A solid-state nanopore-based single-molecule approach for label-free characterization of plant polysaccharides

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ABSTRACT

Polysaccharides are important biomacromolecules existing in all plants, most of which are integrated into a fibrillar structure called the cell wall. In the absence of an effective methodology for polysaccharide analysis that arises from compositional heterogeneity and structural flexibility, our knowledge of cell wall architecture and function is greatly constrained. Here, we develop a single-molecule approach for identifying plant polysaccharides with acetylated modification levels. We designed a solid-state nanopore sensor supported by a free-standing SiNx membrane in fluidic cells. This device was able to detect cell wall polysaccharide xylans at concentrations as low as 5 ng/μL and discriminate xylans with hyperacetylated and unacetylated modifications. We further demonstrated the capability of this method in distinguishing arabinoxylan and glucuronoxylan in monocot and dicot plants. Combining the data for categorizing polysaccharide mixtures, our study establishes a single-molecule platform for polysaccharide analysis, opening a new avenue for understanding cell wall structures, and expanding polysaccharide applications.

Key words: solid-state nanopore, single-molecule, polysaccharides, acetylation, ion current

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INTRODUCTION

Saccharides are one of the four life-building biomacromolecules that exist in all living beings, and they have attracted widespread interest due to their biological importance such as in determining organism development and influencing adaptation to various environments (Scheller and Ulvskov, 2010). The commercial value of saccharides in organic matter, pharmaceutical production, and energy use further reflects their importance in various applications (Scheller and Ulvskov, 2010; Lakshminarayanan et al., 2018). Saccharides are generally categorized as monosaccharides, oligosaccharides, polysaccharides, and glycoconjugates, and they possess anomers, regioisomers, and epimers. In contrast to DNA and proteins that are synthesized in a template-directed manner, the synthesis of polysaccharides is not template-dependent. All these factors result in heterogeneity and flexibility in sugar composition and structure. Considering the diversities in the configuration, crosslinking, and anomeric stereochemistry of saccharides, unraveling their properties remains a great challenge (Yang et al., 2019).

Combinatorial mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) techniques are widespread methods applied to probe cumulative characteristics from a collection of saccharide analytes. For instance, ion mobility MS is one of the main techniques for isomeric species discrimination, scanning tunneling microscopy is used for imaging of single...
Ramsay and Bayley, 2018; Ma et al., 2019). Protein nanopores single-molecule approaches have been developed to identify carbohydrate isomers (Im et al., 2016; Fennouri et al., 2018; Ramsay and Bayley, 2018; Ma et al., 2019). Protein nanopores have been used to determine oligosaccharide length and to investigate the kinetics of enzyme-mediated polysaccharide degradation (Fennouri et al., 2012, 2013). As the size of solid-state nanopores (SSNs) can be tailored for passage of single molecules by different approaches, such as ion or electron beam milling, chemical etching, and multilevel pulse dielectric breakdown (Siwy, 2009; Kwok et al., 2014; Deng et al., 2015), SSNs work as an updated nanoscale technique with improved flexibility. As the analytes translocate through the insulated membrane, which contains a size-matched aperture under a certain external voltage, the transient resistive pulse and the blockage duration resulting from the physical occupation of the nanopore are detected, providing important information on the analytes with regard to their geometric size and surface charge at the single-molecule level. Due to its high spatial and temporal resolution and label-free features, the SSN platform has been applied in studies of polysaccharide determination, e.g., for hyaluronan and heparin. These findings offer an exciting view for glycomics studies and provide specific information for individual molecules in heterogeneous samples, which is not possible with the currently available analytical methods (Belser et al., 2018; Cressiot et al., 2019; Im et al., 2019). Although these nanopore-based approaches have been applied to detect mono-/oligosaccharides and acidic glycans, a method for exploring intact plant polysaccharides that enrich neutral sugars is still unavailable.

