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Increased enzyme production under liquid culture conditions in the industrial fungus *Aspergillus oryzae* by disruption of the genes encoding cell wall α-1,3-glucan synthase

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Under liquid culture conditions, the hyphae of filamentous fungi aggregate to form pellets, which reduces cell density and fermentation productivity. Previously, we found that loss of α-1,3-glucan in the cell wall of the fungus *Aspergillus nidulans* increased hyphal dispersion. Therefore, here we constructed a mutant of the industrial fungus *A. oryzae* in which the three genes encoding α-1,3-glucan synthase were disrupted (tripleΔ). Although the hyphae of the tripleΔ mutant were not fully dispersed, the mutant strain did form smaller pellets than the wild-type strain. We next examined enzyme productivity under liquid culture conditions by transforming the cutinase-encoding gene *cutL1* into *A. oryzae* wild-type and the tripleΔ mutant (i.e. wild-type-cutL1, tripleΔ-cutL1). *A. oryzae* tripleΔ-cutL1 formed smaller hyphal pellets and showed both greater biomass and increased CutL1 productivity compared with wild-type-cutL1, which might be attributable to a decrease in the number of tripleΔ-cutL1 cells under anaerobic conditions.

Key words: cell wall; α-1,3-glucan; *Aspergillus*; enzyme; production

Many filamentous fungi, such as those in genus *Aspergillus*, secrete commercially valuable enzymes and metabolites and are therefore of high value to the fermentation industry.[1–3] Although filamentous fungi secrete higher levels of these valuable enzymes and metabolites than yeasts and bacteria, under liquid culture conditions the hyphae of filamentous fungi often clump together to form large pellets,[4] which prevents the establishment of high cell densities under liquid culture conditions. Therefore, preventing pellet formation is crucial for improving the productivity of commercial fermentation processes.

The aggregation of hyphae and formation of pellets is thought to be related to the biochemical properties of the outer layer of the hyphae,[5] which is composed mainly of cell wall polysaccharides and cell wall-associated proteins.[6] Therefore, further elucidation of the relationship between hyphal aggregation and the chemical components, particularly the cell wall polysaccharides, of the hyphal cell surface is needed.

Several chemical analyses of the components of the cell wall of the human pathogen *Aspergillus fumigatus* have been conducted.[7,8] The major components of the *A. fumigatus* cell wall are polysaccharides, which can be fractionated into those that are alkali soluble (AS) and those that are alkali insoluble (AI). The AS fraction mainly contains α-1,3-glucan with some galactomannan, whereas the AI fraction mainly contains galactomannan, chitin, and highly branched β-1,3-glucan with β-1,6 linkages (β-1,3/1,6-glucan).[7] In the hyphal cells of *A. fumigatus* grown under liquid culture conditions, the major component of the AI fraction is β-1,3/1,6-glucan, which forms a three-dimensional network with numerous non-reducing ends[7]; chitin, galactomannan, and β-1,3/1,4-glucan are covalently bonded to the non-reducing ends of the β-1,3/1,6-glucan, producing a large hetero-polysaccharide network.[7] Consistent with these findings, Yoshimi et al. reported that the components of the cell wall of the model filamentous fungus *Aspergillus nidulans* are similar to those of *A. fumigatus*,[4] and Mizutani et al. reported that the components of the cell wall of the industrial filamentous fungus *A. oryzae* are similar to those of *A. fumigatus*.[9]

Cell wall α-1,3-glucan was first recognized as a virulence factor in pathogenic fungi such as the human pathogenic dimorphic yeast *Histoplasma capsulatum*,[10–12] the human pathogen *A. fumigatus*,[13,14] and the plant pathogenic filamentous fungus *Magnaporthe grisea*.[15,16] α-1,3-Glucan in the cell wall of pathogenic

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yeasts and filamentous fungi is thought to be distributed in the outer layer of the cell wall and to cover β-1,3-glucan or chitin to prevent immune recognition by host cells.[12,15]

