Glucanase-Induced Stipe Wall Extension Shows Distinct Differences from Chitinase-Induced Stipe Wall Extension of *Coprinopsis cinerea*

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ABSTRACT This study reports that a high concentration of the endo- β -1,3-glucanase ENG (200 $\mu\text{g ml}^{-1}$) induced heat-inactivated stipe wall extension of *Coprinopsis cinerea*, whereas a high concentration of the extracellular β -glucosidase BGL2 (1,000 $\mu\text{g ml}^{-1}$) did not; however, in combination, low concentrations of ENG (25 $\mu\text{g ml}^{-1}$) and BGL2 (260 $\mu\text{g ml}^{-1}$) induced heat-inactivated stipe cell wall extension. In contrast to the previously reported chitinase-reconstituted stipe wall extension, β -1,3-glucanase-reconstituted heat-inactivated stipe cell wall extension initially exhibited a fast extension rate that quickly decreased to zero after approximately 60 min; the stipe cell wall extension induced by a high concentration of β -1,3-glucanase did not result in stipe breakage during measurement, and the inner surfaces of glucanase-reconstituted extended cell walls still remained as amorphous matrices that did not appear to have been damaged. These distinctive features of the β -1,3-glucanase-reconstituted wall extension may be because chitin chains are cross-linked not only to the nonreducing termini of the side chains and the backbones of β -1,6 branched β -1,3-glucans but also to other polysaccharides. Remarkably, a low concentration of either the β -1,3-glucanase ENG or of chitinase ChiE1 did not induce heat-inactivated stipe wall extension, but a combination of these two enzymes, each at a low concentration, showed stipe cell wall extension activity that exhibited a steady and continuous wall extension profile. Therefore, we concluded that the stipe cell wall extension is the result of the synergistic actions of glucanases and chitinases.

IMPORTANCE We previously reported that the chitinase could induce stipe wall extension and was involved in stipe elongation growth of the mushroom *Coprinopsis cinerea*. In this study, we explored that β -1,3-glucanase also induced stipe cell wall extension. Interestingly, the extension profile and extended ultra-architecture of β -1,3-glucanase-reconstituted stipe wall were different from those of chitinase-reconstituted stipe wall. However, β -1,3-glucanase cooperated with chitinase to induce stipe cell wall extension. The significance of this synergy between glucanases and chitinases is that it enables a low concentration of active enzymes to induce wall extension, and the involvement of β -1,3-glucanases is necessary for the cell wall remodeling and the addition of new β -glucans during stipe elongation growth.

KEYWORDS glucanases, β -1,3-glucan, stipe elongation, wall extension, *Coprinopsis cinerea*

During the growth and development of fruiting bodies of the basidiomycete *Coprinopsis cinerea*, the stipe needs to elongate quickly to lift up the pileus for more effective spore dispersal (1, 2). Stipe elongation primarily results from manifold cell elongation rather than cell division (2–6). This cell elongation requires cell wall

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extension to create space for the enlarging protoplast (2, 5–10). Fungal cell walls are mainly composed of various glucans, chitin, additional cell wall polysaccharides, and proteins. These chitin chains associate with each other via interchain hydrogen bonds to form chitin microfibrils and are covalently linked to the nonreducing ends of β -1,6-branched β -1,3-glucans by a β -1,4-glycoside linkage to form the central core structure of the cell wall (8, 11–15). In filamentous fungi and yeast, hydrolysis of the cell wall by β -1,3-glucanases and chitinases is thought to play a major role in cell wall softening during morphogenesis and growth (11, 14, 16–18). As early as the 1980s, stipe cell elongation was suggested to be the result of the hydrolysis of matrix polysaccharides in cell walls by glucanases and chitinases, which increased wall plasticity (7, 19, 20). However, given the lack of direct evidence for enzymatic hydrolysis-mediated cell elongation, Mol et al. (21) proposed that stipe cell elongation resulted from the creeping of polymers caused by the continuous breakage and reformation of intra- and interchain hydrogen bonds among the glucan chains and the chitin chains under turgor pressure.

We recently attempted to isolate and purify an active fraction with stipe wall extension activity from *C. cinerea* fruiting body tissues using extensometer analysis but failed because of the very low abundance of the active components. However, we found that cell wall protein extracts from growing apical stipes of *C. cinerea* restarted extension in heat-inactivated walls, which released glucose, *N*-acetylglucosamine, and chitinbiose; further, the stipe wall protein extracts contained chitinase activity (9). Thus, we purified natural or recombinant chitinases from *C. cinerea* and determined their cell wall extension-inducing activities. We found that the *C. cinerea* chitinases ChiE1 (CC1G_04870) and ChiIII (CC1G_04407) degraded insoluble crystalline chitin powder and reconstituted heat-inactivated apical stipe wall extension. Both heat-inactivated stipe cell walls extended by chitinase and native stipe cell walls extended by acid induction exhibited a partially broken parallel microfibril architecture, and double knockdown of ChiE1 and ChiIII resulted in reductions in stipe elongation and in the heat-sensitive cell wall extension of native stipes. Therefore, we proposed a chitinase-hydrolyzing mechanism of stipe cell wall extension (9).