The plant cell wall, the building of which requires the consumption of more than 70% of the CO\textsubscript{2} assimilative products in plants, is a rigid and dynamic network composed of polysaccharides, aromatic substances, and glycoproteins, and it represents the most abundant renewable natural resource on earth. In different plant species, from 50% to more than 90% of cell wall components are polysaccharides (Scheller and Ulvskov, 2010). The cell wall, as a characteristic structure of plant cells, plays many fundamental roles in plant growth and development, including providing mechanical support, facilitating cell adhesion, determining cell-to-cell communication, and mediating defense responses (McNeil et al., 1984; Carpita and Gibeaut, 1993). The plant cell wall is also critical for human and animal nutrition, energy consumption, and industrial production, e.g., textile and paper production. The cell wall is comprised of a complicated polysaccharide pool with great structural diversity. At least 14 sugars are arranged into linear chains via more than four linkages; these chains can be substituted by distinct sugars and modified by functional groups, such as acetyl and feruloyl groups, which influence the physicochemical and rheological properties of polysaccharides (Scheller and Ulvskov, 2010). Cell wall polysaccharides with diverse substitution and modification profiles are further assembled into functional architectures by folding and crosslinking with each other (Grantham et al., 2017). For example, xylan binds to cellulose via a 2-fold conformer and interacts with lignin via a 3-fold conformer (Kang et al., 2019). Gas chromatography–liquid chromatography (GC-LC)–MS is a mainstream technology used to characterize monosaccharide and oligosaccharide profiles (Lerouxel et al., 2002; Pettolino et al., 2012), while NMR provides information on polysaccharide polymorphisms, dynamics, and intermolecular packing (Kang et al., 2019; Zhao et al., 2020), which have helped us to understand cell wall networks. In addition to sugar composition, further studies have revealed that the geometric information of polysaccharides due to their diversities in hydrodynamic volumes and surface charges is also important for the assembly of the functional architecture of polysaccharides (Kang et al., 2019; Zhang et al., 2019). However, the currently available analytical platforms cannot provide this information. Due to this intractability, the development of a nanoscale method that can determine the molecular structure of plant polysaccharides is of great importance.

In this study, we exploited an SSN sensor for the identification of cell wall polysaccharides by using xylans, a major type of hemicellulose in rice (Oryza sativa L.). This platform revealed high sensitivity and advantages over the conventional methods in discriminating xylans with differences in acetylation modifications, substitutions, and hydrodynamic volumes. Hence, our data provide information at single-molecule and modification levels, thereby bridging the gap in the analytical methodology of intact polysaccharide molecules and offering an opportunity to better understand the intricate structure of the plant cell wall.

RESULTS

Construction of a solid-state nanopore sensor for xylan analysis

To explore whether SSNs can be used in plant polysaccharide analysis, an SiN membrane that carries a nanoscale orifice was embedded in a flow cell to assemble a nanopore sensor. The principle of nanopore sensing is that in the chambers that are separated by an insulated membrane containing a nanoscale aperture, the analyte in buffer solution is injected and forced to translocate through the pore by applied bias. The physical occupation of the pore produces a transient resistive pulse, and the amplitude of the ion current change and dwell time are closely correlated to the geometric size and surface charge properties of the analytes (Figure 1A). As nanopore sensors are used to discern molecules in a buffered solution, and xylan, the second most abundant polysaccharide next to the insoluble component, cellulose, in plant cell walls, can be dissolved in aqueous medium (Scheller and Ulvskov, 2010), we selected xylan as the representative plant polysaccharide to develop the nanopore analytical technology. The intact native xylans were extracted from the rice cultivar Nipponbare (NP), and their compositions were confirmed by GC-MS analysis (Supplemental Table 1). The molecular skeleton of xylans from NP is displayed in Figure 1D. Because lithium ions have been confirmed to improve the capture rate and to cause little ionic current noise (Kowalczyk et al., 2012; Hu et al., 2019; Yan et al., 2019), lithium chloride (LiCl) was loaded as an electrolyte for nanopore translocation measurements. To evaluate the translocation behavior of xylans, ion currents were recorded as sample molecules passed through the nanopore embedded in the buffer-filled flow cell. The translocation events...
were observed at a voltage of 150 mV, while xylan was loaded at a concentration of 5 ng/μL (Figure 1C). Most event spikes exhibited a typical sharp current amplitude peak, representing straightforward translocation of one analyte molecule (Supplemental Figure 1A). A few xylan molecules that displayed slightly wide current peaks likely indicated interactions between analytes and the inner surface of the nanopore (Supplemental Figure 1B). The events with rather long blockage durations were probably caused by adsorption at the nanopore wall (Supplemental Figure 1C), and the events caused by bumping were filtered and not subjected to data analysis. Furthermore, a much larger number of rice xylan molecules passed through the cis-chamber supplied with positive bias voltages than that supplied with negative voltages (Figure 1C), suggesting that the surface of rice native xylans adsorbed some anions in the electrolyte solution and, therefore, exhibited negative charges in the test buffer. We also performed zeta potential tests to verify that the rice native xylans could hold negative potential (Supplemental Figure 1D).