We previously reported that transcription of the two genes encoding α-1,3-glucan synthase (i.e. *agsA* and *agsB*) in *A. nidulans* is mainly regulated via the cell wall integrity signaling pathway.[17] To determine the function of *agsA* and *agsB* other than as stealth factors against recognition by host cells, we constructed three strains of *A. nidulans* in which *agsA*, *agsB*, or both were disrupted (i.e. *agsA*Δ, *agsB*Δ, and *agsA*Δ*agsB*Δ).[4] We found that the *agsB*Δ and *agsA*Δ*agsB*Δ strains had no α-1,3-glucan in their cell wall, whereas the *agsA*Δ strain had the same amount of α-1,3-glucan in its cell wall as did the wild-type strain, suggesting that AgsB is the major α-1,3-glucan synthase in *A. nidulans*. Furthermore, whereas the hyphae of the wild-type and *agsA*Δ strains aggregated to form pellets under liquid culture conditions, the hyphae of the *agsB*Δ and *agsA*-Δ*agsB*Δ strains were dispersed.[4] Together, these results suggest that under liquid culture conditions α-1,3-glucan is a hyphal cell aggregation factor in *A. nidulans*.

In industrial fermentation using single-cell microorganisms such as bacteria and yeasts, cultures of high cell density are necessary for high productivity. However, most industrial filamentous fungi, including aspergilli, form hyphal pellets under liquid culture conditions, leading to low cell density and limited productivity. Because hyphal cells of *A. nidulans* mutants lacking α-1,3-glucan in their cell wall are dispersed,[4] we hypothesized that this may also be true of other industrial fungi, such as *A. oryzae*, and therefore disruption of the genes involved in α-1,3-glucan biosynthesis represents a possible strategy for reducing the pellet formation and increasing cell density and fermentation productivity under liquid culture conditions.

In the present study, we constructed an *A. oryzae* mutant in which we disrupted all three genes encoding α-1,3-glucan synthases (i.e. *agsA*, *agsB*, *agsC*; *A. oryzae* tripleΔ) and examined the effects of this disruption on pellet formation. Recently, two novel α-1,3-glucan synthesis-related genes, *amyD* and *amyG*, which putatively encode a GPI-anchored glycosidase and an intracellular glycosidase, respectively, were reported in *A. nidulans*.[18] These two glycosidase-encoding genes together with *agsB* form a gene cluster, the transcription of which is coordinately regulated in *A. nidulans*.[18] Therefore, we also constructed an *A. oryzae agsA*Δ*agsB*Δ*agsC*Δ*amyD*Δ*amyG*Δ quintuple disruptant (*A. oryzae* quintupleΔ). To examine enzyme productivity, we transformed a single copy of the *A. oryzae cutL1* gene encoding the cutinase CutL1, a model secretory enzyme that degrades biodegradable aliphatic polyester,[19] into the wild-type, tripleΔ, and quintupleΔ strains.

Materials and methods

Strains and growth media. Strains used in this study are listed in Table 1. *A. nidulans* ABPU1 (*biA1*, *pyrG89*, *wA3*, *argB2*, *pyroA4*) with *ligD*Δ (*ligD*Δ::*ptrA*)[4] and *A. oryzae* NS4 (*sC*$^{-}$, *niaD*$^{-}$) with *ligD*Δ (*ligD*Δ*::sC*, *adeA*Δ*::ptrA*) were used for all genetic manipulations.[20] *A. nidulans agsA*Δ*agsB*Δ, a strain in which the two genes encoding α-1,3-glucan synthases (i.e. *agsA*, *agsB*) were disrupted, was produced as previously described.[4] All *A. nidulans* strains were cultivated in Czapek-Dox (CD) medium containing 0.6% (70 mM) $NaNO_3$, 0.052% KCl, 0.152% KH_2PO_4, 0.059% $MgSO_4{\cdot}7H_2O$, 0.1% trace elements solution [0.1% $FeSO_4{\cdot}7H_2O$, 0.88% $ZnSO_4{\cdot}7H_2O$, 0.04% $CuSO_4{\cdot}5H_2O$, 0.015% $MnSO_4{\cdot}4H_2O$, 0.01% $Na_2B_4O_7{\cdot}10H_2O$, 0.005% $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$], and 2% glucose. The CD medium was adjusted to pH 6.5. To fulfill the auxotrophic requirements of *A. nidulans*, 200 μg of arginine/mL, 0.02 μg of biotin/mL, 0.5 μg of pyridoxine/mL, 1.22 mg of uridine/mL, and 1.12 mg of uracil were added to the CD medium.

