefore could facilitate our knowledge of plant development and evolution, and also biotechnology approaches to improve plant growth performance as well as biomass-orientated applications.

Flavonoids confer plant fertility, resistance towards biotic and abiotic stresses and pigmentation in flowers and fruits. They are also an important part of human diet due to their antioxidant, anti-cancer and anti-inflammatory properties. In grasses, the predominant flavonoids in vegetative tissues are tricin *O*-conjugates and flavone *C*-glycosides, while anther additionally contains flavonols [1]. The biosynthesis of flavonoid skeleton from *p*-coumaroyl-CoA in the phenylpropanoid pathway requires two highly conserved enzymes, chalcone synthase (CHS) and chalcone isomerase (CHI) (Figure 1). However, the downstream enzymes, in particular those involved in tricin biosynthesis, remained largely uncharacterized.

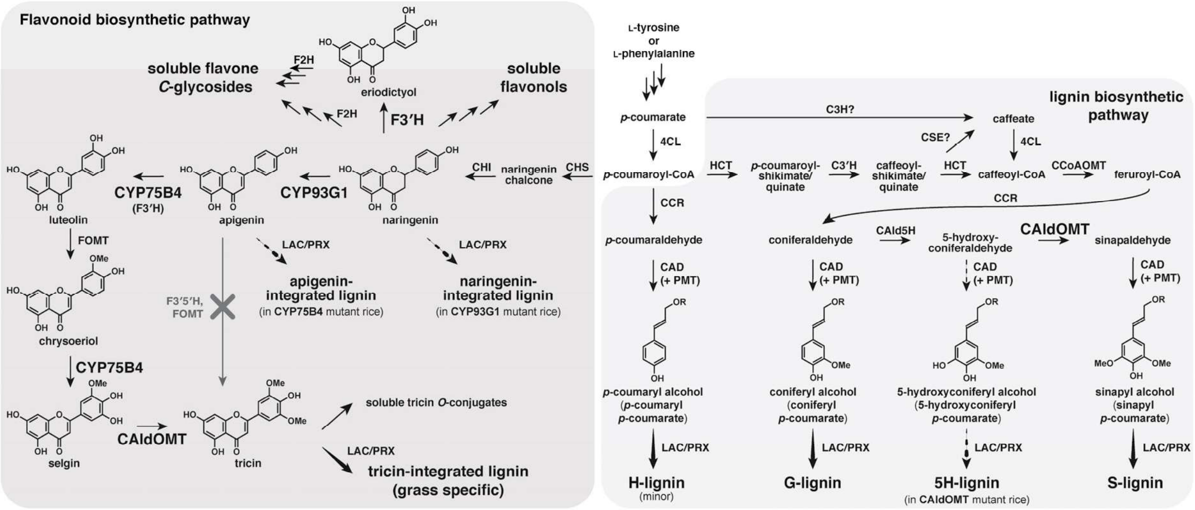

Figure 1. Flavonoid and lignin biosynthetic pathway in rice. Grey arrow: previously proposed tricin biosynthetic pathway.

On the other hand, lignins, which mainly deposit in the secondary cell walls of vascular plants, provide plant mechanical support, conductivity of water and nutrients and resistance towards pathogen attack. Lignins are considered a major component of plant biomass invaluable for the sustainable production of biofuels and biochemicals. However, lignins have long been viewed as a major recalcitrance for chemical pulping and production of fermentable sugars for polysaccharide-derived biofuels and biochemicals. Therefore, modification of lignin content and composition by bioengineering to improve plant biomass utilization properties has become a major research focus [2]. In general, lignins are generated in plant cell walls by polymerization of monolignols, i.e. coniferyl alcohols, sinapyl alcohols and small amount of *p*-coumaryl alcohols, which respectively generate guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units of lignins (Figure 1). With *p*-coumaroyl-CoA as a substrate, monolignols were generated after undergoing aromatic hydroxylations, *O*-methylations as well as side chain reductions (Figure 1). In grasses, a portion of the monolignols are acylated by *p*-coumarate (Figure 1) [3]. Interestingly, it was discovered that flavonoid tricin is also a canonical lignin monomer in grasses [4,5]. However, not much is known about the physiological functions and biosynthesis of such tricin-integrated lignins (tricin lignins). It is also intriguing to know whether manipulating tricin lignins could improve biomass utilization properties of grasses.

Here, the author's recent research activities related to the biosynthesis and bioengineering of flavonoids and lignins are summarized [6-12]. My research focus was particularly directed to elucidation of the tricin biosynthetic pathway, as well as analysis of tricin-depleted rice mutants with emphasis on their cell wall properties and biomass utilization properties. Other flavonoid- and lignin-related studies which the author conducted with many collaborators are also presented here.

Cytochrome P450 (CYP) enzymes involved in tricin biosynthesis

Tricin biosynthetic pathway in rice was elucidated by the identification of two grass-specific CYP enzymes. The first step of tricin biosynthesis involves the formation of flavone skeleton. Several enzymes in rice have been shown to harbor such *in vitro* activities, but our results suggested that CYP93G1 is the sole flavone synthase II (FNSII) enzyme catalyzing this reaction step, leading to the formation of soluble and lignin-bound tricin (Figure 1) [7,8].

It was long believed that the hydroxylation steps in tricin biosynthesis are catalyzed by flavonoid 3′,5′-hydroxylase (F3′5′H), an enzyme that catalyzes the same reaction steps with other classes of flavonoids as substrates [15]. However, in contrast to this general belief, we have identified CYP75B4 as a novel enzyme that function as apigenin 3′-hydroxylase/chrysoeriol 5′-hydroxylase (A3′H/C5′H) catalyzing hydroxylations specifically in tricin biosynthetic pathway [9,10]. The identification of CYP75B4 as an A3′H/C5′H has reconstructed the tricin biosynthetic pathway with chrysoeriol instead of tricetin as the intermediate (the previously proposed pathway that utilizes F3′5′H instead of A3′H/C5′H is indicated in grey in Figure 1) [9,10]. On the other hand, we have also found out that the canonical flavonoid 3′-hydroxylase (F3′H) does not play a significant role in hydroxylation step in tricin biosynthesis, but it is the predominant hydroxylase involved in flavone *C*-glycoside biosynthesis [10]. Collectively, our work suggested that grasses have recruited two closely related hydroxylases for two parallel pathways leading to the formation of tricin type metabolites and flavone *C*-glycoside [10].

Improvement of biomass utilization properties by manipulating tricin biosynthesis

Tricin was discovered as a canonical lignin monomer in grasses but not much is known about its *in planta* functions and how it affects biomass utilization properties. To address these issues, we have carried out in-depth cell wall analysis using rice mutants deficient in CYP93G1 or CYP75B4. Both CYP93G1 and CYP75B4 mutants generated altered lignins depleted in tricin and incorporated with novel flavonoids, naringenin and apigenin, respectively (Figure 1) [8,10]. Biomass digestibility was improved in both CYP93G1 and CYP75B4 mutants compared with wild-type controls without any growth penalty, suggesting molecular manipulation of these tricin biosynthetic genes could increase the production of glucose from rice biomass for further biorefinery applications [8,10].

Bifunctional enzyme involved in both tricin and monolignol biosynthesis

5-hydroxyconiferaldehyde *O*-methyltransferase (CAldOMT) is well known for its function in the biosynthesis of S lignins (Figure 1) [16]. We have further shown that CAldOMT is also involved in the biosynthesis of soluble and lignin-bound tricin (Figure 1) [9,11]. Rice CAldOMT mutant was depleted in tricin lignins and S lignins, and produced atypical 5-hydroxyguaiacyl (5H) lignins [11]. Together with the results of *in vitro* enzymatic assays of recombinant CAldOMT, our results suggested that CAldOMT is a bifunctional enzyme catalyzing the key methylation steps in the two parallel pathways leading to the formations of S lignins and tricin-type metabolites including tricin lignins (Figure 1) [11].

Tricin biosynthesis in *Medicago* species

Although tricin is ubiquitously accumulated in grass species, it is only accumulated in isolated and unrelated dicot lineages including *Medicago* species. The tricin biosynthetic pathway in *Medicago* species was explored by the identification of novel *Medicago*-specific flavonoid B-ring hydroxylases which harbor similar *in vitro* and *in planta* A3′H/C5′H activities as rice CYP75B4 (a study led by Dr. Clive Lo's research group of the University of Hong Kong, Hong Kong, China) [12]. This finding also suggests that *Medicago* species have evolved and recruited another set of enzymes for tricin biosynthesis independent from those in grass species [12].

Involvement of flavonoids in fertility in rice

Flavonoids, more specifically those belong to the flavonoid class flavonols (Figure 1), were reported to be essential for fertility in some but not all plant species. We have found out that flavonoids were also essential for fertility in rice; rice chalcone synthase (CHS; entry enzyme for flavonoid biosynthesis as indicated in Figure 1) mutant which was completely depleted in the accumulation of all kinds of flavonoids was sterile [1]. However, in contrast to the essential roles primarily played by flavonols for fertility in other plant species, our finding suggested that a combination of different classes of flavonoids (flavonols, flavones and flavone *C*-glycosides) confer fertility in rice [1].

Improve *Populus* biomass properties by regulating lignin biosynthesis in fibre cells

Wood biomass is widely used for generating fuels, timber products, chemicals and papers. A new strategy to improve *Populus* biomass properties without any growth penalty was developed by fibre-specific suppression of lignification through down-regulating lignin-biosynthesis related transcription factor 1 (LTF1) (a study led by Prof. Laigeng Li's research group of the Shanghai Institutes for Biological Sciences, China) [13]. The cell-type specific suppression of lignification employed in this study also revealed that fibre cells are more enriched in S lignins, whereas vessel cells are more enriched in G lignins [13].

Lignin-inspired modification of nanocellulose surface properties

A new strategy to improve surface properties of nanocellulose was proposed as inspired by lignification in plant cell walls (a study led by Prof. Takuya Kitaoka's research group of Kyushu University, Japan) [14]. Using pickering emulsion system, lignin was uniformly polymerized on the surface of nanocellulose, modulating the hydrophilicity of nanocellulose [14]. In addition, the presence of nanocellulose at oil-water interface in the pickering emulsion system also influenced the structures of the synthetic lignins generated [14]. This work has provided new insight on modifications of surface properties of nanocellulose which could be useful for industrial applications.

Conclusions

In the author's recent research activities, flavonoid biosynthetic pathway, especially tricin biosynthetic pathway, in rice was elucidated. These works also suggested that manipulating tricin lignins in rice could improve biomass utilization properties that could be beneficial in cellulose-oriented biorefinery applications. The author's studies were also extended to explore tricin biosynthesis in non-grass species, functions of flavonoids in fertility in rice as well as other lignin-related research including improving biomass utilization properties of *Populus* and modifying surface properties of nanocellulose by *in vitro* lignification. In RISH, Kyoto University, the author is currently conducting studies aiming to bioengineer grass biomass plants by introducing new flavonoid and lignin features for further improving their biorefinery applications.

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