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ORIGINAL ARTICLE

Characterization of an antifungal β-1,3-glucanase from *Ficus microcarpa* latex and comparison of plant and bacterial β-1,3-glucanases for fungal cell wall β-glucan degradation

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Abstract

Main conclusion **Each β-1,3-glucanase with antifungal activity or yeast lytic activity hydrolyzes different structures of β-1,3-glucans in the fungal cell wall, respectively.**

Abstract Plants express several glycoside hydrolases that target chitin and β-glucan in fungal cell walls and inhibit pathogenic fungal infection. An antifungal β-1,3-glucanase was purified from gazyumaru (*Ficus microcarpa*) latex, designated as GlxGluA, and the corresponding gene was cloned and expressed in *Escherichia coli*. The sequence shows that GlxGluA belongs to glycoside hydrolase family 17 (GH17). To investigate how GlxGluA acts to degrade fungal cell wall β-glucan, it was compared with β-1,3-glucanase with different substrate specificities. We obtained recombinant β-1,3-glucanase (designated as CcGluA), which belongs to GH64, from the bacterium *Cellulosimicrobium cellulans*. GlxGluA inhibited the growth of the filamentous fungus *Trichoderma viride* but was unable to lyse the yeast *Saccharomyces cerevisiae*. In contrast, CcGluA lysed yeast cells but had a negligible inhibitory effect on the growth of filamentous fungi. GlxGluA degraded the cell wall of *T. viride* better than CcGluA, whereas CcGluA degraded the cell wall of *S. cerevisiae* more efficiently than GlxGluA. These results suggest that the target substrates in fungal cell walls differ between GlxGluA (GH17 class I β-1,3-glucanase) and CcGluA (GH64 β-1,3-glucanase).

Keywords Antifungal activity · *Ficus* · Fungal cell wall · GH17 · Latex · Pathogenesis-related protein

Abbreviations

CcGluA	*Cellulosimicrobium cellulans* β-1,3-Glucanase
CM-curdlan	Carboxymethyl curdlan
GlxGluA	Gazyumaru latex glucanase-A
GH	Glycoside hydrolase family
IFC	Insoluble fraction of fungal cell
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
PDA	Potato dextrose broth with agar
*p*NP	*para*-Nitrophenol

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Introduction

Fungal cell walls are important for cell morphogenesis and protection against external stresses (Yun et al. 1997; Latgé and Beauvais 2014). These cell walls contain chitin, chitosan, β-1,3-glucan, β-1,6-glucan, mixed β-1,3-/β-1,4-glucan, α-1,3-glucan, mannan, mannoprotein, and melanin as major constituents. A comparison of these cell walls shows that there is a great variability in fungal cell wall composition and organization. The *Saccharomyces cerevisiae* cell wall is composed of 1–2% chitin, 50–55% β-1,3-glucan, 10–15% β-1,6-glucan, and 10–20% mannan (mannoprotein) (Lesage and Bussey 2006). In contrast, the cell wall of filamentous fungi such as *Trichoderma viride* (*T. viride*) is composed of 12–22% chitin, about 20% β-1,3-glucan, 7–12% β-1,6-glucan and 15–20% protein (Benítez et al. 1975). These structural polysaccharides are important in maintaining the strength of fungal cell walls. Chitinases and β-1,3-glucanases could potentially inhibit fungal growth, and plants are thought to express these enzymes as defense

mechanisms against fungal pathogens. Indeed, some chitinases and β-1,3-glucanases from plants have been shown to exhibit antifungal activity (Schlumbaum et al. 1986; Mauch et al. 1988; Moravčíková et al. 2016). Although there are several reports on the relationship between the structural, biochemical, and enzymatic characteristics and antifungal activities of chitinases (Iseli et al. 1993; Suarez et al. 2001; Taira et al. 2002; Taira 2010), the corresponding information on β-1,3-glucanases is limited. To understand the role of β-1,3-glucanases in plant defense systems against fungal pathogens, it is imperative to elucidate these relationships.

Although plant β-1,3-glucanases have antifungal activity, they are also thought to play roles in plant development such as seed germination (Leubner-Metzger and Meins 2000; Branco et al. 2011), pollination, and elongation of pollen tube (Doxey et al. 2007; Wan et al. 2011). To find antifungal β-1,3-glucanase, we searched in plant latex, a known biological defense system. Plant latex, the cytosol of special cells called laticifers, is generally considered to play a protective role against herbivores and insects (Konno 2011), and we have presented evidence to suggest that latex also plays a role in the defense against fungal pathogens (Kitajima et al. 2018). In comparison with other tissues, plant latex is enriched in defense-related proteins (Chye and Cheung 1995; Konno et al. 2004). In a previous study, we found that the latex from gazyumaru (*Ficus microcarpa*), a woody flowering plant distributed in the subtropical/tropical regions of Asia, exhibited strong antifungal activity. We previously purified an antifungal chitinase from gazyumaru latex (Taira et al. 2005). Furthermore, we have confirmed the possession of a vacuolar-targeting signal peptide by the cloned gene, and substantiated the antifungal activity of the resultant recombinant protein (Takashima et al. 2021). In this study, we found that a latex β-1,3-glucanase, designated GlxGluA, exhibits antifungal activity. We successfully purified GlxGluA and cloned and expressed the corresponding gene in *Escherichia coli*. Sequence analysis showed that GlxGluA belongs to glycoside hydrolase family 17 (GH17). All β-1,3-glucanases are classified into GH16, GH17, GH55, GH64, GH81, and others in the CAZy database (http://www.cazy.org/) (Henrissat and Davies 1997) based on their amino acid sequences. Most plant β-1,3-glucanases belong to the GH17 enzyme family. Several GH17 β-1,3-glucanases have been shown to exhibit antifungal activity in vivo and in vitro (Mondal et al. 2007; Sridevi et al. 2008; Liu et al. 2009).

In this study, we compared the enzymatic characteristics, effects on fungal cell walls, and antifungal activities against filamentous fungus and yeast between GlxGluA and GH64 β-1,3-glucanase (designated as CcGluA) from *Cellulosimicrobium cellulans* (*C. cellulans*, also known as *Arthrobacter luteus*). The combination of GH64 β-1,3-glucanase and an alkaline protease from *C. cellulans* has been reported to exert strong lytic activity against living yeast cell walls to produce yeast cell protoplasts (Kitamura 1982a). Mannan protein, a cell wall component of the outer layer of yeast cells, is denatured and solubilized by heat treatment. Heat-treated yeast cells were lysed using only β-1,3-glucanase. Therefore, GH64 β-1,3-glucanase is a key enzyme involved in the degradation of yeast cell wall β-glucan. Comparative analysis of both enzymes was beneficial for understanding the substrate specificity of plant β-1,3-glucanases exhibiting antifungal activity. The results obtained in this study suggest that GlxGluA (GH17 class I β-1,3-glucanase) and CcGluA (GH64 β-1,3-glucanase) target different substrates in fungal cell walls. We further discuss the relationship between the substrate specificity of these enzymes and their antifungal and lytic activities.

Materials and methods

Materials

Gazyumaru latex and leaves were harvested from a gazyumaru tree on the University of Ryukyu campus. Carboxymethyl curdlan, laminari-oligosaccharides, and *p*-nitrophenyl-laminari-oligosaccharides were purchased from Megazyme (Wicklow, Ireland). Q-Sepharose Fast Flow, HiTrap SP HP, and HiTrap Phenyl HP were obtained from GE Healthcare. YMC-BioPro SP was supplied by YMC Co. Ltd. (Kyoto, Japan). *E. coli* BL21(DE3) cells and the expression vector pET-22b were procured from Novagen (Madison, WI, USA) and the SHuffle T7 chaperone expression vector pGro7 was supplied by Takara Bio (Kyoto, Japan). A synthetic gene encoding glucanase from *C. cellulans* (GenBank: AAA25520.1) was obtained from Integrated DNA Technologies. All other reagents used were of analytical grade and commercially available. *S. cerevisiae* S288C strain was used.