A. oryzae niaD^{-} strains were cultivated in CDE medium, which was CD medium in which the nitrogen source was 70 mM of sodium hydrogen L(+)-glutamate monohydrate instead of 70 mM $NaNO_3$. *A. oryzae niaD* (CutL1-expressing) strains were cultured in CD medium described above. The strain of *A. oryzae* requiring adenine was cultivated in CDE medium supplemented with 0.01% adenine (CDEA). To construct *A. oryzae* tripleΔ (*agsA*Δ*agsB*Δ*agsC*Δ) strain, we used the *adeA*-marker recycle method as described below. The *adeA* marker of the gene disruption cassette integrated in the chromosome of *A. oryzae* was excised by Cre-*loxP* system,[21,22] resulting in the generation of *adeA*$^{-}$ strains that were grown in CDEA medium.

Conidia of *A. oryzae* used to inoculate flask cultures were isolated from cultures grown on malt medium containing 9% malt extract (Becton Dickinson and Company, Sparks, USA), 0.5% yeast extract (Becton Dickinson and Company), and 0.1% trace elements solution. YPD medium containing 2% peptone (Becton Dickinson and Company), 1% yeast extract, and 2% glucose was used as the medium for the flask cultures used to analyze the growth characteristics of the strains. YPM medium containing 2% peptone, 1% yeast extract, and 2% maltose was used as the medium for the flask cultures to analyze the secretion of CutL1. YPDS medium containing 6% peptone, 1% yeast extract, 6% glucose, and 20 mM succinate buffer (pH 7.0) was used for the flask cultures to analyze CutL1 secretion under conditions of suppressed amylase production.

Construction of A. oryzae *tripleΔ and quintupleΔ strains.* The disruption cassette for each of the three *ags* genes was constructed as shown in Fig. S1(A). The pYES2-based vector[17] containing the disruption cassette for each *ags* gene was constructed with following amplicons (A, L, R, B). Amplicon A: the yeast-*Escherichia coli* shuttle vector pYES2 was digested with *Bam*HI and *Eco*RI. Amplicon L: the DNA fragment corresponding to −1500 to −500 bp upstream the translation initiation site was amplified with wild-type genomic DNA template and primers (agsA-LU [or agsB-LU or agsC-LU] and agsA-LL-loxP [or agsB-LL-loxP or agsC-LL-loxP]) by polymerase chain reaction (PCR). Amplicon R: the DNA fragment corresponding to +2500–3500 bp downstream the translation initiation site was amplified with wild-type genomic DNA and the primers (agsA-RU-loxP [or agsB-RU-loxP or

agsC-RU-loxP] and agsA-RL [or agsB-RL or agsC-RL]) by PCR. Amplicon B: *A. nidulans adeA* marker and Cre-expression cassette encompassed with two *loxP* fragment were amplified with pAAAXP-Cre (Zhang et al., in submission) as a template and the primers (adeA-Cre-loxP-Fw and adeA-Cre-loxP-Rv) by PCR. The amplicons A, L, R, and B were fused in order to obtain each *ags* gene disruption vector (Fig. S1(A)) by homologous recombination in *S. cerevisiae* BY4741.[23] The disruption cassette fragment was amplified with the disruption vector for each *ags* gene by PCR. Primers for PCR are listed in Table S1.