In this study, we report that β -1,3-glucanase also reconstituted heat-inactivated apical stipe cell wall extension. Remarkably, the extension profile of β -1,3-glucanase-reconstituted stipe wall is different from that of chitinase-reconstituted stipe wall during measurement, and heat-inactivated stipe cell walls extended by β -1,3-glucanase did not exhibit a partially broken parallel microfibril architecture on the inner wall surface. However, β -1,3-glucanase cooperated with chitinase to induce stipe cell wall extension. The detailed results are as follows.

RESULTS

Characteristics of β -glucanase-induced stipe wall extension. During the isolation and purification of active fractions from *C. cinerea* pilei extracts that induced stipe wall extension, a fraction (I-2) showing both glucanase hydrolysis activity and stipe wall extension activity was obtained by CM-Sepharose cation-exchange chromatography after ammonium sulfate precipitation, dialysis, and Bio-Gel P-6 column desalination. However, when the active fraction was further isolated into two fractions (II-2-1 and II-2-2) with glucanase hydrolysis activity (see Table S1 in the supplemental material) using TSKgel phenyl-5PW high-performance liquid chromatography (HPLC), neither showed stipe wall extension activity. Interestingly, when combined, these two glucanase fractions exhibited stipe wall extension activity (Fig. S1A₄). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption ionization–time of flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis determined that these two active fractions were the previously reported endo- β -1,3-glucanase ENG (CC1G_04051) (22) and extracellular β -glucosidase BGL2 (CC1G_08724) (23), respectively. Thus, the characteristics and mechanisms of stipe wall extension induced by recombinant ENG (24) and BGL2 (R. J.

FIG 1 Heat-inactivated stipe wall extension of *C. cinerea* reconstituted *in vitro* by recombinant glucanases on a constant load extensometer. Heat-inactivated apical stipe fragments were clamped to the extensometer and incubated in 50 mM sodium acetate (pH 4.5) for 30 min, and then, after the buffer was replaced by the indicated solution of glucanase in 50 mM sodium acetate (pH 4.5) (indicated by arrows), the fragments were incubated for 120 min. The curves are the average of 9 measurements. (A) The change in wall extension rate. (B) The change in wall length.

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As shown in Fig. 1, a high concentration of ENG (100 $\mu\text{g ml}^{-1}$) or BGL2 (1,000 $\mu\text{g ml}^{-1}$) alone did not exhibit apparent cell wall extension activity; however, in combination, low concentrations of ENG (25 $\mu\text{g ml}^{-1}$) and BGL2 (260 $\mu\text{g ml}^{-1}$) induced stipe cell wall extension. When the ENG concentration was fixed at 25 $\mu\text{g ml}^{-1}$, the enzyme-reconstituted stipe wall extension increased as the BGL2 concentration increased from 30 to 260 $\mu\text{g ml}^{-1}$ but did not increase further at 500 $\mu\text{g ml}^{-1}$ BGL2 (Fig. 2A). When the BGL2 concentration was fixed at 260 $\mu\text{g ml}^{-1}$, the enzyme-reconstituted stipe wall extension increased as the ENG concentration increased from 6.25 to 25 $\mu\text{g ml}^{-1}$ but did not increase further over a range of 25 to 100 $\mu\text{g ml}^{-1}$ of ENG (Fig. 2B). Remarkably, at 200 $\mu\text{g ml}^{-1}$, ENG alone induced stipe wall extension, and at 1,000 $\mu\text{g ml}^{-1}$ ENG, the stipe cell wall extension rate increased to 4 $\mu\text{m min}^{-1}$, which is 2.46 times the rate induced by the combination of ENG (25 $\mu\text{g ml}^{-1}$) and BGL2 (260 $\mu\text{g ml}^{-1}$) (Fig. 1). It is important to notice that 1,000 $\mu\text{g ml}^{-1}$ of ENG induced a strong wall extension but did not cause stipe wall breakage.

ENG/BGL2-reconstituted stipe cell wall extension showed an acidic pH dependence; extension activity decreased as pH increased over a range of pH of 3.0 to 7.0 (Fig. 2C).

FIG 2 Protein concentration, reaction pH, and stipe region dependence of glucanase-reconstituted heat-inactivated stipe cell wall extension. Apical stipe fragments (A, B, C) or different stipe region fragments (D) from 60-mm fruiting bodies were analyzed for their wall extension activities as described in Fig. 1 but with the following changes in protein concentration, reaction pH, and stipe regions. (A) When the ENG concentration was fixed as $25 \mu\text{g ml}^{-1}$, the wall extension rate varied with BGL2 concentrations. (B) When the BGL2 concentration was fixed as $260 \mu\text{g ml}^{-1}$, the wall extension rate varied with ENG concentrations. (C) The wall extension rate varied with reaction pH. After incubation of the stipe fragments in 50 mM sodium acetate (pH 4.5) for 30 min, the bathing solution was replaced with 50 mM sodium acetate (pH 3.0, 4.5, and 5.0) or Bis-tris (pH 6.0 and 7.0) containing ENG ($25 \mu\text{g ml}^{-1}$) and BGL2 ($260 \mu\text{g ml}^{-1}$). (D) The wall extension rate was different for fragments from the apical, medium, and basal stipe regions. The concentrations of ENG and BGL2 were $25 \mu\text{g ml}^{-1}$ and $260 \mu\text{g ml}^{-1}$, respectively.