Assay of β-1,3-glucanase activity against β-glucan

The β-1,3-glucanase activity of the samples was determined colorimetrically. Five microliters of the enzyme solution was added to 250 µL of 0.2% (w/v) carboxymethyl curdlan (CM-curdlan) in 0.1 M sodium acetate buffer (pH 5.5). The reaction mixture was incubated at 37 °C. The ferriferrocyanide reagent was added to the reaction mixture to determine its reducing power. Absorbance of the reaction mixture was measured at 420 nm. The β-1,3-glucanase activity against laminarin or soluble β-glucan was measured using the same method. Curdlan, an insoluble β-glucan, was homogenized and dispersed in 0.1 M sodium acetate buffer (pH 5.0). Enzymatic reactions and reducing sugar color reactions were performed in the same manner, and insoluble debris was removed by centrifugation at 12,000*g* for 10 min before measuring the absorbance.

Purification of GlxGluA from gazyumaru latex

β-1,3-Glucanase activity was observed in the soluble fraction of gazyumaru latex, and the β-1,3-glucanase was purified. In initial studies, we attempted to purify proteins using hydrophobic interaction column chromatography; however, this method was unsuitable. High concentrations of ammonium sulfate contributed to the loss of glucanase activity. The antifungal β-1,3-glucanase was predicted to have a basic isoelectric point and was purified using ion-exchange column chromatography. This was pre-treated by chitin column chromatography to remove a large amount of the basic chitinase that is contained in gazyumaru latex. The following procedures were performed: 10 mL of 5 M NaCl solution was added to the 50 mL of gazyumaru latex at a final concentration of 1 M. The sample was centrifuged at 12,000*g* for 20 min at 4 °C and the about 40 mL of supernatant was recovered. The supernatant was dialyzed against a 20 mM sodium acetate buffer (pH 5.0). After dialysis, the sample was re-centrifuged and the precipitate was removed. The supernatant was applied to a chitin-packed column (1.6 × 10 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The flow-through fraction was collected and dialyzed against 20 mM Tris–HCl buffer (pH 7.5). After dialysis, the sample was applied onto a Q-Sepharose Fast Flow packed column (1.6 × 5 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The flow-through fraction was dialyzed against 20 mM sodium acetate buffer (pH 5.0). After dialysis, the samples were applied to a HiTrap SP HP 1 mL column equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed with the same buffer and the adsorbed proteins were eluted using a linear gradient of 0–0.5 M NaCl with 50 column volumes. The eluted fractions with β-1,3-glucanase activity were dialyzed against 20 mM sodium acetate buffer (pH 5.0). This fraction exhibited chitinase activity in addition to β-1,3-glucanase activity. It was further purified using a column with higher resolution. The sample was applied to a YMC-BioPro SP column equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was washed with the same buffer and the adsorbed proteins were eluted using a linear gradient of 0–0.5 M NaCl with 50 column volumes. The eluted fractions with β-1,3-glucanase activity were collected as purified β-1,3-glucanase, and designated as GlxGluA.

N-terminal and internal amino acid sequence analysis

Purified GlxGluA, mixed with or without *Staphylococcus aureus* V8 protease, was subjected to SDS-PAGE. To digest GlxGluA with protease, electrophoresis was interrupted for 18 h once the sample reached the bottom of the stacking gel. Electrophoresis was then restarted. The separated protein bands were blotted onto polyvinylidene difluoride membranes and stained with Coomassie Brilliant Blue. To determine the N-terminal amino acid sequences, protein bands were subjected to amino acid sequence analysis using a protein sequencer (PPSQ-23A; Shimadzu, Kyoto, Japan). The protein sequencer PPSQ-23A carries out Edman degradation of proteins and separation of the produced phenylthiocarbamyl amino acids by liquid chromatography.

cDNA cloning of GlxGluA-coding gene

A contig containing the β-1,3-glucanase-coding region was constructed from the RNA-sequencing data of *Ficus microcarpa* leaves (SRX5394268) obtained from NCBI Sequence Read Archive. Based on the contig sequence, we designed primers P1 and P2 to amplify the mature GlxGluA-coding gene with 5′ and 3′ sequences for in-fusion cloning. The 5′ and 3′ terminal nucleotide sequences of GlxGluA were predicted from the contig sequence. Total RNA was isolated from gazyumaru leaves using an RNeasy kit (Qiagen). First-strand cDNA was synthesized from the isolated RNA using SuperScript IV VILO (Thermo Fisher Scientific) with oligo-dT primers. The cDNA of GlxGluA was amplified by PCR using primers P1 and P2, and the cDNA as the template. The amplified cDNA of GlxGluA was inserted into a vector which was amplified by PCR from pET-22b using primers P3 and P4. The plasmid constructed by in-fusion cloning was designated pET-GlxGluA. To establish the inactive mutant, the predicted catalytic glutamate residue was mutated to an alanine residue. pET-GlxGluA-E94A was constructed using pET-GlxGluA as a template and primers P5 and P6.

Expression and purification of recombinant GlxGluA and GlxGluA-E94A proteins

E. coli harboring pGro7 was transformed with pET-GlxGluA or pET-GlxGluA-E94A. The transformants were cultured in Luria Bertani (LB) medium containing 50 mg/L ampicillin, 200 μg/L chloramphenicol, and 2 mg/L L-arabinose at 37 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1 mM) was added to induce protein expression when the optical density of the medium reached 0.4–0.8. The medium was then incubated for 24 h at 18 °C. The cells were collected by centrifugation and suspended in 20 mM sodium phosphate buffer (pH 7.4). The cells were then homogenized using a sonicator. The insoluble fraction was removed by centrifugation (12,000*g*, 10 min) and the soluble fraction was dialyzed against 20 mM sodium phosphate buffer (pH 6.0). After dialysis, the samples were applied to a HiTrap SP HP column (1 mL) equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The column was washed with the same buffer, and the adsorbed proteins were eluted using

a linear gradient of 0–0.3 M NaCl. Fractions containing GlxGluA were dialyzed against 20 mM sodium phosphate buffer (pH 7.4). After dialysis, 1.5 M ammonium sulfate was added to the dialysate. The sample was applied to a HiTrap phenyl HP column (1 mL) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 1.5 M ammonium sulfate. The column was washed with the same buffer. The adsorbed proteins were eluted using a linear gradient of 1.5–0 M ammonium sulfate. The eluted fractions yielding a single band corresponding to the molecular weight of GlxGluA were collected as purified recombinant GlxGluA or GlxGluA-E94A.

Plasmid construction for CcGluA and CcGluA-E117A expression

The gene encoding CcGluA (GenBank: AAA25520.1) (Shen et al. 1991) with 5′ and 3′ sequences for in-fusion cloning and a 6×histidine-coding tag were synthesized by Integrated DNA Technologies. The vector was amplified from pET-22b as a template using primers P3 and P4. The synthesized gene was introduced into the amplified vector by in-fusion cloning. The constructed plasmid was designated pET-CcGluA.

To produce CcGluA without glucanase activity, we mutated the glutamate residue predicted to be the catalytic residue to alanine. pET-CcGluA-E117A was amplified by PCR using pET-CcGluA as a template and primers P7 and P8.

Expression and purification of CcGluA protein

E. coli SHuffle T7 was transformed with pET-CcGluA, and the transformants were cultured in LB medium containing 50 mg/L ampicillin at 37 °C. IPTG was added to induce protein expression when the optical density of the medium reached 0.4–0.8. The medium was then incubated for 24 h at 18 °C. The cells were collected by centrifugation and suspended in 20 mM sodium phosphate buffer (pH 7.4). The cells were then homogenized using a sonicator. The insoluble fraction was removed by centrifugation at 12,000*g* for 10 min and the soluble fraction was dialyzed against 20 mM sodium phosphate buffer (pH 7.4). After dialysis, the sample was applied to a cOmplete His-Tag Purification Resin-packed column (1.6×5 cm) (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with 10 mM sodium phosphate buffer (pH 7.4). The column was washed with the same buffer, and the adsorbed proteins were eluted using a linear gradient of 0–0.3 M imidazole. The fractions with glucanase activity were dialyzed against 20 mM sodium phosphate buffer (pH 7.4). After the addition of 1.5 M ammonium sulfate to the dialysate, the sample was loaded onto a HiTrap Phenyl HP column (1 mL) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 1.5 M ammonium sulfate. The adsorbed proteins were eluted using a linear gradient of 1.5–0 M ammonium sulfate. The eluted fractions giving a single band corresponding to the molecular weight of CcGluA were collected as purified recombinant CcGluA or CcGluA-E117A.

Thin-layer chromatography (TLC) analysis of curdlan and oligosaccharide degradation

Curdlan (0.2% w/v) and GlxGluA (0.2 μM) were incubated in 10 mM sodium acetate buffer (pH 5.0) at 37 °C for 0, 1, 4, or 16 h. In addition, curdlan (0.2% w/v) and CcGluA (1.0 μM) were incubated in 10 mM Tris–HCl buffer (pH 7.4) at 37 °C for 0, 1, 4, or 16 h. Each reaction product was subjected to TLC analysis.

Further, 5 mM laminari-oligosaccharides or 4 mM *para*-nitrophenol (*p*NP) glycoside and 2 μM GlxGluA were incubated in 0.1 M sodium acetate buffer (pH 5.0) for specific time period at 25 °C. The reaction mixture was boiled for 5 min and then subjected to TLC.

The TLC plates were chromatographed three times with n-BuOH:AcOH:H_2O (3:1:1, by vol.). The chromatographic spots of *p*-nitrophenol and its derivatives were visualized using ultraviolet lamps emitting light at 254 nm. Glucose, laminari-oligosaccharide, and *p*NP-glycosides were stained with methanol containing 1% (v/v) sulfuric acid (H_2SO_4) and baked at 180 °C for 3 min.

Antifungal activity assay against *T. viride*

An agar disk (4 mm in diameter) containing *T. viride* was prepared from a culture of this fungus growing actively on potato dextrose broth containing 1.5% (w/v) agar (PDA). This disk was placed at the center of a new PDA plate, and the wells were punched into the agar at a distance of 20 mm from the center of the PDA plate. Five microliters of each test sample were placed in each well. The plates were incubated for 24 h at 25 °C.

Quantitative antifungal activity assay (Taira et al. 2002)

An agar disk (4 mm in diameter) containing *T. viride* was prepared from a culture of this fungus growing actively on a PDA plate. This disk was placed on a new PDA plate and overlaid with 5 μL of sterile water or each of the protein solutions. The plates were incubated at 25 °C for 12 h and then photographed. We measured the mycelial growth area of *T. viride*. The protein concentrations required to inhibit the growth of the fungus by 50% were determined by constructing dose–response curves (percentage of growth inhibition versus protein concentration).

Lytic activity assay against *S. cerevisiae*

S. cerevisiae was cultured in yeast extract peptone dextrose (YPD) broth at 30 °C. The cells were collected by centrifugation in the log phase of growth and washed with distilled water. The cells were resuspended in distilled water and autoclaved at 121 °C for 10 min. Heated water solubilizes mannoproteins, which are the major components of the yeast cell wall. Insoluble residues, which probably contained chitin and β-glucan, were collected by centrifugation and washed with distilled water. The heat-treated cells were suspended in 0.1 M sodium phosphate buffer (pH 7.4) or 0.1 M sodium acetate buffer (pH 5.0), and the optical density was adjusted to between 0.9 and 1.1 at 800 nm. The reaction mixture containing 1.8 mL of cell suspension and 0.2 mL of 10 µM enzyme solution was incubated at 25 °C. The yeast lytic activity of the enzyme was evaluated based on the decrease in the turbidity of the reaction mixture at 800 nm.

Preparation of insoluble fraction from *T. viride* and *S. cerevisiae* cells

T. viride spores were added to the potato dextrose broth at a final concentration of 1×10^4 spores/mL. *T. viride* was grown at 25 °C for 48 h. The hyphae of *T. viride* were collected using a filter paper and washed with water. The cells were suspended in water and autoclaved at 121 °C for 10 min. The autoclaved sample was filtered through filter paper and washed with water. The insoluble fraction was collected and lyophilized.

S. cerevisiae was cultured in yeast extract peptone dextrose (YPD) broth at 30 °C for 48 h. The cells were collected by centrifugation at 5000*g* for 10 min, resuspended in water, and autoclaved at 121 °C for 10 min. The autoclaved samples were centrifuged at 5000*g* for 10 min. The insoluble fraction was washed with water, collected by centrifugation, and lyophilized.

Hydrolysis of insoluble fractions from *T. viride* and *S. cerevisiae* by β-1,3-glucanases

The reaction mixtures contained 0.2% (w/v) insoluble fraction of fungal cells (IFCs; major components of cell wall polysaccharides) and 2.0 µM enzyme in 0.1 M sodium acetate buffer (pH 5.0). The enzyme reactions were performed at 37 °C for 24 h. The reducing sugar content in the reaction mixture was determined using a ferriferrocyanide reagent, according to the method described above. The residue of the enzymatically hydrolyzed IFC was used as a substrate for the second enzymatic hydrolysis. The sample was centrifuged 24 h after the enzymatic reaction to recover the insoluble residue. The insoluble residue was washed with water and resuspended in 0.1 M sodium acetate buffer (pH 5.0). The second enzymatic hydrolysis step was performed under the same conditions as the first enzymatic treatment except for the enzyme used. In the second reaction, the GlxGluA-hydrolyzed IFC residue was used as a substrate for CcGluA and the CcGluA-hydrolyzed IFC residue was used as a substrate for GlxGluA.