The *agsA* gene in *A. oryzae* NS4 (*sC*$^-$, *niaD*$^-$) with *ligD*Δ (*ligD*Δ*::sC*, *adeA*Δ*::ptrA*) was disrupted with the *agsA*-disruption cassette and the *agsA* disrutants were selected as *adeA*$^+$ transformants and confirmed by Southern blot analysis. The *adeA* marker gene in the *agsA* disruptant was excised by induction of Cre-recombinase with 1% xylose (instead of glucose) in the CDEA medium (Fig. S1(A)). The *agsA*Δ*agsB*Δ strains were obtained from the *agsA*Δ strain by using the *agsB* disruption cassette containing *adeA* marker. The *agsA*-Δ*agsB*Δ*agsC*Δ strains were constructed from the double mutant by using the *adeA* marker recycle method.

To construct the dual disruption cassette for the *amyD* and *amyG* genes, gene fragments containing the 5′ non-coding region of *amyD* (amplicon 1), the 3′ non-coding region of *amyG* (amplicon 2) derived from the *A. oryzae* genomic DNA template, and the *adeA* gene (amplicon 3) from the plasmid TOPO-2.1-adeA (described below) were amplified by means of PCR (Table S1). Amplicon 1 was amplified with the primers amyD-LU and amyD-LL + Ade, amplicon 2 with the primers amyG-RU + Ade and amyG-RL, and amplicon 3 with the primers amyD-AU and amyG-AL. The primers amyD-LL + Ade, amyG-RU + Ade, amyD-AU, and amyG-AL were chimeric oligonucleotides; each contained a reverse-complement sequence for PCR fusion. The PCR products were gel-purified and then used as the substrates for a second round of PCR using the primers amyD-LU and amyG-RL to fuse the three fragments. The resulting major PCR product was gel-purified and used to transform *A. oryzae* tripleΔ (Fig. S1(B)). Disruption of the *amyD* and *amyG* genes was confirmed by means of Southern blot analysis (Fig. S1(C)).

The plasmid TOPO-2.1-adeA was constructed by following methods. The DNA fragment containing *A. oryzae adeA* marker gene was amplified with *A. oryzae* wild-type genomic DNA as a template by PCR. The amplified fragment containing the *adeA* gene was cloned into pCR2.1-TOPO vector (Invitrogen, Tokyo, Japan) by TA cloning according to the manufacturer's instructions. This plasmid was used for the PCR template to construct the *amyD* and *amyG* gene disruption cassette and for complementation of *A. oryzae adeA*$^-$ strains.

Scanning electron microscopy. Samples for scanning electron microscopy analysis were prepared by following the method of Mizutani et al. with slight modification.[24] Colonies of *A. oryzae* wild-type, tripleΔ, and quintupleΔ strains grown on CDE agar plates were fixed, dehydrated, and lyophilized in tert-butanol. The lyophilized colonies were coated with platinum–vanadium and observed under a Hitachi SU8000 scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 3 kV.

Construction of CutL1-expressing strains of A. oryzae. To allow comparative analysis of enzyme productivity, we constructed *A. oryzae* wild-type, tripleΔ, and quintupleΔ strains overexpressing cutinase CutL1 as a model secretory enzyme (i.e. *A. oryzae* wild-type-cutL1, tripleΔ-cutL1, and quintupleΔ-cutL1). To construct the CutL1 overexpression system, plasmid pNGA-gla-cut[25] expressing the *A. oryzae cutL1* gene under the control of the *A. oryzae* glaA142 promoter was transformed into *A. oryzae* wild-type, tripleΔ, and quintupleΔ by following the methods of Gomi et al.[26] CutL1-overexpressing transformants were selected by using the *niaD* gene as a selectable marker. We confirmed that a single copy of the overexpression construct was integrated at the *niaD* locus by means of Southern blot analysis (Fig. S2).