ENG/BGL2-reconstituted cell wall extension showed maximal activity in the apical stipe region and minor activity in the basal stipe region (Fig. 2D).

Characterization of soluble sugars released during β -glucanase-induced stipe wall extension. The high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis showed that $25 \mu\text{g ml}^{-1}$ ENG alone released the soluble sugars P1, P2, P3, and P4 from stipe cell walls but did not induce stipe cell wall extension (Fig. 3A). The electrospray ion quadrupole time of flight mass spectrometry (ESI-Q/TOF-MS) analysis showed that P1 was a monosaccharide, P2 and P3 were disaccharides, and P4 was a trisaccharide (Fig. 4A). The ESI-Q/TOF-MS/MS analysis showed that P2 (Fig. 4B1) produced not only B_1 and C_1 fragment ions from glycosidic bond cleavages but also $^{0.4}A_2$, $^{0.3}A_2$, and $^{0.2}A_2$ fragment ions, which represent diagnostic, cross-ring cleavage ions arising exclusively from the (1 $\rightarrow$ 6)-linkage; therefore, it is gentiobiose. P3 (Fig. 4B2) generated only typical B_1 and C_1 fragment ions from glycosidic bond cleavages; thus, it is laminaribiose. P4 (Fig. 4B3) produced B_1 , C_1 , B_2 , and C_2 fragment ions from glycosidic bond cleavages, as well as $^{0.4}A_2$, $^{0.3}A_2$, and $^{0.2}A_2$ fragment ions from cross-ring cleavage of the (1 $\rightarrow$ 6)-linkage; therefore, it is 3-O- β -D-gentiobiosyl-D-glucose. As shown in the HPAEC-PAD analysis, P1 had the same retention time as the glucose standard on the Pac PA-100 column (Fig. 3A), and its retention time was also identical to that of the glucose standard but not to those of the galactose and mannose standards on the Pac PA1 column (Fig. 4C); therefore, it is glucose. In

FIG 3 The β -glucanase-reconstituted heat-inactivated stipe wall released soluble sugars into the medium during wall extension. (A) HPAEC-PAD analysis of the soluble sugars released from extending heat-inactivated stipe wall induced by $25 \mu\text{g ml}^{-1}$ ENG, $260 \mu\text{g ml}^{-1}$ BGL2, or $25 \mu\text{g ml}^{-1}$ ENG and $260 \mu\text{g ml}^{-1}$ BGL2 in 50 mM sodium acetate (pH 4.5). The sodium acetate (pH 4.5) buffer without protein was used as the control. P1 to P4 are the product peaks. The glucose, laminaribiose, and laminaritriose standards are dp1, dp2, and dp3, respectively. The ordinate scale is 200 nC. (B) HPAEC-PAD analysis of the soluble sugars released from extending heat-inactivated stipe wall induced by $1,000 \mu\text{g ml}^{-1}$ ENG in 50 mM sodium acetate (pH 4.5). P1 to P5 are the product peaks. The glucose, laminaribiose, and laminaritriose standards are dp1, dp2, and dp3, respectively. The ordinate scale is 1,000 nC. (C) The DNS analysis of reduced sugars released from the alkali-insoluble component of apical stipe cell walls by either $0.4 \mu\text{g ml}^{-1}$ ENG, $35 \mu\text{g ml}^{-1}$ BGL2, or $0.4 \mu\text{g ml}^{-1}$ ENG and $35 \mu\text{g ml}^{-1}$ BGL2 in 50 mM sodium acetate buffer (pH 4.5).

contrast, $260 \mu\text{g ml}^{-1}$ BGL2 alone did not induce stipe cell wall extension, and it released only glucose from stipe cell walls (Fig. 3A). Remarkably, a combination of $25 \mu\text{g ml}^{-1}$ ENG and $260 \mu\text{g ml}^{-1}$ BGL2 only released a large amount of glucose while inducing stipe cell wall extension (Fig. 3A). It seems that BGL2 synergistically degraded laminarioligosaccharides released from stipe cell walls by ENG during cell wall extension. However, we found that although the high concentration of ENG ($1,000 \mu\text{g ml}^{-1}$) alone induced stipe wall extension, it released glucose (P1), gentiobiose (P2), laminaribiose (P3), and 3-O- β -D-gentiobiosyl-D-glucose (P4), but the amount of these laminarioligosaccharides was greater than that released by the low concentration of ENG ($25 \mu\text{g ml}^{-1}$) (Fig. 3B). The high concentration of ENG ($1,000 \mu\text{g ml}^{-1}$) alone also generated an additional soluble oligosaccharide, P5, during stipe cell wall extension

FIG 4 Characterization of the soluble sugars released from glucanase-reconstituted heat-inactivated stipe wall during wall extension. (A) ESI/Q-TOF-MS spectra of P1 (A1), P2 (A2), P3 (A3), P4 (A4), and P5 (A5). (B) ESI/Q-TOF-MS/MS spectra of P2 (B1), P3 (B2), P4 (B3), and P5 (B4). (C) HPAEC-PAD analysis of P1.