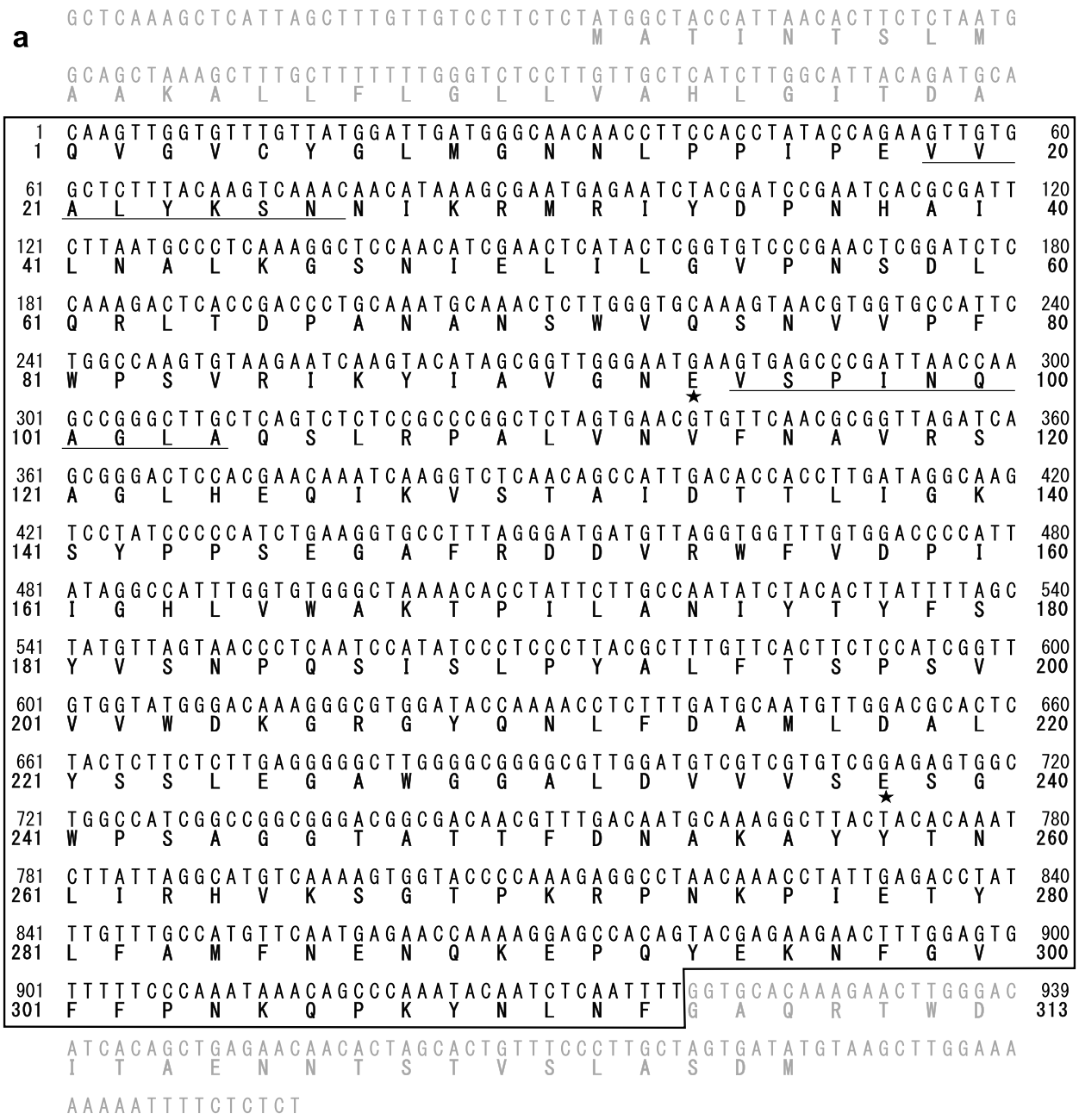
Results

Characterization of purified GlxGluA

GlxGluA was purified from the latex of gazyumaru using several column chromatography techniques. About 100 µg of GlxGluA was purified from 50 mL of gazyumaru latex. The molecular mass of the purified GlxGluA was estimated to be 31.3 kDa by SDS-PAGE (Fig. S1a). The N-terminal sequence of GlxGluA could not be identified, probably because of post-translational modifications. The N-terminal sequences of the GlxGluA fragments obtained by digestion with V8 protease were VXPINXAGL and VVALYKXN ("X" indicates "undetermined amino acid residue").

Cloning and sequence analysis of GlxGluA

RNA-seq data (SRX5394268) of gazyumaru leaves containing latex were obtained from the NCBI Sequence Read Archive. Some reads were mapped to the template nucleotide sequence encoding endo-1,3-beta-glucosidase (XM_010099494.2) from *Morus notabilis* (Supplementary Fig. S2). A contig was constructed by de novo assembly with 15 reads, each containing 301 bases. The contig contains an open reading flame encoding a β-1,3-glucanase candidate. Analysis using SignalP (http://www.cbs.dtu.dk/services/SignalP/) revealed that the deduced amino acid sequence of the candidate contains an N-terminal signal peptide (Supplementary Fig. S2; gray font at the N-terminal end in Fig. 1a and b). The C-terminal sequence AQRTWDITAENNTSTVSLASDM (gray font at the C-terminal end in Fig. 1a) is highly similar to the vacuolar-targeting signal sequences from tobacco (Shinshi et al. 1988) and rubber tree β-1,3-glucanases (Chye and Cheung 1995) (Fig. 1c). These analyses suggested that the sequence without both signal sequences encodes a mature β-1,3-glucanase. Using the primers designed from the candidate gene sequence and cDNA obtained from the total RNA of gazyumaru latex, we attempted to amplify the nucleotide sequence encoding the mature region of the candidate gene by PCR. The amino acid sequence deduced from the nucleotide sequence of the PCR product contained the N-terminal sequences of GlxGluA fragments. The size of recombinant GlxGluA on SDS-PAGE matched that of native GlxGluA. From these analyses, we confirmed that


a
b
ER-targeting signal
GH17 β-1,3-glucanase
Vacuole-targeting signal
Mature GlxGluA
Q1
F313
RNA-seq data
GlxGluA cDNA obtained by RT-PCR using total RNA of gazyumaru
RNA-seq data
c
Tobacco VSGGVWDSSVETNAT-ASLISEM
Rubber tree -AEKNWDISTEHNATILFLKSDM
Gazyumaru -AQRTWDITAENNTSTVSLASDM

◂**Fig. 1** Primary structure of GlxGluA. **a** Nucleotide sequence of GlxGluA cDNA with its deduced amino acid sequence. Black fonts in solid box indicate mature GlxGluA-coding regions obtained by RT-PCR using total RNA of gazyumaru leaves containing latex. Gray fonts indicate the sequences obtained from the contig constructed using RNA-seq data. Stars indicate catalytic residues predicted by a homolog search. Amino acid sequences with underlined amino acid sequences of peptide fragments from V8 protease-digested native GlxGluA. **b** Schematic representation of GlxGluA. Gray boxes and a black box indicate signal sequence coding regions and mature GlxGluA-coding region, respectively. **c** Comparison of vacuole-targeting signal sequences among tobacco, rubber tree, and gazyumaru β-1,3-glucanases

the nucleotide sequence we obtained encoded GlxGluA. Nucleotide and deduced amino acid sequences are shown in Fig. 1a (black font in a solid box). GlxGluA comprises a single catalytic domain belonging to GH17 β-1,3-glucanase (Fig. 1b) and contains 313 amino acid residues (Gln1-Phe313). The molecular weight and theoretical isoelectric point were calculated as 34,708.55 and 8.94, respectively, using the ProtParam tool (https://web.expasy.org/protparam/). Nucleotide sequence data for GlxGluA are available in the GenBank database under the accession number as LC618823.1. The nucleotide sequence of CcGluA (AAA25520.1) was obtained from NCBI (Shen et al. 1991).

β-Glucan hydrolysis activities of GlxGluA and CcGluA

We successfully achieved heterologous expression of GlxGluA using *Escherichia coli*. We then investigated whether it possesses activity equivalent to that obtained from the gazyumaru latex. Purified native GlxGluA from latex and purified recombinant GlxGluA showed the same level of β-1,3-glucanase activity against CM-curdlan at various pH values and temperatures (Fig. S3a and b). The optimum pH and temperature for both native and recombinant GlxGluA were 5.5 and 60 °C, respectively. Based on these results, the native and the recombinant enzymes were judged to be equivalent, and the following experiments were performed using the recombinant enzyme. GlxGluA was stable between pH 3.0 and 9.0 but was unstable under pH 2.0 and over pH 10.0 (Fig. S3c). It was stable at 60 °C and unstable at temperatures over 70 °C (Fig. S3d).

Furthermore, to comprehend substrate specificity of GlxGluA, we initiated a comparative analysis with CcGluA, a representative yeast lytic enzyme. The β-1,3-glucanase activities toward several substrates are summarized in Table 1. GlxGluA showed approximately 5% and 0.2% activity against curdlan and laminarin, respectively, compared to the activity against CM-curdlan. CcGluA showed the highest β-1,3-glucanase activity against CM-curdlan. It showed approximately 45% and 7% activity against curdlan and laminarin, respectively.