Fractionation of cell wall components and quantitative analysis of the carbohydrate composition of the cell wall. Conidia (final concentration, 1×10^5/mL) of individual strains were inoculated into 200 mL of YPD liquid medium and rotated at 160 rpm at 30 °C for 48 h in a 500-mL Erlenmeyer flask. Cultured mycelia were collected by filtration through Miracloth (Merck Millipore, Darmstadt, Germany), washed twice with 20 mL of distilled water, and lyophilized. Lyophilized mycelia were pulverized by using a MM400 bench-top mixer mill (Retsch, Haan, Germany), and 0.5 g of the resulting powder was suspended in 40 mL of 0.1 M Na phosphate buffer (pH 7.0). Fractionation of the cell wall components by alkali-treatment was performed as described previously.[4] Carbohydrate composition of each cell wall fraction was quantitatively determined by following the methods of Yoshimi et al.[4]

Analysis of the growth characteristics of A. nidulans *and* A. oryzae *under liquid culture conditions.* Conidia (final concentrations, 1×10^5/mL) of *A. nidulans* wild-type or *agsA*Δ*agsB*Δ were inoculated into 200 mL of CD liquid medium in 500-mL Erlenmeyer flasks and rotated at 160 rpm at 30 °C for 60 h. Mycelia were then collected and dried as described below. Conidia (final concentrations, 1×10^5/mL) of *A. oryzae* wild-type, tripleΔ, quintupleΔ, wild-type-cutL1, tripleΔ-cutL1, or quintupleΔ-cutL1 were inoculated into 200 mL of YPDS liquid medium and rotated at 160 rpm at 30 °C for 72 h in 500-mL Erlenmeyer flasks. Samples (5 mL) were withdrawn from the culture broth every 12 h and filtered through Miracloth to isolate the mycelia. The mycelia were washed twice with 20 mL of distilled water, dried at 70 °C for 24 h, and then weighed. Filtrates were stored in glass vials in a freezer (−20 °C) until use. Immediately after defrosting, the pH was measured and the concentrations of

glucose and CutL1 (see below) in the filtrate were determined. The amount of glucose remaining in the culture broth was measured by using a glucose C II test kit (Wako, Osaka, Japan). The mean diameter of the hyphal pellets was determined by measuring 10 randomly selected pellets under a stereomicroscope (M125; Leica Microsystems, Wetzlar, Germany).

Assay of enzyme productivity under liquid culture conditions. Conidia (final concentration, 1×10^5/mL) of *A. oryzae* wild-type-cutL1, tripleΔ-cutL1, and quintupleΔ-cutL1 were inoculated into 50 mL of YPM liquid medium and rotated at 100 rpm at 30 °C for 24 h in 200-mL Erlenmeyer flasks. The culture broth was then filtered through Miracloth to remove the mycelia, and the filtrates were collected and stored as described above. To quantify the amounts of the proteins secreted, 100% (w/v) trichloroacetic acid was added to 200 µL of filtrate to precipitate the proteins. The precipitated proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 17.5% SDS polyacrylamide gel) and stained with Coomassie Brilliant Blue. Gel images were captured by using a flatbed scanner (GT X-750; Seiko Epson Corp., Nagano, Japan) and the amount of CutL1 secreted in the broth was quantified densitometrically by using the United States National Institutes of Health's ImageJ software (http://rsb.info.nih.gov/nih-image/about.html).