FIG 4 (Continued)

(Fig. 3B). Based on ESI-Q-TOF-MS analysis, P5 was a tetrasaccharide (Fig. 4A5). In the ESI-Q-TOF-MS/MS spectrogram, P5 generated B_1 , C_1 , B_2 , C_2 , and C_3 fragment ions from glycosidic bond cleavages, as well as $^{0.4}A_2$, $^{0.3}A_2$, and $^{0.2}A_2$ fragment ions from cross-ring cleavage of the (1→6)-linkage; therefore, it is a 3-O- β -D-gentiobiosyl-D-laminaribiose.

The above results suggested that BGL2 cooperated with ENG to induce stipe cell wall extension by promoting cleavage of the backbones of β -1,6 branched β -1,3-glucans by ENG rather than by degrading the laminarioligosaccharides released by ENG. As shown in Fig. 3C, the hydrolytic activity of the combination of ENG and BGL2 on the alkali-insoluble cell wall fraction of *C. cinerea* stipes was 5.7 times the sum of the individual hydrolytic activities of ENG and BGL2, indicating a strong synergy between their hydrolytic activities on stipe cell walls.

The differences and synergy between glucanase-induced stipe wall extension and chitinase-induced stipe wall extension. Fig. 5 shows that the profile of glucanase-induced stipe wall extension is different from that of chitinase-induced stipe wall extension during measurement. When heat-inactivated apical stipe fragments were shifted to a bath solution containing $120 \mu\text{g ml}^{-1}$ chitinase ChiE1 in 50 mM sodium acetate (pH 4.5), stipe cell walls were immediately induced to extend, and this reconstituted wall extension continued at a nearly constant rate until the end of the measurement. However, when the heat-inactivated apical stipe fragments were shifted to a bath solution containing $200 \mu\text{g ml}^{-1}$ ENG in 50 mM sodium acetate (pH 4.5), stipe cell walls were immediately induced to a maximal extension rate that then gradually decreased to zero after approximately 60 min of extension (Fig. 5). When the heat-inactivated apical stipe fragments were shifted to a bath solution containing both $120 \mu\text{g ml}^{-1}$ ChiE1 and $200 \mu\text{g ml}^{-1}$ ENG in 50 mM sodium acetate (pH 4.5), stipe wall was induced to extend at an additive extension rate and often broke during the measurement (Fig. 5). This stipe breakage might be due to excessive hydrolysis by the enzymes. A high concentration of ChiE1 ($400 \mu\text{g ml}^{-1}$) alone caused stipe breakage when inducing heat-inactivated apical stipe cell wall expansion (Fig. 5). Low concentrations of ChiE1 and ENG were therefore used to study their synergistic action on stipe wall extension. As shown in Fig. 5, individually, $50 \mu\text{g ml}^{-1}$ ENG or $60 \mu\text{g ml}^{-1}$ ChiE1 showed little wall extension activity, but a combination of $50 \mu\text{g ml}^{-1}$ ENG and $60 \mu\text{g ml}^{-1}$ ChiE1 exhibited apparent wall extension activity. The combined ENG/ChiE1-reconstituted heat-inactivated stipe wall extension showed a steady and continuous extension profile (Fig. 5), which was similar to the extension profile of the high concentration of ChiE1 alone ($120 \mu\text{g ml}^{-1}$).

Ultra-architecture of β -glucanase-induced extended stipe walls. A field emission scanning electron microscope (FESEM) was used to observe the ultra-architecture of β -glucanase-induced extended stipe walls. As described previously (9), both native stipes after acid-induced extension (Fig. 6A) and heat-inactivated stipes after chitinase-reconstituted extension (Fig. 6B) showed partially broken chitin microfibril architecture on the inner surfaces of the cell walls; however, after glucanase-reconstituted extension, heat-inactivated stipes did not show this partially broken chitin microfibril architecture (Fig. 6D and E).

DISCUSSION

This study shows that a low concentration of endo- β -1,3-glucanase ENG ($25 \mu\text{g ml}^{-1}$) alone did not induce heat-inactivated stipe cell wall extension but did release glucose, laminaribiose, gentiobiose, and 3-O- β -D-gentiobiosyl-D-glucose from stipe cell walls; however, when combined with $260 \mu\text{g ml}^{-1}$ extracellular β -glucosidase BGL2, $25 \mu\text{g ml}^{-1}$ ENG induced stipe wall extension and released a large amount of glucose only from the cell walls. Remarkably, a high concentration of ENG ($\geq 200 \mu\text{g ml}^{-1}$) alone induced stipe wall extension and released more glucose, laminaribiose, gentiobiose, and 3-O- β -D-gentiobiosyl-D-glucose, as well as 3-O- β -D-gentiobiosyl-D-laminaribiose. In contrast, a high concentration of BGL2 ($1,000 \mu\text{g ml}^{-1}$) alone did not induce stipe wall extension, although it did release more glucose from stipe cell walls than the combination of $25 \mu\text{g ml}^{-1}$ ENG and $260 \mu\text{g ml}^{-1}$ BGL2 did. β -Glucosidases

FIG 5 Comparison of heat-inactivated stipe cell wall extensions induced by glucanase ENG or by chitinase ChiE1 individually or by a combination of ENG and ChiE1. The curves are the average of 9 measurements. (A) The change in wall extension rate. (B) The change in wall length.