Curdlan hydrolysis products by GlxGluA or CcGluA

Curdlan hydrolysis products were analyzed using TLC (Fig. 2a). GlxGluA did not produce glucose from curdlan but yielded laminari-oligosaccharides with various degrees of polymerization. In particular, pentamers and hexamers accumulate during this reaction. Dimers, trimers, tetramers, and laminari-oligosaccharides with a higher degree of polymerization than hexamers were produced at lower levels. Laminari-oligosaccharides larger than the hexamer, which were produced early in the reaction, were degraded over time. The major curdlan hydrolysis product of CcGluA was pentaose, which was produced at the beginning of the reaction, and its amount increased over the course of the reaction. Laminari-oligosaccharides larger than pentaose were produced by CcGluA, whereas those smaller than pentaose were not.

TLC analysis of GlxGluA hydrolytic oligosaccharides

The oligosaccharide hydrolysis products of GlxGluA were analyzed by TLC (Fig. 2b–j). GlxGluA did not hydrolyze laminaribiose and *p*NP-β-glucose (Fig. 2f and j), but hydrolyzed other laminari-oligosaccharides and *p*NP-labeled laminari-oligosaccharides. GlxGluA produced laminaribiose, laminaritriose, and laminaritetraose from laminarihexaose in a short time (Fig. 2b). The quantity of these products increased over time. GlxGluA slightly produced glucose and laminaripentaose from laminarihexaose in a long time. GlxGluA hydrolyzed laminaripentaose mainly to laminaribiose and laminaritriose, and slightly to glucose and laminaritetraose (Fig. 2c). Laminaribiose, laminaritriose, and glucose were produced from laminaritetraose by GlxGluA (Fig. 2d). Laminaribiose and glucose were produced from laminaritriose (Fig. 2e). Laminaritriose and *p*NP-β-glucose were produced from *p*NP-laminaritetraose by GlxGluA (Fig. 2g). This result indicates that GlxGluA hydrolyzes the third glycosidic bond from the non-reducing end of *p*NP-laminaritetraose. *p*NP-β-glucose and laminaribiose were produced from *p*NP-laminaritriose by GlxGluA (Fig. 2h). Small amounts of *p*-nitrophenol and laminaritriose were produced from *p*NP-laminaritriose. These results suggest that GlxGluA hydrolyzes the second or third glycosidic bond from the non-reducing end of *p*NP-laminaritriose. *p*-Nitrophenol and laminaribiose were produced from *p*NP-laminaribiose by GlxGluA (Fig. 2i), indicating that GlxGluA hydrolyzes the second glycosidic bond from the non-reducing end of *p*NP-laminaribiose. GlxGluA preferentially hydrolyzes internal, rather than external, glycosidic bonds. GlxGluA, belonging to the GH17 family with an anomeric

Table 1 The β-1,3-glucanase activity of GlxGluA and CcGluA

Enzyme	Substrate	Specific activity(U/mol)	Relative activity (%)
GlxGluA	CM-curdlan	1.21×10^{10}	100.0 ± 3.98
	Curdlan	6.27×10^{8}	5.17 ± 0.12
	Laminarin	1.97×10^{7}	0.16 ± 0.02
CcGluA	CM-curdlan	2.61×10^{8}	100.00 ± 2.89
	Curdlan	1.19×10^{8}	45.47 ± 5.76
	Laminarin	1.73×10^{7}	6.63 ± 1.31

One unit of β-1,3-glucanase activity is defined as 1 µmol of glucose equivalents released per minute. Data are mean ± standard error of triplicate

retention-type hydrolysis mechanism, may potentially exhibit transglycosylation reactions. However, no transglycosylation products were detected in the TLC experiments. To confirm whether GlxGluA possesses glycosyltransferase activity, it is necessary to utilize high-resolution and quantitative methods.

Antifungal activities of GlxGluA and CcGluA against *T. viride*

Both GlxGluA and CcGluA inhibited the extension of *T. viride* hyphae (Fig. 3). A small amount of GlxGluA (200 pmol) inhibited hyphal growth more strongly than a large amount of CcGluA (1000 pmol) (compare wells 2 and 4 in Fig. 3). The GlxGluA-E94A and CcGluA-E117A mutants, in which the catalytic residue was mutated, did not inhibit the extension of *T. viride* hyphae (wells 3 and 5 in Fig. 3). The antifungal activities of GlxGluA and CcGluA were investigated quantitatively (Fig. 4). Growth inhibition was stronger depending on the amount of these enzymes. From the dose–response curves, GlxGluA was estimated to inhibit fungal growth by 50% at 30 µM. Even at the highest enzyme concentration (565 µM) used in this experiment, the growth-inhibitory activity of CcGluA was less than 50% inhibit the growth of the fungus by 50%.

Lytic activities of GlxGluA and CcGluA against *S. cerevisiae*

We tested the ability of GlxGluA and CcGluA to inhibit the growth of *S. cerevisiae*, but found that neither enzyme inhibited the yeast growth. Instead, the lytic activity against heat-treated yeast cells, in which mannan protein was removed, was measured. We measured the turbidity of the heated yeast cell suspensions after enzyme treatment (Fig. 5). GlxGluA hardly reduced the turbidity of the cell suspension, while CcGluA reduced the turbidity by 30% and 40% at pH 7.4 and 5.0, respectively. At the beginning of the reaction at pH 7.4, CcGluA rapidly decreased the turbidity. After 20 min, the turbidity decreased gradually. At pH 5.0, the initial decrease in turbidity was slightly slower than that at pH 7.4. However, the turbidity dropped sharply between 20 and 40 min.

Enzyme degradation of cell wall fractions of *T. viride* and *S. cerevisiae*

To characterize the enzymatic properties of GlxGluA and CcGluA in relation to their lytic and/or growth-inhibitory activities on fungi, the fungal cell wall-degrading activities of two enzymes were determined using the insoluble fraction of fungal cells (IFC) as a substrate. The reducing sugars from the IFCs treated with GlxGluA or CcGluA were measured (white bars with "G" or "C" in Fig. 6). GlxGluA produced more reducing sugars from *T. viride* IFC than CcGluA. In contrast, CcGluA produced more reducing sugars from *S. cerevisiae* IFC than GlxGluA.

Next, we examined whether specific substrates of the enzyme were remained in the insoluble fraction of the fungal cell wall treated with the other enzyme. The IFC reaction mixture treated with one enzyme was centrifuged and washed thoroughly with water. This step is to remove soluble substrates resulting from first enzyme treatments. The insoluble residue was obtained as "pre-treated IFC." The pre-treated IFC obtained with one enzyme processing was then treated with the other enzyme, and the resulting reducing sugars were measured (gray bars in Fig. 6). "GC" indicates that the first enzyme treatment was performed with GlxGluA and the subsequent enzyme treatment was performed with CcGluA. In the same way, "CG" indicates that the first and the subsequent enzyme treatments were performed with CcGluA and GlxGluA, respectively. The amount of reducing sugars obtained from *T. viride* IFC by GC treatments was 88% of that obtained by GlxGluA treatment. Amounts of the reducing sugars ("GC") were almost equal to that of the reducing sugar produced from IFC treated with CcGluA. These results suggest that the substrates for GlxGluA and CcGluA in the cell walls of *T. viride* differ. The amount of reducing sugars obtained from *T. viride* IFC by CG treatments was 68% of that obtained by CcGluA treatment. The ratio of reducing sugar of CG/C (68%) was lower than that of GC/G (88%). In addition, the sum of the reducing sugars of C and CG was lower than that of G and GC. These results suggest that the carbohydrates solubilized by CcGluA from *T. viride* IFC contain substrates that can be degraded by GlxGluA.

The amount of reducing sugars obtained from *S. cerevisiae* IFC by GC treatments was 76% of that obtained by GlxGluA treatment. CcGluA produced a high amount of reducing sugars from *S. cerevisiae* IFC. The amount of reducing sugars was approximately fourfold higher than that of the reducing sugars produced by GC treatment. These results

suggest that carbohydrates solubilized by GlxGluA from *S. cerevisiae* IFC contain substrates that can be degraded by CcGluA. Surprisingly, GlxGluA did not produce reducing sugars from the residue of *S. cerevisiae* IFC pre-treated with CcGluA. This suggests that the carbohydrates solubilized by CcGluA from *S. cerevisiae* IFC contain many substrates that can be degraded by GlxGluA.

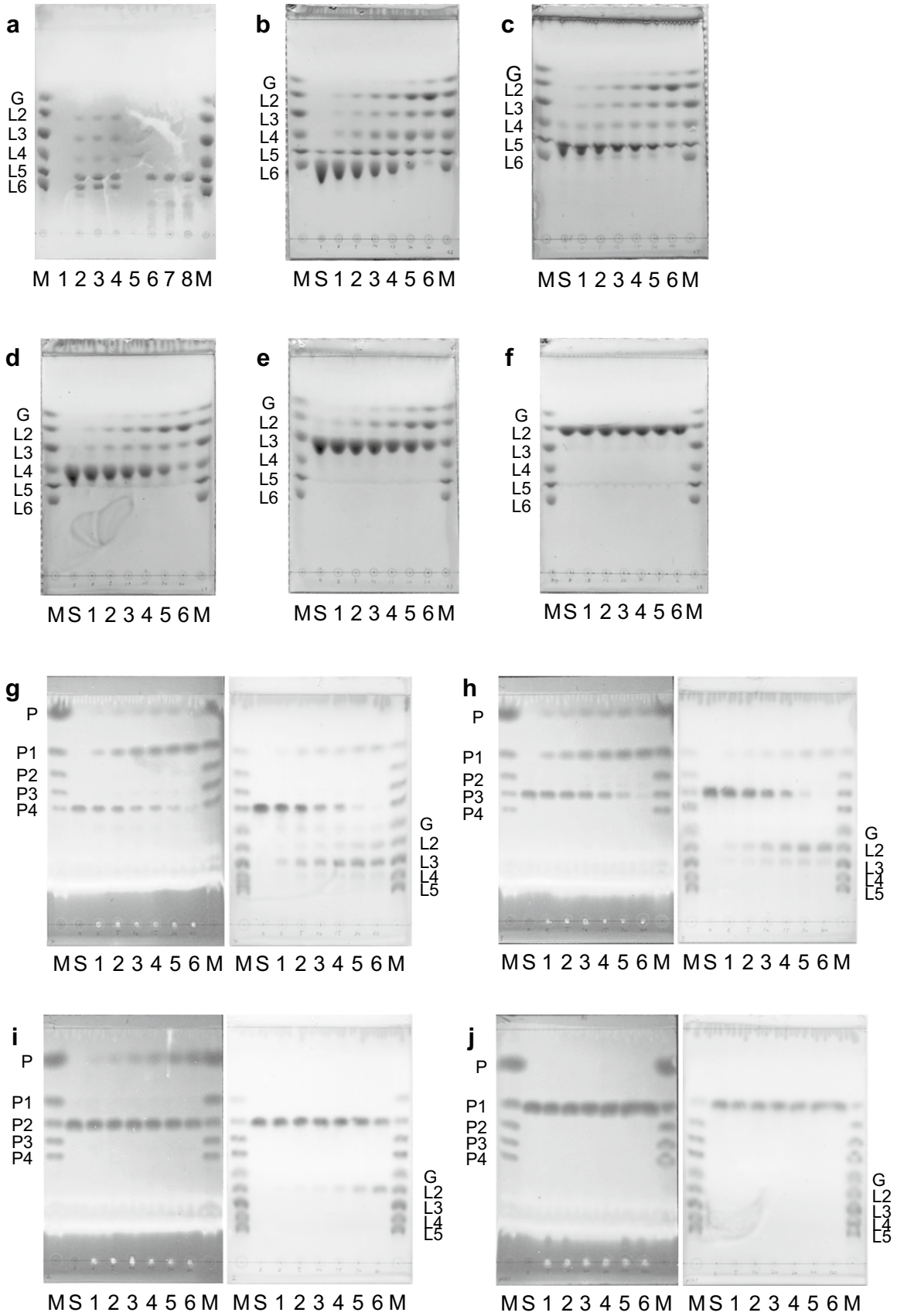
Discussion

RNA-seq data and cDNA cloning results suggested that the GlxGluA precursor contains N-terminal and C-terminal signal peptides that mediate transportation of the protein to the endoplasmic reticulum and vacuole, respectively. Latex is the cytoplasm of specialized cells known as laticifers, which contain organelles of vacuolar origin called lutoids (Moir 1959). Therefore, GlxGluA may be located in the lutoids of laticifers. GlxGluA has a basic isoelectric point (theoretical pI = 8.94). These characteristics (localization and isoelectric point) are similar to those of tobacco class I β-1,3-glucanase (Sela-Buurlage et al. 1993). Plant β-1,3-glucanases can be subdivided into several classes. Class I β-1,3-glucanases have a basic isoelectric point and are localized in vacuoles. Several reports have shown that class I β-1,3-glucanases inhibit the growth of filamentous fungi (Leah et al. 1991; van Kan et al. 1992; Chye and Cheung 1995). Amino acid sequence comparisons revealed that GlxGluA was 60%, 61%, 49%, and 71% identical to tobacco (P15797.2), tomato (AAA03618.1), barley (AAA32939.1), and rubber tree (AAA87456.1) β-1,3-glucanases, respectively. In the present study, recombinant GlxGluA inhibited the growth of *T. viride*. We have previously found that chitinase in gazyumaru latex exhibits strong antifungal activity; it is located in the lutoid. Some reports have shown that a combination of chitinase and β-1,3-glucanase exerts synergistic effects on fungal growth. We quantitatively evaluated and characterized the antifungal activities of chitinase and β-1,3-glucanase in gazyumaru latex. These enzymes will be good models for elucidating the antifungal systems of plant-produced fungal cell wall-degrading enzymes. We are currently investigating the synergistic effects of chitinase and GlxGluA on antifungal activity.

To understand the substrate specificity of the antifungal GlxGluA discovered in gazyumaru latex, its substrate specificity and mode of degradation were characterized. In addition, we compared these characteristics with those of CcGluA, which has yeast cell lysing activity (Table 1). The degradation of β-glucans with different structures showed that GlxGluA and CcGluA have different substrate specificities. Curdlan forms a triple-helical structure with inter-β-1,3-glucan chain hydrogen bonds (McIntosh et al. 2005). CM-curdlan is a water-soluble β-1,3-glucan in which carboxymethyl groups are introduced to unwind its triple-helical structure. Laminarin is a β-1,3-glucan with a low frequency of short β-1,6-glycosidic bond branching structures (Kim et al. 2000). Among the β-glucans tested, GlxGluA showed the highest activity against CM-curdlan. Its hydrolytic activity against curdlan was remarkably weak. These results suggest that GlxGluA preferentially hydrolyzes linear β-1,3-glucan without a triple-helical structure. The three-dimensional structures of several GH17 β-1,3-glucanases have revealed that these enzymes have narrow substrate-binding grooves that can recognize single β-glucan chains (Wojtkowiak et al. 2013). In contrast, CcGluA showed about half the activity of CM-curdlan against curdlan. Some reports have shown that GH64 glucanases have a wide substrate-binding cleft and can hydrolyze the triple-helical β-1,3-glucan (Wu et al. 2009; Kumagai et al. 2016). Although laminarin has few branched structures, each enzyme could hardly hydrolyze it. The branched structure is thought to hinder the recognition of β-1,3-glucan by both enzymes.

To determine which glycosidic linkages of the substrate are preferentially hydrolyzed by GlxGluA, the hydrolysis products of laminari-oligosaccharides and *p*NP-labeled oligosaccharides were analyzed (Fig. 2). During oligosaccharide degradation by GlxGluA, the degradation rate decreased as the degree of substrate polymerization decreased (Fig. 2b–e). GlxGluA did not hydrolyze laminaribiose (Fig. 2f). Analysis of the laminarihexaose degradation product revealed that GlxGluA randomly hydrolyzed the glycosidic bonds inside the laminarihexaose (Fig. 2b). The results of the hydrolysate of *p*NP-glycosides (Fig. 2g–j) suggest that GlxGluA is an endo-type enzyme that preferentially hydrolyzes the second or third glycosidic bond from the non-reducing end of the laminari-oligosaccharide substrates.

GlxGluA released various laminari-oligosaccharides as curdlan products (Fig. 2a). In contrast, CcGluA releases laminaripentaose as the predominant product of curdlan. In other words, GlxGluA hydrolyzes β-glucan as an endo-type enzyme, whereas CcGluA releases a product similar to that of an exo-type enzyme. CcGluA is a major component of zymolyase, a commercially available crude enzyme produced by submerged cultures of *C. cellulans* (Kaneko et al. 1969). Zymolyase has strong lytic activity against living yeast cell walls (Kitamura et al. 1971) and produce protoplasts or spheroplasts from various strains of yeast. β-1,3-glucan laminari-pentaohydrolase is an essential enzyme for the lytic activity of zymolyase. It hydrolyzes β-1,3-glucan and releases laminaripentaose as the main and minimum product unit (Kitamura and Yamamoto 1972; Kaneko et al. 1973; Kitamura 1982a, b). In this study, we confirmed the enzymatic characteristics of recombinant CcGluA. It was of interest to determine whether the differences in substrate


a
G
L2
L3
L4
L5
L6
M 1 2 3 4 5 6 7 8M
b
G
L2
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MS 1 2 3 4 5 6M
c
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MS 1 2 3 4 5 6M
d
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◂**Fig. 2** TLC analysis of curdlan and oligosaccharide degradation products. **a** Curdlan (0.2%, w/v) degradation by GlxGluA (0.2 µM) was performed at pH 5.0 and 37 °C for 0, 1, 4, or 16 h (lanes 1, 2, 3, or 4). Curdlan (0.2%, w/v) degradation by CcGluA (1 µM) was performed at pH 7.4 and 37 °C for 0, 1, 4, or 16 h (lanes 5, 6, 7, or 8). Lane M, marker containing glucose and laminari-oligosaccharides (DP = 2–6). **b–f** Laminari-oligosaccharide degradation by GlxGluA was performed at pH 5.0 and 25 °C for 0, 2, 5, 10, 15, 30, or 60 min (lanes S, 1, 2, 3, 4, 5, or 6). Reaction mixture containing 10 µM GlxGluA and 5 mM substrates: laminarihexaose (**b**); laminaripentaose (**c**); laminaritetraose (**d**); laminaritriose (**e**); laminaribiose (**f**). **g–j** *p*-Nitrophenyl-β-laminari-oligosaccharide degradation by GlxGluA was performed at pH 5.0 and 25 °C for 0, 2, 5, 10, 15, 30, or 60 min (lanes S, 1, 2, 3, 4, 5, or 6). The reaction mixture contained 10 µM GlxGluA and 4 mM substrates: *p*-nitrophenyl-β-laminaritetraose (**g**); *p*-nitrophenyl-β-laminaritriose (**h**); *p*-nitrophenyl-β-laminaribiose (**i**); *p*-nitrophenyl-β-glucose (**j**). In **g–j**, *p*-nitrophenol derivatives, and carbohydrates were detected on the left and right plates, respectively, where Glc, L2, L3, L4, L5, and L6 represent glucose, laminaribiose, laminaritriose, laminaritetraose, laminaripentaose, and laminarihexaose, respectively. P, P1, P2, P3, and P4 represent *p*-nitrophenol, *p*-nitrophenyl-β-glucose, *p*-nitrophenyl-β-laminaribiose, *p*-nitrophenyl-β-laminaritriose, and *p*-nitrophenyl-β-laminaritetraose, respectively

specificity and recognition between GlxGluA and CcGluA were related to their antifungal and lytic activities.

GlxGluA and CcGluA are β-1,3-glucan-degrading enzymes of different GH families, and their natural substrates are thought to be β-1,3-glucans in the fungal cell wall. GlxGluA inhibited *T. viride* growth, but did not lyse *S. cerevisiae* cells (Figs. 3 and 4). CcGluA lysed *S. cerevisiae* cells, but showed only weak inhibitory activity against *T. viride* growth. We considered that this difference was associated with the difference in substrate specificity and degradation mode between GlxGluA and CcGluA, and the difference in the structures of β-1,3-glucan in the cell walls between yeast and filamentous fungi. Based on the substrate specificity of GlxGluA and CcGluA, we speculate that the structure of the natural substrate of each enzyme is as follows. The substrate of CcGluA is a triple-helical β-glucan composed of linear β-1,3-glucans, and the substrate of GlxGluA is an amorphous β-glucan near a branch of β-glucan and near the cross-link between chitin and β-glucan. Fungal cell wall β-glucan is synthesized by the FKS family, with GTPase acting as a regulatory subunit on the plasma membrane (Douglas et al. 1994; Beauvais et al. 2001; Dijkgraaf et al. 2002). The synthesized β-glucans are modified by several enzymes produced by fungi. Fungal GH17 enzymes degrade β-1,3-glucan chain and transfer the fragment to another β-1,3-glucan to form a β-1,6-branching structure (Goldman et al. 1995; Kalebina et al. 2003; Gastebois et al. 2010). In addition, fungal GH16 enzymes degrade β-1,3-glucan chains and transfer the fragments to chitin to form crosslinks (Cabib 2009; Cabib et al. 2012; Fang et al. 2019). These β-1,3-glucan synthase and β-glucan remodeling enzymes are conserved in both yeast and filamentous fungi, even though the composition of the cell wall polysaccharides differ significantly between *S. cerevisiae* and *T. viride*. The *S. cerevisiae* cell wall components include 1–2% chitin, 60% β-glucan (β-1,3:50% + β-1,6:10%), and 40% mannoprotein (Lipke and Ovalle 1998). It has been suggested that about half of the chitin in the cell wall is linked to glucan in *S. cerevisiae* (Kollár et al. 1995). Our preliminary studies showed that the *T. viride* cell wall contained approximately 20% chitin and 32% β-glucan. Assuming half of *T. viride* cell wall chitin is cross-linked with β-glucan like *S. cerevisiae* cell wall chitin, this cross-linked structure in the cell wall of *T. viride* should be present in higher amounts than that in *S. cerevisiae*. In contrast, the ratio of triple-helical β-1,3-glucan in the cell wall of *S. cerevisiae* could be higher than that in the cell wall of *T. viride*.