To further examine the details of CutL1 productivity, *A. oryzae* wild-type-cutL1 and tripleΔ-cutL1 strains were cultured for 72 h in YPDS medium containing 6% glucose to suppress amylase production. Conidia (final concentration, 1×10^5/mL) of each strain were inoculated into 200 mL of YPDS medium in 500-mL Erlenmeyer flasks and rotated at 120 rpm at 30 °C for 72 h. An aliquot (5 mL) of the culture broth was withdrawn, and the concentration of secreted CutL1, pH, amount of glucose remaining in the filtrate, and mycelial dry weight, were determined. The amount of glucose remaining in the filtrate was determined by using the phenol/H_2SO_4 method.[27,28]

Results

Growth characteristics of A. nidulans *wild-type and* agsAΔagsBΔ *and of* A. oryzae *tripleΔ and quintupleΔ*

Yoshimi et al. previously reported that the hyphae of *A. nidulans agsB*Δ and *agsA*Δ*agsB*Δ, which both lack α-1,3-glucan in their cell wall, were well dispersed under liquid culture conditions.[4] Here, we compared the dry weight of mycelia of *A. nidulans* wild-type and *agsA*Δ*agsB*Δ grown in CD liquid medium and found that the dry weight of *A. nidulans agsA*Δ*agsB*Δ mycelia was 1.5-fold higher than that of the wild-type mycelia (Fig. S3), which suggests that the increased hyphal dispersion in *A. nidulans agsA*Δ*agsB*Δ resulted in greater biomass production under liquid culture conditions. To investigate whether removal of α-1,3-glucan from the cell wall of the industrial fungus *A. oryzae* would also result in increased hyphal dispersion and an increase in biomass, we constructed a strain of *A. oryzae* in which the three genes encoding α-1,3-glucan synthases (i.e. *agsA*, *agsB*, *agsC*) were genetically disrupted (Fig. S1(A)).

To observe the mycelial growth of the *A. oryzae* strains on plate medium, conidia (1×10^4/mL) of the wild-type and tripleΔ strains were inoculated at the center of CDE agar plates and cultured at 30 °C for four days. Both strains showed normal radial growth and conidiation (data not shown). To compare the morphology of the wild-type, tripleΔ, and quintupleΔ strains, we observed the conidiophores and mycelia of these strains under a scanning electron microscope. No significant differences in the morphologies of the conidiophores and mycelia were observed among the three strains (Fig. S4). These results suggest that deletion of the three *ags* genes did not affect the growth of *A. oryzae* under plate culture conditions. This result is consistent with that previously reported for *A. nidulans agsB*Δ and *agsA*Δ*agsB*Δ, which were also found to grow normally under plate culture conditions.[4]

To examine the characteristics of mycelial growth in *A. oryzae* wild-type and tripleΔ under liquid culture conditions, conidia were inoculated into YPD liquid medium and rotated (160 rpm) at 30 °C for 48 h. At 36 h, the mean diameter of the hyphal pellets of *A. oryzae* tripleΔ (mean diameter, 2.2 mm) was 30% smaller than that of the hyphal pellets of wild-type *A. oryzae* (mean diameter, 3.2 mm) ($p < 0.01$; Fig. 1(A) and (B)). This suggests that loss of α-1,3-glucan in the cell wall caused by disruption of the three *ags* genes led to a weakening of the adhesive forces among the hyphae of *A. oryzae* tripleΔ. No differences in the branching of hyphae or the morphology of the hyphal tip were detected between the *A. oryzae* wild-type and tripleΔ strains in the microscopic assessment (data not shown). Similarly, *A. nidulans* wild-type and *agsA*Δ*agsB*Δ also have comparable microscopic hyphal morphologies.[4]

Whereas the hyphae of *A. nidulans agsA*Δ*agsB*Δ were well dispersed under liquid culture conditions, those of *A. oryzae* tripleΔ were not and small pellets still formed. We hypothesized that the cells must still be producing a small amount of α-1,3-glucan and that it was causing the formation of the small hyphal pellets in the tripleΔ strain. He et al.[18] recently reported that *A. nidulans amyD* and *amyG*, which encode a GPI-anchored glycosidase and an intracellular glycosidase, respectively, were involved in the biogenesis of α-1,3-glucan in *A. nidulans*. Therefore, to try to further abolish the production of α-1,3-glucan in *A. oryzae* we constructed a strain in which the *amyD* and *amyG* genes were also disrupted (i.e. *agsA*Δ*agsB*Δ*agsC*Δ*amyD*Δ*amyG*Δ); however, the quintupleΔ strain still formed small hyphal pellets under liquid culture conditions (Fig. 1(A)).