FIG 6 Representative FESEM images of the architecture of the inner surfaces of the cell walls of apical stipes, which were prepared for FESEM after their wall extension was measured as described in Fig. 1A.

(Continued on next page)

usually trim nonreducing terminal glucose residues off polysaccharides and oligosaccharides, while they are unable to cleave glucose residues containing branch sites (25–27). In fungal cell walls, β -glucan chains are long and branched, and the nonreducing termini of the side chains of these branched β -glucans are covalently linked to chitin chains, other carbohydrate polymers, or cell wall proteins (12, 13, 28). Therefore, the cleavage of backbone of β -1,3-glucan by endo- β -1,3-glucanases should cause a softening of the cell wall (18), whereas hydrolysis of the backbones of β -1,6 branched β -1,3-glucans by only β -glucosidases would be difficult to accomplish, and it is hard to imagine any significant effect of limited exohydrolysis on stipe wall extension. Degradation of β -glucans is thought to be accomplished by the synergistic actions of both endo- and exo- β -glucanases; i.e., endo- β -glucanases degrade long-chain β -glucans to oligosaccharides, and then exo- β -glucanases further degrade these oligosaccharides to glucose (25, 26). However, our results indicated that BGL2 activity acts in synergy with ENG by enhancing the ability of ENG to hydrolyze the backbone of β -1,6 branched β -1,3-glucans rather than by only degrading released oligosaccharides, which is also supported by the fact that the amount of reducing sugars released from stipe cell walls by a combination of ENG and BGL2 was 5.7 times the sum of the amounts of reducing sugars released by ENG or BGL2 individually. Perhaps, after each cleavage, ENG may disassociate from the cleavage site of a β -1,6 branched β -1,3-glucan and bind to another site on the same β -1,6 branched β -1,3-glucan, to a new β -1,6 branched β -1,3-glucan, or to the released oligosaccharide to continue hydrolysis (29). Preferential binding of BGL2 to the oligosaccharides released from β -1,6 branched β -1,3-glucans by ENG would enable more ENG to bind to β -1,6 branched β -1,3-glucans, enhancing the capacity of ENG to degrade the backbone of β -1,6 branched β -1,3-glucans for wall extension.

This study explored the features of β -1,3-glucanase-reconstituted extension of heat-inactivated stipe cell walls that differ from those of chitinase-reconstituted extension reported previously by Zhou et al. from our lab (9). First, the β -1,3-glucanase-reconstituted heat-inactivated stipe cell wall extension initially exhibited a fast extension rate at acidic pH that quickly decreased to zero after approximately 60 min of extension. In contrast, the chitinase-reconstituted stipe cell wall extension had a steady and continuous rate for 120 min during the measurement of wall extension. Second, a high concentration of ENG alone or a combination of ENG and BGL2 induced strong stipe wall extension that never caused stipe cell wall breakage; in contrast, a high concentration of chitinase usually resulted in stipe cell wall breakage. Third, in FESEM images, the inner surfaces of cell walls after glucanase-reconstituted extension still remained amorphous matrices and did not appear to have been damaged. In contrast, the inner surfaces of heat-inactivated stipe cell walls after chitinase-reconstituted extension exhibited fewer amorphous matrices, and some broken parallel microfibril architectures were observed (also see reference 9). A logical explanation is that chitin chains are not only cross-linked to the nonreducing termini of the side chains and backbones of β -1,6 branched β -1,3-glucans but are also cross-linked to other polysaccharides (13, 30–32). ENG could degrade β -1,3-glucan side chains and backbones of β -1,6 branched β -1,3-glucans but not other types of β -glucans linked to chitin chains. Consequently, ENG would only partially loosen the cell wall, which would result in an

FIG 6 Legend (Continued)

(A) Acid-induced extended native apical stipe fragments that were first incubated in 50 mM HEPES-NaOH (pH 6.8) for 30 min and were then incubated in 50 mM sodium acetate (pH 4.5) for 120 min during the measurement of wall extension. (B) Heat-inactivated apical stipe fragments after ChiE1-reconstituted extension. (C) Heat-inactivated apical stipe fragments that were not extended. (D) Heat-inactivated apical stipe fragments after ENG/BGL2-reconstituted extension. (E) Heat-inactivated apical stipe fragments after high concentration ENG-reconstituted extension. (B to E) All heat-inactivated apical stipe fragments were first incubated in 50 mM sodium acetate (pH 4.5) for 30 min and then were incubated in 50 mM sodium acetate (pH 4.5) without enzyme (C) or with 120 $\mu\text{g ml}^{-1}$ ChiE1 (B), 25 $\mu\text{g ml}^{-1}$ ENG and 260 $\mu\text{g ml}^{-1}$ BGL2 (D), or 1,000 $\mu\text{g ml}^{-1}$ ENG (E) for the 120 min during the measurement of wall extension. Arrows indicate partially broken areas in the transversely arranged microfibril structure on the inner surfaces of stipe cell walls. Scale bar, 500 nm.