Cell wall degradation experiments suggested that the *T. viride* cell wall contains a substrate preferred by GlxGluA over CcGluA, whereas the cell wall of *S. cerevisiae* contains a substrate that is much preferred by CcGluA than GlxGluA. The hydrolytic experiments against insoluble residues from pre-treated fungal cell walls with GlxGluA suggest that there is a β-glucan substrate that could be degraded by only CcGluA in both *T. viride* and *S. cerevisiae* cell walls. Even if GlxGluA hydrolyzes amorphous β-glucan, triple-helical β-glucan is itself insoluble. Based on these results and predictions, we speculate that the reaction of CcGluA with the cell wall of *S. cerevisiae* results in the efficient decomposition of triple-helical β-1,3-glucan containing a small amount of the amorphous region and without any cross-linking to chitin. Therefore, CcGluA exhibits strong lytic activity against *S. cerevisiae*. There is also a substrate for GlxGluA in the insoluble residue from *T. viride* cell wall, which is pre-hydrolyzed by CcGluA. Although reducing sugars were detected in the reaction mixture of GlxGluA and the cell wall of *S. cerevisiae*, we failed to observe any reducing sugars in the reaction between GlxGluA and the insoluble residues from *S. cerevisiae* cell walls pre-treated with CcGluA. Amorphous β-glucan with a β-glucan branching structure may be solubilized by the hydrolysis of triple-helical β-glucan by CcGluA. In contrast, amorphous β-glucan with cross-linked chitin and β-glucan is insoluble because it is covalently bound to insoluble chitin.

However, the reason for the strong antifungal activity of GlxGluA remains unclear. We speculate that GlxGluA could recognize cross-linking between β-1,3-glucan and chitin chains and cleave the β-1,3-glucan linkage around the cross-linked region. Deletion of genes involved in cross-linking between chitin and β-1,3-glucan and branching of β-glucan leads to increased sensitivity to dyes that bind to the cell wall in yeast (Pardini et al. 2006; Cabib et al. 2007; Aimanianda et al. 2017). The cross-linked regions are thought to be important for structural maintenance of filamentous fungal cell walls. To understand the relationship between

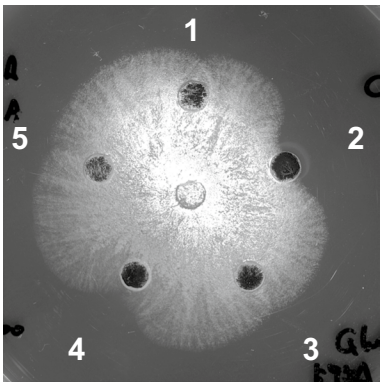

Fig. 3 Antifungal activity of β-1,3-glucanase against *T. viride*. The antifungal activity was measured using a hyphal extension inhibition assay. An agar disk with *T. viride* hyphae was placed at the center of the PDA plate. Well 1 contained distilled water; well 2 contained 200 pmol GlxGluA; well 3 contained 200 pmol GlxGluA-E94A; well 4 contained 1000 pmol CcGluA; and well 5 contained 1000 pmol CcGluA-E117A

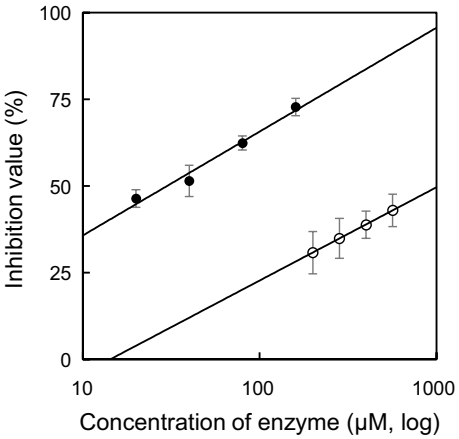

Fig. 4 Quantitative evaluation of the antifungal activity of GlxGluA and CcGluA against *T. viride*. An agar disk containing mycelia of *T. viride* was placed on a PDA plate and overlaid with 5 μL of the sample. The area of mycelial regrowth was measured after incubation for 12 h at 25 °C. Closed circles, GlxGluA; open circles, CcGluA. Each data point represents the mean ± standard error of triplicates

the enzymatic characteristics of β-1,3-glucanase and its antifungal activity, we are currently analyzing fungal cell wall structures containing cross-linked regions. In addition, analysis of substrate specificity for the characterized branched β-glucans, measurement of antifungal activity against more diverse species of fungi, and structural analysis of the cell walls prepared from them are required.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00425-023-04271-4.

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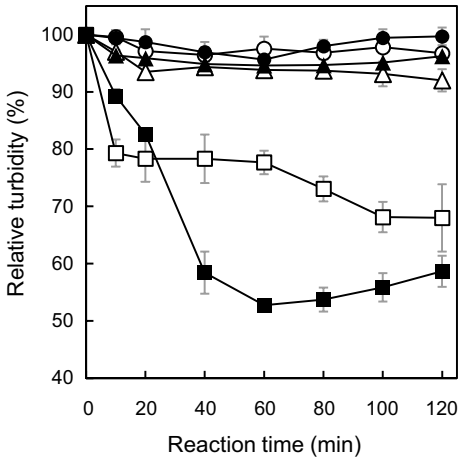

Fig. 5 Lytic activities of β-1,3-glucanases against *S. cerevisiae*. The reaction mixture contained 1 μM enzyme and an appropriate amount of yeast cells (Abs 800 nm = 0.95–1.05). The lytic reaction was performed in 0.1 M sodium phosphate buffer (pH 7.4) or 0.1 M sodium acetate buffer (pH 5.0) at 25 °C. Open circles, blank (pH 7.4); closed circles, blank (pH 5.0); open triangles, GlxGluA (pH 7.4); closed triangles, GlxGluA (pH 5.0); open squares, CcGluA (pH 7.4); closed squares, CcGluA (pH 5.0). Each data point represents the mean ± standard error (SE) of triplicates

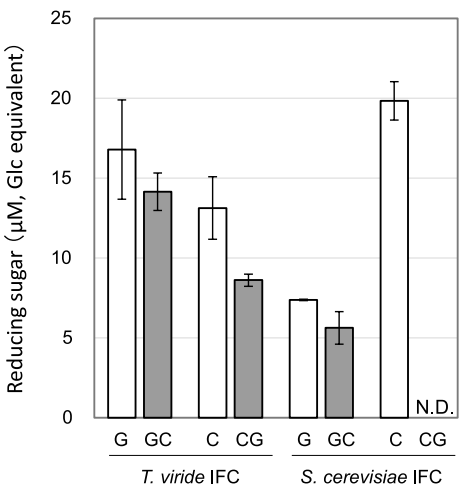

Fig. 6 Reducing sugar production by β-1,3-glucanase from fungal cell-insoluble fraction. Enzymatic degradation of the cell-insoluble fraction (IFC) was performed using sodium acetate buffer (pH 5.0) at 37 °C for 24 h. The reaction mixture contained 2 μM enzyme and 0.2% (w/v) IFC. White bars indicate reducing sugars from the reaction mixture of one enzyme and IFC; gray bars indicate reducing sugars from the reaction mixture of the other enzyme and pre-treated IFC with one enzyme. G, IFC hydrolysate by GlxGluA; GC, hydrolysate of GlxGluA-treated IFC by CcGluA; C, IFC hydrolysate by CcGluA; CG, hydrolysate of CcGluA-treated IFC by GlxGluA. *N.D.* not detected. Data are mean ± standard error of triplicate

Author contributions TTak and NK: performed the experiments. TTak and TTai: wrote the manuscript. TTak, TTai and KU: designed the experiments and thoroughly revised the manuscript. TTai: coordinated the research project. All the authors read and approved the manuscript.

Data availability The sequence of the cDNA containing the GlxGluA gene has been deposited in the GenBank database under accession no. LC618823. The authors confirm that other experimental data are available and accessible via the main text and/or the supplemental data.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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