Further characterization showed that the mycelial dry weights of *A. oryzae* tripleΔ and quintupleΔ were both greater than that of *A. oryzae* wild-type at 12–48 h of culture (Fig. 1(C)) and also that the two mutant strains consumed glucose slightly faster than did the wild-type strain (Fig. 1(D)). The pH of the culture broth in which tripleΔ and quintupleΔ was cultured was slightly higher than that of the culture broth in which the wild-type strain was cultured at 24–48 h of culture (Fig. 1(E)). Since the smaller pellets of *A. oryzae* tripleΔ and quintupleΔ (Fig. 1(B)) produced a larger total hyphal pellet surface area than that of the wild-type strain, fewer hyphal cells in the mutant strains would have been

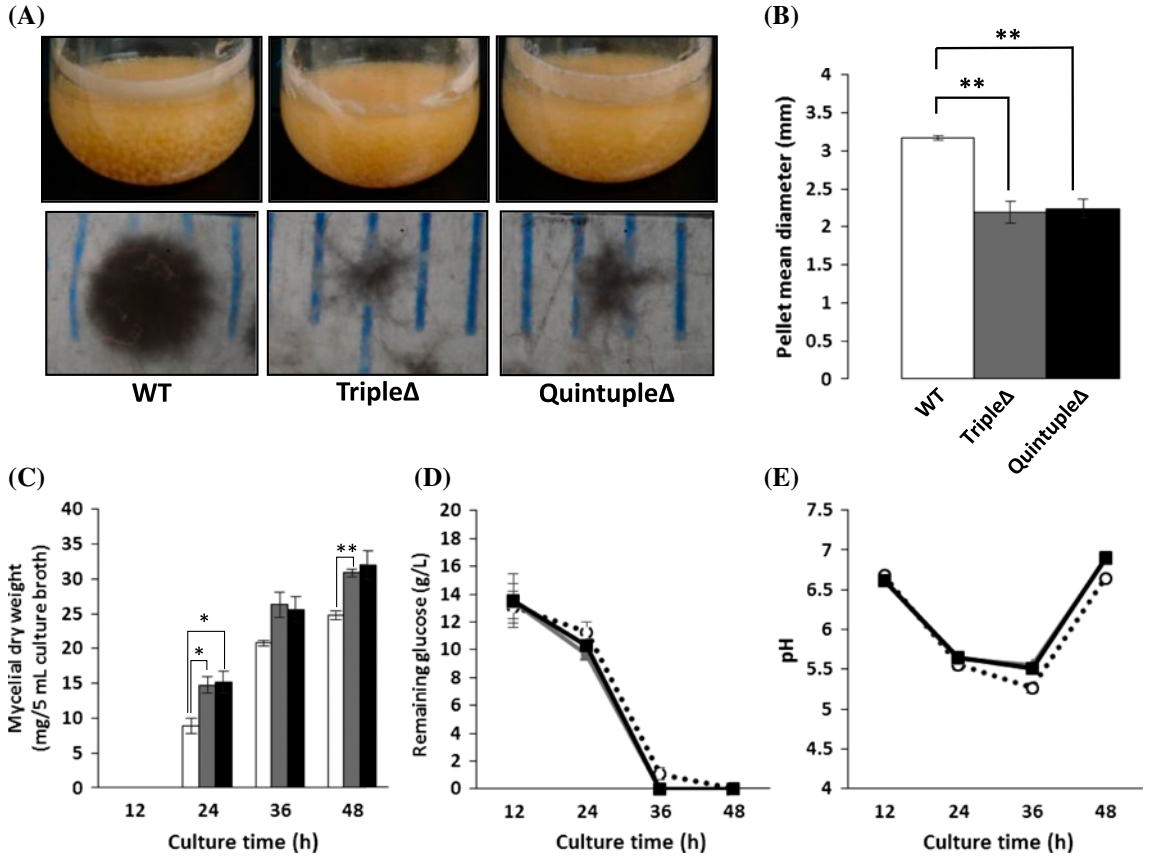

Fig. 1. Phenotypes of *Aspergillus oryzae agsAΔagsBΔagsCΔ* triple mutant (tripleΔ) and *agsAΔagsBΔagsCΔamyDΔamyGΔ* quintuple mutant (quintupleΔ) under liquid culture conditions.

Notes: (A) Growth characteristics of the wild-type (WT), tripleΔ, and quintupleΔ strains. Photographs of cultures in Erlenmeyer flasks (upper) and of representative hyphal pellets of each strain as seen under a stereomicroscope (bottom; scale = 1 mm) at 36 h of culture. Conidia (final concentration, 1×10^5/mL) of each strain were inoculated into YPD medium and rotated at 160 rpm at 30 °C for 48 h. (B) The mean diameter of hyphal pellets for each strain was determined by measuring 10 randomly selected pellets under a stereomicroscope. Wild-type, white bar; tripleΔ, grey bar; quintupleΔ, black bar. Error bars represent the standard deviation of the mean calculated from three replicates (**$p < 0.01$). (C) Mycelial dry weight of each strain. Samples were withdrawn every 12 h and filtered through Miracloth. The mycelia were washed twice with water and dried at 70 °C. Wild-type, white bar; tripleΔ, grey bar; quintupleΔ, black bar. Error bars represent the standard error of the mean calculated from three replicates (*$p < 0.05$, **$p < 0.01$). (D) Amount of glucose remaining in the culture supernatant, as quantified by using a glucose C II test (Wako, Osaka, Japan). Wild-type, dotted line/circles; tripleΔ, grey line/triangles; quintupleΔ, black line/squares. Error bars represent the standard error of the mean calculated from three replicates. None of the differences were statistically significant. (E) pH of the culture supernatant recorded every 12 h. Wild-type, dotted line/circles; tripleΔ, grey line/triangles; quintupleΔ, black line/squares. Error bars represent the standard error of the mean calculated from three replicates. None of the differences were statistically significant.

subject to anaerobic conditions. Thus, the larger surface area of the hyphal pellets of *A. oryzae* tripleΔ and quintupleΔ mean that these strains may be useful for improving the productivity of fermentation processes.

Analysis of the monosaccharide composition of the A. oryzae *cell wall*

To examine the composition of the cell wall in *A. oryzae* wild-type, tripleΔ, and quintupleΔ, lyophilized hyphal cells of each strain were fractionated according to their solubility in alkali, and each fraction was hydrolyzed to determine the monosaccharide composition in the fraction. It has been reported that in *A. fumigatus* and *A. nidulans* the AS fraction of the cell wall contains mainly α-1,3-glucan with some galactomannan,[4,7] and that the AI fraction is composed of chitin, β-1,6-branched β-1,3-glucan, and galactomannan.[4,8] In the present study, the components of the cell wall from each strain were separated into four fractions: hot-water-soluble fraction (HW), water-soluble components after dialysis of the AS fraction (AS1), AS fraction (AS2), and AI fraction (AI), and the weight ratios of the fractions were then calculated (Table S2). The total recovery rate of the four fractions (HW + AS1 + AS2 + AI) was 55–65% of the total weight of lyophilized mycelia derived from each strain.

In *A. oryzae* wild-type, tripleΔ, and quintupleΔ, the HW fraction mainly contained galactose and mannose (Fig. 2(A)) and the AS1 fraction mainly contained glucose and mannose (Fig. 2(B)). The AS2 fraction derived from *A. oryzae* wild-type mainly contained glucose (approx. 20% of the AS2 fraction) with a small amount of mannose; however, those derived from *A. oryzae* tripleΔ and quintupleