Journal Pre-proofs

Short Communication

Insights into contrastive cellulose nanofibrils assembly and nanocrystals catalysis from dual regulations of plant cell walls

Yuanhang Ai, Hailang Wang, Peng Liu, Hua Yu, Mengdan Sun, Ran Zhang, Jingfeng Tang, Yanting Wang, Shengqiu Feng, Liangcai Peng

PII: S2095-9273(24)00409-2
DOI: https://doi.org/10.1016/j.scib.2024.06.013
Reference: SCIB 2816

To appear in: *Science Bulletin*

Please cite this article as: Y. Ai, H. Wang, P. Liu, H. Yu, M. Sun, R. Zhang, J. Tang, Y. Wang, S. Feng, L. Peng, Insights into contrastive cellulose nanofibrils assembly and nanocrystals catalysis from dual regulations of plant cell walls, *Science Bulletin* (2024), doi: https://doi.org/10.1016/j.scib.2024.06.013

Insights into contrastive cellulose nanofibrils assembly and nanocrystals catalysis from dual regulations of plant cell walls

Yuanhang Ai[a,b,1], Hailang Wang[a,b,1], Peng Liu[a,b], Hua Yu[a,b], Mengdan Sun[b], Ran Zhang[b], Jingfeng Tang[a], Yanting Wang[a,b]*, Shengqiu Feng[b]*, Liangcai Peng[a,b]*

[a]Key Laboratory of Fermentation Engineering (Ministry of Education), National "111" Center for Cellular Regulation & Molecular Pharmaceutics, Cooperative Innovation Center of Industrial Fermentation (Ministry of Education & Hubei Province), Hubei Key Laboratory of Industrial Microbiology, School of Life & Health Sciences, Hubei University of Technology, Wuhan 430068, China.
[b]College of Plant Science & Technology, Huazhong Agricultural University, Wuhan 430070, China.

[1] Equal contributor.

*Correspondent author; Tel: +86-27-87281765; Fax: +86-27-87280016.
Email: lpeng@mail.hzau.edu.cn (L.P, lead contact), wyt@mail.hzau.edu.cn (Y.W), fengsq@mail.hzau.edu.cn(S.F);

Plant cell walls are the most abundant sources of biomass for biofuels and bioproducts [1]. In general, there are two major types of plant cell walls: a pectin-rich primary cell wall that surrounds growing cells and a lignin-rich secondary cell wall that provides structural support to mature cells [2]. In particular, cellulose is the most characteristic component of plant cell walls, and plays a central role in plant morphogenesis and biomass production [3]. In higher plants, cellulose is synthesized by cellulose synthase (CESAs) complexes, requiring a large CesA family for its synthesis [4]. Among primary wall *AtCesAs*, only the overexpression of *AtCesA6*-like genes can accelerate primary wall synthesis and enhance secondary wall deposition for high cellulose production in *Arabidopsis* [5]. Conversely, the overproduction of secondary wall *CesAs* leads to various defects in plant growth and development in transgenic plants [6]. Although β-1,4-glucan chains are deposited as crystalline cellulose microfibrils, the characteristic assembly of these microfibrils in genetically engineered cell walls remains elusive [7].

Given that cellulose microfibrils are increasingly applied to generate diverse cellulose nanofibers (CNFs) and nanocrystals (CNCs) as enzyme-digestible and

chemical-convertible substrates, we have recently developed atomic force microscopy (AFM) technology to observe the assembly of length-reduced cellulose nanofibrils *in situ* and the production of dimension-reduced CNCs *in vitro* in a natural rice OsCESA9-site-mutant and site-mutants of three OsCESA4, 7, and 9 isoforms essential for secondary wall synthase complexes [8-10]. Although the CNCs are of great interest owing to their unique physical and chemical properties, specific biodigestibility and broad applications, achieving desirable CNCs by integrating genetically modified CNFs with green-efficient and cost-effective preparation is challenging [11-13].

In this study, we selected two types of transgenic rice lines overexpressing the rice *OsCesA5* and *Arabidopsis AtCesA7* genes, which are respectively involved in cellulose biosynthesis for primary and secondary wall formation (Fig. 1a). Using our previously established genetic method, we identified four independent homozygous transgenic rice lines, designated AtA7, AtA7-2, OsA5, and OsA5-2 (Figs. S1 and S2 online). In two-year field experiments, we did not observe any evident defects in plant growth and development in the transgenic rice lines relative to the wild type (WT). However, we detected contrasting extension forces in the AtA7/AtA7-2 and OsA5/OsA5-2 lines (Fig. S3 online). Since plant cell walls determine their mechanical strength [10], the AtA7 and OsA5 lines also showed contrasting alterations in cellulose and non-cellulosic polysaccharide levels with similar lignin contents (Fig. S4 online). Additionally, we found that the AtA7 line contained significantly higher soluble sugars, while the OsA5 line had reduced ones at $P < 0.01$ (Table S1 online), consistent with our previous assumption that soluble sugars accumulation is derived from altered cellulose biosynthesis and carbon partitioning in transgenic plants [14]. Furthermore, this study determined either a similar pentose composition of hemicellulose or three similar monolignol constitutions of lignin among the transgenic lines and WT, suggesting that the biosynthesis of hemicellulose or lignin was not affected in the transgenic lines (Tables S2 and S3 online).

As a consequence, we observed contrasting primary and secondary wall deposition between the AtA7 and OsA5 lines by measuring the average width of the cell walls (Fig. 1b). The AtA7 line had relatively narrower primary and secondary walls than the WT, whereas the OsA5 had wider walls, supporting their distinctively altered wall-polymer compositions (Fig. S4 online). We also calculated the width proportion

between the primary and secondary walls, and the AtA7 and OsA5 lines remained a contrast relative to the WT (Fig. S5 online), indicating that the primary walls should be more altered than those of the secondary walls in both transgenic lines examined. In addition, we identified a T-DNA insertion *Oscesa5* mutant and examined its major phenotypes in contrast to the OsA5 line, including plant height, wall polymer composition, and cell wall width, providing dual evidence for OsCesA5 regulation in cellulose biosynthesis (Fig. S6 online).

Using a well-established AFM approach [8, 9], we observed CNFs assembly in transgenic lines and measured the average distances between two amorphous/non-crystalline/defective chains on the surfaces of cellulose microfibrils from 40 randomly selected samples (Fig. 1c). Since CNFs lengths are accounting for their assembly, we calculated that the AtA7 line had shorter nanofibrils (98 nm), while OsA5 had significantly longer nanofibrils (296 nm) compared to the WT (236 nm) (Fig. 1d). SOAX analysis revealed a disordered CNFs orientation in the AtA7 line attributed to shorter nanofibril assembly (Fig. S7 online), whereas the OsA5 exhibited a normal orientation with major angles at 90° and 150°, probably due to its longer nanofibrils. Such distinct CNFs assembly was also observed between the AtA7-2 and OsA5-2 lines (Fig. S8 online), confirming the genetic stability of *AtCesA7* and *OsCesA5* overexpression in transgenic rice lines. Because cellulose features are accountable for CNFs assembly [8, 9], this study detected significantly reduced cellulose crystalline index (CrI) and degree of polymerization (DP) values by 15%-20% in the three types of AtA7 substrates. In contrast, the OsA5 line had significantly increased CrI and DP values by 6%-13% (Fig. S9 online). Therefore, these data consistently indicate a contrasting CNFs assembly between the AtA7/ AtA7-2 and OsA5/ OsA5-2 transgenic lines.

Since CNFs assembly can determine CNCs production [8, 9, 13], we attempted to generate desirable CNCs using acid catalysis with crude cellulose substrates of transgenic lines. While sulfuric acid (H_2SO_4) is commonly used to generate CNCs, we initially co-supplied green-like acetic acid to reduce the H_2SO_4 dosage in four proportions (Table S4 online). Both AtA7 and OsA5 lines produced CNCs of varying sizes from across all mixed-acid treatments (Fig. S10 online), consistent with their distinct CNFs assemblies. We then used RSM-based modeling to optimize mixed-acid