initially rapid wall extension that would proceed until all of the β -1,3-glucan chains linked to chitins were disrupted. In *Saccharomyces cerevisiae*, chitins are cross-linked to the nonreducing terminal glucose of β -1,3-glucan chains by a β -1,4-glucosidic linkage and are also cross-linked to β -1,6-glucans through a β -1,3-linked glucose branch (13, 31). In *Aspergillus fumigatus*, β -1,6-glucan is absent in the cell walls, while galactomannan and the linear β -1,3/1,4-glucan are covalently cross-linked to the nonreducing termini of β -1,6 branched β -1,3-glucan side chains and are also possibly cross-linked to chitins (12, 33). In the basidiomycete *Agaricus bisporus*, it was reported that the alkali-insoluble wall fraction contained chitins and β -1,6 branched β -1,3-glucans and that some of the glucans were cross-linked to chitins (21). In contrast, the molecular structure of the *C. cinerea* cell wall has not been studied in detail. In the future, the types of β -glucans in the alkali-insoluble wall fraction and their linkages to chitins need to be studied to elucidate the distinctive features of β -1,3-glucanase-reconstituted stipe cell wall extension.

Although β -1,3-glucanase-reconstituted stipe cell wall extension showed features that were different from those of chitinase-reconstituted stipe cell wall extension, glucanases and chitinases acted synergistically to induce stipe cell wall extension. First, when tested individually, neither $60 \mu\text{g ml}^{-1}$ ChiE1 nor $50 \mu\text{g ml}^{-1}$ ENG induced heat-inactivated stipe wall extension, but a combination of these two enzymes, each at a low concentration, showed stipe wall extension activity. Second, the combined ENG/ChiE1-reconstituted heat-inactivated stipe wall extension showed a steady and continuous extension profile that was similar to the extension profile of high-concentration ChiE1-reconstituted heat-inactivated stipe wall extension. In fact, in preliminary experiments, crude protein extracts from fruiting bodies also induced heat-inactivated stipe cell wall extension in the same steady and continuous extension profile (Fig. S1A₁). Third, at increased concentrations, e.g., $200 \mu\text{g ml}^{-1}$ ENG or $120 \mu\text{g ml}^{-1}$ ChiE1, each enzyme alone induced stipe cell wall extension, and combining them not only resulted in a synergistic wall extension activity but also caused stipe breakage due to excessive hydrolysis. Excess chitinase individually also led to stipe breakage when inducing stipe cell wall extension at $400 \mu\text{g ml}^{-1}$. This stipe breakage caused by overtreatments with hydrolases was similar to the cell wall autolysis in fungal cells (9, 34, 35). Furthermore, as reported previously, when native growing apical stipe fragments were induced by acidic pH to extend, they released glucose, *N*-acetylglucosamine, and chitinbiose into the bath solution (9). Therefore, we concluded that stipe cell wall extension is the result of the synergistic activities of glucanases and chitinases. The significance of this synergy between glucanases and chitinases is that it enables a low concentration of active enzymes to induce wall extension during stipe elongation growth, which is critical to maintaining the delicate balance between cell wall plasticity and cell wall integrity to limit the breakage of cell walls for stipe elongation growth (9, 34, 35). This combination of multiple enzymes at low concentrations may explain why it has been difficult for us to purify and to characterize a single active component with wall extension activity from *C. cinerea* fruiting bodies (9). In addition, the evolution of synergy between glucanases and chitinases may be related to the structural composition of fungal cell walls. In fungal cell walls, β -1,6-branched β -1,3-glucans are covalently cross-linked to other wall polysaccharides, such as β -1,6-glucans, β -1,3/1,4-glucans, and galactomannans, besides chitins, which are responsible for the shape of the cell (12, 14, 30, 32, 36). Therefore, the involvement of β -1,3-glucanases is necessary for the cell wall remodeling and the addition of new β -glucans that promote wall extension during stipe elongation growth.

According to the Carbohydrate-Active enZymes (CAZY) glycoside hydrolase family classification (<http://www.cazy.org/Glycoside-Hydrolases.html>), fungal β -1,3-glucanases are included in different glycoside hydrolase (GH) families, such as the GH2, 3, 5, 16, 17, 55, and 81 families (18, 34–37). In yeast cells, although *S. cerevisiae* and *Schizosaccharomyces pombe* contain many putative β -1,3-glucanases (38–40), only one or two endo- β -1,3-glucanases belong to the GH81 family (38, 40, 41). Deletion of endo- β -1,3-

glucanase Eng1 or Eng2 of the GH81 family in yeast cells resulted in defects in cell separation (38, 41) and ascus wall degradation (40). In the filamentous fungus *Neurospora crassa*, a single deletion of β -1,3-glucanase CGL-1 belonging to the GH55 family resulted in a defect in conidia cell separation (42). Interestingly, a single deletion of an exochitinase NAG-1 of *Neurospora crassa* also led to a defect in conidia cell separation. These authors suggested that both exochitinase NAG-1 and β -1,3-glucanase CGL-1 are needed to digest the cell wall glucan/chitin matrix in the septa connecting adjacent conidia during their maturation to allow for conidia separation and dissemination (42). ENG from *C. cinerea* belongs to the GH16 family (34, 43). There are 15 putative β -1,3-glucanase genes in the GH16 family in *C. cinerea*. Reverse transcription-quantitative PCR (qRT-PCR) analysis showed that ENG among 15 GH16 family members was expressed at higher levels in the growing apical stipe region than in the nongrowing basal and the basal swollen regions, consistent with distribution of elongation regions in the growing stipe (43). In the preliminary experiments, we constructed plasmid pCcengdsRNA containing the hairpin double-stranded RNA (dsRNA) of *eng*, which was cotransformed with marker plasmid pCc α 1 into the haploid oidia to generate the single knockdown transformant ENG-RNAi. All detected transformants of ENG-RNAi did not exhibit any apparent phenotype associated with fruiting body development or mycelium growth (Fig. S2A and B). The presence of multiple members in one GH family may seriously limit functional analysis by gene knockdown (32, 39). qRT-PCR analysis also proved that some GH16 family members were upregulated in ENG-RNAi knockdown strains (Fig. S2C), indicating that the function of silenced single ENG is compensated for by upregulation of other, unsilenced GH16 family member expression. In filamentous fungi, *Aspergillus fumigatus* contains one GH81 endo- β -1,3-glucanase, ENG1, and seven GH16 endo- β -1,3-glucanases (ENG2 to 8), and single deletion of ENG1 or ENG2 of *Aspergillus fumigatus* did not lead to any phenotypic defect (44, 45) due to functional redundancy of multiple endo- β -1,3-glucanases belonging to different GH families (27). Thus, Mouyna et al. (27) undertook a successive sequential deletion of members of these glucanases (ENG2 to 5), leading to a defect in conidial separation as well as abnormal morphology during conidial swelling. In the future, some technical barriers need to be overcome to perform a successive knockdown or knockout of 15 GH16 family members in *C. cinerea* to provide genetic evidence for involvement of endo- β -1,3-glucanase in stipe cell wall extension *in vivo*.

MATERIALS AND METHODS

Strains and culture conditions. The *C. cinerea* ATCC 56838 isolate used in this study was purchased from the American Type Culture Collection (ATCC). The culture of mycelia and fruiting bodies was performed as described by Kamada et al. (19).

Isolation and purification of the native proteins with extension activity in *Coprinopsis cinerea*.

A total of 200 g of the pilei opening to about 75° were collected, added to 200 ml of precooled grinder buffer (2 mM Na₂S₂O₅, 25 mM HEPES [pH 7.0], and 0.1% Triton X-100), and broken in a homogenizer. The fractions were filtered and centrifuged, and then the supernatant was precipitated by ammonium sulfate with the saturation between 40 and 60%. The precipitate was dissolved in sodium acetate buffer (50 mM, pH 4.5) and dialyzed. Then the fraction was applied to a Bio-Gel P-6 open column, and the active fraction gathered was subsequently applied to CM-Sepharose fast flow open column (20 × 1 cm; Amersham), with the active I-2 fraction eluted in a linear gradient of 0 to 0.5 M sodium chloride dissolved in sodium acetate buffer (50 mM, pH 4.5). After the active I-2 fraction with extension activity was collected, lyophilized, and dissolved in 1 ml of 1 M (NH₄)₂SO₄ in sodium acetate buffer (50 mM, pH 4.5), it was applied to a TSK gel phenyl-SPW HPLC column (10 μ m, 10 mm × 250 mm; Tosoh) from which two elution peaks, II-2-1 fraction and II-2-2 fraction, were eluted in a linear gradient of 1.0 to 0 M (NH₄)₂SO₄ in sodium acetate buffer (50 mM, pH 4.5). The II-2-1 fraction was collected, lyophilized, and dissolved in 1 ml of 0.1 M sodium chloride in sodium acetate buffer (50 mM, pH 4.5), and then it was applied to a TSK gel G2000SW HPLC column (13 μ m, 21.5 mm × 600 mm; Tosoh) for further purification. The detailed procedures were described by Zhou et al. (22). During the whole process, the content of protein in each fraction was monitored by a UV detector at 280 nm; the β -1,3-glucanase activity of fractions was determined with the substrate laminarin (Sigma), and the inducing cell wall extension activity of fractions was determined using an extensometer (6).

Heterologous expression and purification of the endo- β -1,3-glucanase ENG and the β -glucosidase BGL2. Heterologous expression, purification, and specific activities of recombinant ENG and BGL2 in *Pichia pastoris* GS115 were performed as described by Liu et al. (24) and Dai et al. (submitted for publication).

Stipe wall extension assays. Apical stipe fragments 10 mm in length were collected from 30-mm fruiting bodies, and fragments from different stipe regions (apical, medium, and basal) were collected from 60-mm-long fruiting bodies as described by Niu et al. (8). The collected stipe fragments were abraded using carborundum and stored at -20°C for less than a week. Stipe wall extension measurements were performed with an extensometer as described by McQueen-Mason et al. (46) and Zhang et al. (6).

HPAEC-PAD and HPAEC-PAD-ESI-Q/TOF-MS/MS analysis of soluble sugars released from extending stipe cell walls. After the measurement of stipe cell wall extension was completed, the bathing solution in the extensometer was added to 1 ml of boiled water, heated at 100°C for 10 min, centrifuged at 12,000 rpm for 10 min, and filtered with a $0.22\text{-}\mu\text{m}$ Millipore filter (Amicon). The resulting samples were applied to a Dionex CarboPac PA-100 column ($4 \times 250\text{ mm}$) with a PA-100 guard column for the HPAEC-PAD analysis of laminarioligosaccharides as described by Liu et al. (24). The P1 fraction eluted from the Dionex CarboPac PA-100 column was collected and applied to the Dionex CarboPac PA-1 column with a PA-1 guard column and eluted with constant-gradient (15 mM) NaOH solution at a flow rate of 1 ml min^{-1} for the HPAEC-PAD analysis of monosaccharide (47). Glucose, galactose, and mannose were used as monosaccharide standards. MagIC Net 3.1 software was used to analyze the PAD data. Samples were also applied to a HPAEC-PAD system coupled with an ESI-Q/TOF-MS (6540; Agilent Technologies) and MassHunter 6.0 (Agilent Technologies) for characterization of each soluble sugar as described by Yi et al. (48) and Liu et al. (24).

Analysis of the hydrolytic activities of ENG and BGL2 on alkali-insoluble cell wall components. For the preparation of alkali-insoluble cell wall components as described by Wang et al. (49), the apical stipes from 60-mm fruiting bodies were collected, ground into flour with liquid nitrogen, and added to precooled 50 mM Tris-HCl (pH 7.5) buffer containing 50 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (4,500 rpm for 10 min), the precipitate was washed two times with the same buffer and then disrupted in a homogenizer at 4°C with 0.5-mm-diameter glass beads. The cell wall pellet was collected by centrifugation, washed twice with the above buffer, washed eight times with distilled water, and lyophilized. Then, 0.1 g of lyophilized cell wall pellet was added to 30 ml of 1 M NaOH and incubated at 60°C for 60 min, during which the NaOH solution was replaced every 20 min. The alkali-insoluble pellet was obtained by centrifugation, washed eight times with distilled water, and lyophilized.

For the determination of the hydrolytic activities of ENG and BGL2 toward alkali-insoluble cell wall components, a 200- μl reaction mixture containing 1 mg of the alkali-insoluble cell wall component and either 0.4 $\mu\text{g ml}^{-1}$ ENG, 35 $\mu\text{g ml}^{-1}$ BGL2, or 0.4 $\mu\text{g ml}^{-1}$ ENG and 35 $\mu\text{g ml}^{-1}$ BGL2 in 50 mM sodium acetate buffer (pH 4.5) was incubated at 37°C for 1 h and then heated at 100°C for 10 min. After centrifugation, the reducing sugar content of the supernatant was measured with 3,5-dinitrosalicylic acid reagent (DNS) (50).

FESEM. After the measurement of stipe cell wall extension was finished, stipe segments clamped in the extensometer were washed with 0.2 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer (phosphate-buffered saline [PBS], pH 7.4) and fixed in 4% paraformaldehyde in PBS for 3 min. Then, samples were taken from the extensometer and fixed in 4% paraformaldehyde in PBS for 4 h at 4°C and rinsed in PBS. The specimens were dehydrated in a graded ethanol series and dried by critical point drying using CO_2 . For observing the cell wall structure with a field emission scanning electron microscope (FESEM), specimens were coated with gold at 37 mA for 45 s with an SCD 500 sputter coater (Bal-Tec). An S4800 cold-cathode FESEM (Hitachi, Japan) was used for examining the cell wall structure.

Construction of *eng*-dsRNA expression vector and screening of transformants. The antisense sequence (bp 750 to 1) and the sense sequence (bp 101 to 750) of *eng* were cloned and, respectively, ligated to the NcoI and the KpnI site of pCcExp with *Agaricus bisporus gpdII* promoter, a nonfunctional “loop” sequence, and *Aspergillus nidulans trpC* terminator, generating the vector pCcengdsRNA. The pCcengdsRNA and pCc*pab-1* or pCcExp and pCc*pab-1* were cotransformed to protoplasts prepared from fresh oidia of the strain AmutBmut, generating the *eng*-RNAi transformants and the mock transformants by screening, respectively. The culture of mycelia and fruiting bodies of transformants were described by Zhou et al. (9). Total RNA of *eng*-RNAi transformants and the mock transformants was extracted, the cDNA was synthesized, and qRT-PCR was performed as described by Zhou et al. (9) and Kang et al. (43).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01345-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We declare no competing financial interests.

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