To optimize the operating parameters, we applied voltages ranging from 50 to 250 mV. Informative data were collected as the voltages were set from 100 to 200 mV (Supplemental Figure 1F–1H and Supplemental Table 2): few translocation events were recorded at 50 mV, and the detection baselines became unstable at 250 mV. We also compared the effects of pore sizes ranging from 3 to 6 nm in diameter (Figure 1B). More translocation events were recorded using 3–4.6-nm nanopores than nanopores at other sizes, which corresponded to the hydrodynamic diameter of xylan in achieving a high signal-to-noise ratio (Armstrong et al., 2004). Therefore, the nanopores used in the following assays were 3–4.6 nm in diameter, and the results were further verified by current recordings (Figure 1B). In addition, to minimize the possible interference derived from the pore size variations, the recorded current blockage amplitude \( I_b \) was normalized by dividing this value by the open pore current \( I_0 \). All the following examinations were performed in the same buffer, which contained 6 M LiCl, 10 mM Tris, and 1 mM EDTA (pH 8.0), as reported previously (Rivas et al., 2018). A translocation test was also performed (data not shown) in 1 M KCl and 1 M LiCl, but few events were captured. Using optimal parameters, more than 1000 translocation events could be recorded per minute, suggesting that this methodology is applicable for the measurement of plant polysaccharide molecules, in this case, native rice xylans.

**Discrimination of xylans with varied acetyl modifications**

Plant xylans often incorporate acetyl esters, which give rise to unique acetylation profiles that facilitate xylan folding and

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**Figure 1. Solid-state nanopore setup for plant polysaccharide xylan detection.**

(A) Schematic graph showing nanopore setup for plant polysaccharide detection. The red dashed arrow is the force direction and single xylan molecule translocation event.

(B) The representative open pore currents generated in the experiments. The insert at the top left corner shows the transmission electron micrograph of a 4-nm nanopore. Scale bar, 2 nm.

(C) Representative raw nanopore current traces and events at the indicated voltages.

(D) Molecular formula of polysaccharide samples. The region between R1 and R2 is composed of repeating units that represent the extension of repeating units at the reducing and nonreducing ends. The red parts represent the acetyl group.
determine cell wall architecture, thereby affecting plant growth and development (Gille and Pauly, 2012; Grantham et al., 2017). Brittle leaf sheath1 (bs1) is our previously reported rice mutant that bears hyperacetylated xylan due to a defect in deacetylase (Zhang et al., 2017). To investigate whether the single-molecule-based nanopore approach can discriminate xylans with altered acetylation profiles, the native xylans prepared from NP (the wild-type plants) and bs1 (the mutants) were examined using SSNs. Before examination, an acetate content assay and heteronuclear single quantum coherence (HSQC) analysis were performed to confirm that the xylan molecules extracted from bs1 were excessively acetylated compared with those isolated from NP (Figure 2A–2C). Furthermore, the distinction in the size distribution of bs1 xylans and wild-type xylans was revealed by gel permeation chromatography (Supplemental Figure 2). Consequently, at a voltage of 150 mV, bs1 xylans showed greatly reduced current blockage compared with wild-type NP xylans, although the dwell time distribution was similar (Figure 2D–2F, Supplemental Figure 3 and Supplemental Table 2). In addition, the translocation frequency (f = number of spike events per second) was significantly decreased from 20 events/s for the wild-type to 4 events/s for bs1 (Figure 2E, Supplemental Figure 3B and Supplemental Table 2). Each dot presented in the scatterplot indicates the translocation performance of one xylan molecule based on the varied blockage and dwell time (Figure 2F). These findings suggest that the distinct acetyl groups on the xylan backbone result in their distinct nanopore translocation behaviors. Furthermore, a series of external voltages ranging from 100 to 200 mV were supplied. The blockage amplitude increased as the voltage increased, while the normalized current blockage remained unchanged, and there were clear differences in the values of bs1 and NP due to their distinct molecular structures and nanopore properties; however, the dwell time was decreased for both samples due to faster translocation under higher electrophoretic force with increased bias voltages.
The event charge deficit (ECD) values (the sum of ECD for NP was $3.98 \times 10^6$, while that for bs1 was $7.20 \times 10^5$) and distribution (Supplemental Figure 3C) also illustrated the lower blockage of bs1 than NP, as demonstrated in Figure 2 (Fologea et al., 2005; Rivas et al., 2018). Hence, xylans with two types of acetylation patterns exhibited different translocation performances. SSNs have application potential for distinguishing plant xylans with different acetylation profiles.

**Comparison between acetylated and deacetylated xylans**

As our knowledge of xylan properties is largely derived from deacetylated xylans (Wu et al., 2015; Li et al., 2013), we next compared the translocation behaviors between acetylated and deacetylated xylans, the structures of which are displayed in Figure 1D. Acetyl group-free xylan was prepared using sodium hydroxide and then subjected to SSN assays. Although the translocation behaviors of acetylated and deacetylated wild-type xylan were similar based on the partially overlapped scatterplot (Figure 3A and 3B), the distribution patterns of NP deacetylated xylan (NP-ac) analytes were more concentrated (Figure 3B and Supplemental Figure 4A). Equally important, the current blockage and dwell time of bs1 deacetylated xylan (bs1-ac) were nicely separated from those of bs1 native xylans (Figure 3C), and the results were largely identical to wild-type deacetylated xylan (Figure 3D), suggesting that acetylation is one of the determinants that impact translocation behaviors of xylan molecules. The similar translocation behavior between bs1-ac and NP-ac xylan confirms our previous finding that the bs1 gene mutation does not significantly affect the xylan substitution pattern (Zhang et al., 2017).

After supplying varied external voltages, the values of both current blockage and dwell time for bs1-ac and NP-ac xylans were found to be higher than those of the corresponding acetylated molecules (Figures 3E and 3F and Supplemental Table 2), and their differences in size distribution are displayed in Supplemental Figure 8. The translocation frequency for bs1-ac xylans was consistently lower than that for NP-ac, while the external voltages changed from 100 to 200 mV (Supplemental Figure 4B). Therefore, the SSNs demonstrated the advantage of being able to identify xylans with or without acetylation.

**Recognition of xylans from distinct cell wall types**

The xylan extracted from rice plants was arabinoxylan (AX), which contains neutral sugar arabinose substitutions, while the xylan derived from dicots was glucuronoxylan (GX), which harbors acidic sugar glucuronic acids as side chains (Figure 4A). These compounds represent distinctive polymers that distinguish type I (dicots) and type II (monocots) plant cell walls (Scheller and.
To determine whether acidic side chains affect the translocation behavior of xylans, commercially available 4-O-methyl-D-glucurono-D-xylan (meGX) was subjected to an SSN system at an external voltage from 100 to 200 mV. We observed translocation events of meGX when a negative bias voltage was applied, which was contrary to the rice xylans examined above (Figures 1C and 4B). The surface of the negatively charged meGX may be neutralized by adsorbed counterions during the measurements. Moreover, the dwell time for the translocation of meGX was increased compared with that of rice AX, probably due to the opposite motion of electroosmotic flow, which slowed the xylan translocation rate (Figure 4B and 4D and Supplemental Figure 5 and Supplemental Table 2). These divergences suggested that the two types of xylan molecules possess distinct surface charge characteristics, which can be discriminated by SSNs. Therefore, SSNs were able to recognize AX and GX, the most important hemicellulose of the two plant cell wall types.

**DISCUSSION**

Single-molecule nanopores have received much attention due to their many advantages such as being label-free, having a single-molecular resolution, and possessing quantifiability. After being successfully applied in DNA and protein characterization (Kowalczyk et al., 2012; He et al., 2018; Hu et al., 2019; Tian et al., 2019; Yan et al., 2019), nanopores have been used to identify structures with more flexible saccharides (Bacri et al., 2011; Im et al., 2016, 2019; Fennouri et al., 2018; Karawdeniya et al., 2018). However, their application in plant polysaccharide characterization has not yet been reported. Plant polysaccharides exist ubiquitously in nature with vast biological significance and commercial importance, but the analytical issues derived from their structural and physicochemical complexity present a long-term challenge. In this study, using major cell wall hemicellulose xylans as experimental samples, we used SSNs as a tool to discriminate individual plant polysaccharide molecules with specific modifications.

Xylan is an important hemicellulose that interacts with cellulose, lignin, and other cell wall polymers to form a load-bearing network of plant cell walls (Scheller and Ulvskov, 2010; Zhang et al., 2019). Xylans produced in different tissues and plant species and at various developmental stages show great structural diversity, and SSNs are an established technique for the discrimination of biomacromolecules with structural...
heterogeneity. Xylans vary in backbone length, side-chain substitutions, and acetyl modifications on the backbone and side chains, which influence their physicochemical and rheological properties, consequently determining cell wall architecture and functions (Scheller and Ulvskov, 2010; Gille and Pauly, 2012; Zhang et al., 2019). Here, we applied native xylan prepared from wild-type rice NP internodes to an SiNmembrane with nanopores of ~3 nm in diameter. The optimal nanopore sizes (3–4.6 nm diameter) and range of bias voltage (100–200 mV) were established after a series of preliminary experiments. The platform was able to automatically record full-set translocation data in a few minutes with xylan concentrations as low as ~5 ng/μL. Based on the changes in current blockage, dwell time, and event frequency, this platform could be used to discriminate excessively acetylated xylans that were extracted from a rice bs1 mutant and unacetylated xylans that were prepared by treating the native xylan with sodium hydroxide. The alterations in translocation behavior suggest that these molecules may undergo varied inter/intramolecular interactions. Hence, acetyl groups represent a key factor affecting xylan geometry.

In addition to the effects of acetylation, distinct substitutions on xylan side chains also affect SSN translocation performances. GX and AX, which contain distinct side-chain sugars, contribute to discrepancies between type I and type II cell walls. GX analytes were discerned from AX analytes based on their reversed voltage polarity, shorter blockage duration, and improved translocation event frequency. The capacity of the SSNs in identifying xylans with varied side-chain substitutions was further corroborated by the finding that deacetylated bs1 xylan, which harbors the wild-type-like substitution, showed largely identical translocation data to NP xylan. Therefore, this study developed an SSN platform as a tool to distinguish individual xylan molecules with diverse acetylation and substitution profiles. Saponification extracts generally include multiple noncellulosic polymers (Zhang et al., 2012). Here, we used 4N-KOH to prepare a noncellulosic polysaccharide mixture. The SSNs categorized this polysaccharide mixture into at least two groups, suggesting its potential to discriminate different plant-derived polysaccharides.

In this work, we used a polar voltage-charged solid nanopore sensor to record perforation events and to generate the current blockage and dwell time profiles of individual polysaccharide molecules. Different from conventional methods, such as NMR, which provides information on sugar units, and MS, which reveals mono- or oligosaccharide mass properties in compositional analysis (Lerouxel et al., 2002; Pettolino et al., 2012; Calabretta et al., 2019; Mallagaray et al., 2019; Sheikh et al., 2019), the SSN system tends to determine polysaccharide structure based on translocation performances. These data are correlated to polysaccharide conformation, as well as to in muro interfacial and intermolecular information. Moreover, SSNs can characterize a single-molecule analyte, which is different from the cumulative signals recorded by MS and NMR approaches. Hence, this approach offers higher-order information that is complementary to that obtained from conventional methods. We believe that, with the development of nanotechnology, SSN systems can be iteratively upgraded to a promising polysaccharide analytical technique. This study opens a gateway to unravel the elaborate nanoscale structures of cell wall polysaccharides for scientific and commercial purposes.
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Collection and analysis of data
Polysaccharide experiments were conducted with external potentials from 50 to 250 mV. The I-V characteristics and ion current blockade of the samples were recorded on a patch-clamp amplifier (Axopatch 200B) sampled at 25 kHz and low-pass filtered at 5 kHz with sensitive electronics, which were housed inside a Faraday cage. Data analysis was performed on Clampfit and Origin for graphing. All the event spikes were collected at an open pore current of 10%, which was approximately four times larger than the noise; all dwell times longer than 200 ms and normalized current blockages lower than 0.05 were excluded from the data analysis.

NMR spectroscopy analysis
The NMR characterizations of NP and bs1 xylans were performed as previously described (Zhang et al., 2017). In brief, ~10 mg of native xylan was dissolved in DMSO-d6 (99.9% D, Sigma) and analyzed using an Agilent DD2 600-MHz NMR spectrometer equipped with a gradient 5-mm 1H/13C/15N triple resonance cold probe, and the measurement time for each sample was 12 h. The spectra were acquired using the Agilent standard pulse sequence gHSQCAD to determine the one-bond 13C-1H correlation. NMR data processing and analysis were conducted using MestReNova 10.0.2 software (Mestrelab Research).

Gel permeation chromatography analysis
For analysis of the xylan molecule size, xylans (1 mg/mL) were dissolved in mixed solvent (V_DMSO:V_H2O = 9:1) and filtered before loading onto the Agilent PL-50 system equipped with a column of PLgel Olexis and detected by a differential detector. The xylan molecules were eluted with 1 mL min⁻¹ DMSO at 35°C.

GC-MS analysis
For GC-MS analysis, 2 mg of destarched alcohol insoluble residue was hydrolyzed in 2 M trifluoroacetic acid at 121°C for 90 min. The supernatants were air-dried and reduced with sodium borohydride (10 mg/mL in 1 M ammonium hydroxide). The generated alditol acetates were extracted in ethyl acetate and analyzed using an Agilent 7890 GC system equipped with a 5977A MSD (Agilent).

ACCESSION NUMBERS
The data that support the findings of this study are available from the corresponding authors upon request.

SUPPLEMENTAL INFORMATION
Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS
L.L., B.Z., Y.Z., and D.W. conceived and planned the study. Y.C. and S.W. performed nanopore detection assays. B.Z. and L.Z. prepared xylan samples. Y.C., B.Z., and L.L. analyzed the data. Y.Z., B.Z., and Y.C. wrote the article. All authors discussed the results and contributed to the final manuscript. Y.C., B.Z., and L.L. share equal first authorship.

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DECLARATION OF INTEREST
The authors declare no competing financial interests.

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