treatment for maximum CNC yield, resulting in a significant regression model with a high coefficient of determination (R^2) of 0.953 at $P < 0.01$ (Tables S5-S8 online). Three-dimensional surface modeling identified optimal conditions for mixed-acid treatment (Fig. S11 online). Under the optimal 1.4-mixed-acid conditions, the AtA7 line consistently produced lower-yield CNCs, while OsA5 generated higher-yield ones, reflecting their contrasting CNCs production. Even with traditional 64% H_2SO_4 treatments, the AtA7 and OsA5 lines maintained contrasting CNCs yields, though these were significantly lower compared to the optimal 1.4-mixed-acid treatments up to 4-fold. By randomly measuring total 100 CNCs samples, this study found that the AtA7 line was of significantly length-reduced CNCs than those of the WT, whereas the OsA5 line had longer ones, in agreement with their contrastive sizes of CNCs examined (Fig. 1e, f and Fig. S12 online). Transmission electron microscopy (TEM) observation confirmed the distinct CNC lengths between AtA7/AtA7-2 and OsA5/OsA5-2 lines (Fig. S13, online). Unexpectedly, the CNCs diameters/widths were individually varied under AFM and TEM observations of all samples examined, suggesting an intricate acid-catalysis for CNCs width-assembly. Compared to previously reported CNCs, however, the AtA7 line produced either the second-shortest or smallest CNCs with less energy input (Table S9 online), providing an option for desirable CNCs production using a green-like mixed-acid process or simple H_2SO_4 treatment.

Furthermore, we conducted thermogravimetric analysis (TGA) to compare the CNCs from AtA7 and OsA5 lines, revealing distinct TGA and DTG curves at specific temperatures (Fig. S14 and Table S10, online). Fourier transform infrared spectroscopy identified a new peak at 1729 cm^{-1} in the CNCs from the optimal 1.4-mixed-acids treatment, indicating the presence of a C=O group from the esterification of cellulose with acetic acid (Fig. S15 online). AtA7 and OsA5 also exhibited contrasting degrees of acetylation and sulfonation, as shown by their $C_{acetoxyl}$ values. Additionally, the CrI values of the CNCs from AtA7 and OsA5 lines were distinct, aligning with other observed physical and chemical differences (Table S11 online).

To understand the differences in CNFs assembly between AtA7 and OsA5 lines, we performed real-time PCR analyses on 14-day-old rice seedlings to measure the transcript levels of *OsCesA4, 7,* and *9* (Fig. S16 online). Compared to the WT, the AtA7 line had significantly reduced transcript levels of *OsCesA7* and *OsCesA9* by 2.3- and 2.7-fold, whereas the OsA5 line showed increased transcript levels for *OsCesA4, A7, A9* genes by 3.3-, 3- and 2.7-fold, correlating with the observed differences in secondary wall formation and cellulose levels. These changes in transcription are assumed to affect the activities of the OsCESA4, 7, and 9 complexes in cellulose biosynthesis [5, 10, 15]. As mutations and knockout lines of *OsCESA4, 7,* and *9* have resulted in inhibited cellulose biosynthesis accounting for increased amorphous regions with extra broken chains [8, 9], those data could explain the length-reduced CNFs assembly in the AtA7 line. We also analyzed the transcript levels of *OsCesA1, 3, 5,* and *8,* which are key isoforms for cellulose biosynthesis in primary cell walls [15]. The AtA7 line showed similar expression levels of these genes compared to the WT, suggesting that the smaller primary walls in AtA7 should be due to reduced OsCESAs activity rather than gene transcription level. In contrast, the OsA5 line showed significantly increased transcript levels of *OsCesA1* and *OsCesA5,* leading to enhanced primary wall formation. Because the raised primary walls enable the deposition of more secondary walls with stronger mechanical strength [5], it is assumed that enhanced secondary wall deposition may play a feedback role in the spatiotemporal control of cellulose microfibril twisting and bending during plant cell wall deposition in the OsA5 line. This also explains why the OsA5 line exhibited cellulose microfibrils with less amorphous regions for length-raised CNFs assembly.

Based on our data and previous findings, we finally propose a mechanism model to elucidate how AtA7 and OsA5 lines develop distinct plant cell walls *in vivo,* leading to contrasting CNCs production *in vitro* (Fig. 2 and Fig. S17 online). In the AtA7 line, cellulose biosynthesis in primary walls is relatively inhibited, while in the OsA5 line, it is significantly enhanced. The reduced primary wall dimensions in AtA7 fail to

provide the mechanical strength and flexibility required for secondary wall deposition. This is due to the downregulation of *OsCesA7* and *OsCesA9*, which represses cellulose biosynthesis in secondary walls, resulting in high-density amorphous regions and broken inner-chains that lead to shorter CNFs assembly and altered cellulose microfibril orientation, as seen in rice site mutants and knockout-lines of *OsCESA4*, *7*, and *9* isoforms [8, 9]. Conversely, although the dimension-raised primary walls could consequently enhance secondary wall deposition in transgenic *Arabidopsis* [5], this study demonstrated for the first time that the OsA5 line has a longer CNFs assembly owing to fewer amorphous chains occurring with cellulose microfibrils. Importantly, this study detected distinct cellulose CrI and DP values between the AtA7 and OsA5 lines, further demonstrating their distinct CNFs assembly in plant cell walls.

As the presence of raised amorphous and broken inner-chains in cellulose microfibrils is a major factor in the acid catalysis of shorter CNCs in *OsCESA4*, *7*, and *9* site mutants and knockout lines [8, 9], this study provides evidence that these amorphous regions act as initial breakpoints responsible for the contrasting CNCs production between AtA7 and OsA5 lines. Furthermore, the inner-broken chains likely contribute to the reduced CNC diameter in the AtA7 line. By examining other critical CNC properties, such as CrI, TGA, and yield under two acid treatments, we further demonstrated the contrasting CNCs conversions between the AtA7 and OsA5 lines. This extends the options for producing CNCs with specific desired properties.

In conclusion, our hypothetical model elucidates how *CesA* engineering regulates primary wall synthesis, thereby controlling secondary wall formation, leading to contrasting CNFs assembly *in vivo* and CNCs production *in vitro* in AtA7 and OsA5 lines. This provides novel insights into cellulose biosynthesis and cell wall construction for high-yield biomass and high-quality bioproduction.

Figure captions

Fig. 1. Cell wall, nanofibrils and CNCs features in transgenic AtA7 and OsA5 lines and Wild type. (**a**) Mature transgenic rice lines and wild type (WT/*Nipponbare*), bar as 25 cm. (**b**) Primary and secondary cell walls (down) of leaf vein at young seedlings under TEM, primary cell wall/PCW, secondary cell wall/SCW, bar as 0.5 μm; The width ratio between PCW and SCW of 30 samples. (**c**) AFM observation of cellulose microfibrils; Dotted lines as representative cellulose microfibrils for measuring the distance between two amorphous cellulose chains on the surfaces of microfibrils, bar as 200 nm. **(d)** The average distance (nm) between two amorphous chains of randomly-selected 40 samples (n = 40) accounting for average length of cellulose nanofibrils, data as mean ± SD (n = 40). (**e**) AFM observations of CNCs, bar as 200 nm. (**f**) Measurements of randomly-selected 100 CNCs samples including average lengths and heights; a, b, c as multiple t-test at $P < 0.05$.

Fig. 2. A mechanism model about how *AtCesA7* and *OsCesA5* overexpression distinctively regulates primary walls syntheses to control secondary wall formations for contrastive CNFs assembly *in vivo* and CNCs production *in vitro* in the transgenic AtA7 and OsA5 lines compared to the WT. The CNCs length measured by the average distance between two amorphous regions; The amorphous regions characterized as initial breakpoints for facilitating acidic catalysis of cellulose microfibrils into CNCs; The red and black H^+ highlighted for initiating acidic catalysis from the amorphous region and inner-broken chain, respectively.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (32170268, 32101701), Hubei Provincial Natural Science Foundation of China for Excellent Young Scientists (2024AFA100), National 111 Project of Ministry of Education of China (BP0820035, D17009), the Initiative Grant of Hubei University of Technology for High-level Talents (GCC20230001).

Author contributions

Yuanhang Ai: methodology, investigation, visualization, writing-original draft; Hailang Wang: methodology, investigation, visualization; Peng Liu, Hua Yu, Mengdan Sun, Ran Zhang: methodology, software, funding acquisition; Jingfeng Tang: project administration, funding acquisition; Yanting Wang, Shengqiu Feng: supervision, methodology, funding acquisition; Liangcai Peng: conceptualization, supervision, methodology, writing–review and editing, project administration, funding acquisition.

Appendix A. Supplementary materials

Supplementary materials to this article can be found online at https://

References

[1] Wang M, Wang Y, Liu J, et al. Integration of advanced biotechnology for green carbon. Green Carbon 2024; DOI: 10.1016/j.greenca.2024.02.006.

[2] Vermerris W, Abril A. Enhancing cellulose utilization for fuels and chemicals by genetic modification of plant cell wall architecture. Curr Opin Biotechnol 2015; 32:104-12.

[3] Desprez T, Juraniec M, Crowell E F, et al. Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 2007; 104:15572-7.

[4] Arioli T, Peng L, Betzner A S, et al. Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science 1998; 279:717-20.

[5] Hu H, Zhang R, Feng S, et al. Three AtCesA6-like members enhance biomass production by distinctively promoting cell growth in *Arabidopsis*. Plant Biotechnol J 2018; 16:976-88.

[6] Joshi C P, Thammannagowda S, Fujino T, et al. Perturbation of wood cellulose synthesis causes pleiotropic effects in transgenic aspen. Mol Plant 2011; 4:331-45.

[7] Hu H, Zhang R, Tao Z, et al. Cellulose synthase mutants distinctively affect cell growth and cell wall integrity for plant biomass production in *Arabidopsis*. Plant Cell Physiol 2018; 59:1144-57.

[8] Zhang R, Hu Z, Wang Y, et al. Single-molecular insights into the breakpoint of cellulose nanofibers assembly during saccharification. Nat Commun 2023; 14:1100.

[9] Zhang R, Hu Z, Peng H, et al. High density cellulose nanofibril assembly leads to upgraded enzymatic and chemical catalysis of fermentable sugars, cellulose nanocrystals and cellulase production by precisely engineering cellulose synthase complexes. Green Chem 2023; 25:1096-106.

[10] Li F, Xie G, Huang J, et al. OsCESA9 conserved-site mutation leads to largely enhanced plant lodging resistance and biomass enzymatic saccharification by reducing cellulose DP and crystallinity in rice. Plant Biotechnol J 2017; 15:1093-104.

[11] Habibi Y, Lucia L A, Rojas O J. Cellulose nanocrystals: chemistry, self-assembly, and applications. Chem Rev 2010; 110:3479-500.

[12] Lu P, Hsieh Y-L. Preparation and properties of cellulose nanocrystals: Rods, spheres, and network. Carbohydr Polym 2010; 82:329-36.

[13] Vanderfleet O M, Cranston E D. Production routes to tailor the performance of cellulose nanocrystals. Nat Rev Mater 2020; 6:124-44.

[14] Wu L, Feng S, Deng J, et al. Altered carbon assimilation and cellulose accessibility to maximize bioethanol yield under low-cost biomass processing in corn brittle stalk. Green Chem 2019; 21:4388-99.

[15] Wang L, Guo K, Li Y, et al. Expression profiling and integrative analysis of the CESA/CSL superfamily in rice. BMC Plant Biol 2010; 10:282.

Yuanhang Ai received his Ph.D. from Huazhong Agricultural University in 2022, and currently he is a postdoctoral fellow at Huazhong University of Science and Technology working on microorganism biotechnology and biomass engineering.

Hailang Wang is a Ph.D. candidate at Huazhong Agricultural University and Hubei University of Technology working on

genetic modification of plant cell walls in bioenergy crops.

Yanting Wang is a Professor at Hubei University of Technology, and she is currently working on plant cell wall biotechnology and biomass biochemistry for biofuels and bioproducts in bioenergy crops.

Shengqiu Feng is an Associate Professor of Huazhong Agricultural University, and she is currently working on the metabolism regulation of secondary metabolites in medicine crops.

Liangcai Peng received his Ph.D. in Biochemistry and Molecular Biology from Australian National University in 1998, and currently he is a distinguished Professor at Hubei University of Technology. His group research includes plant cellulose biosynthesis, cell wall structure and function,

bioenergy crop breeding and microorganism engineering, biomass green chemistry and nanotechnology for biofuels and nanomaterials.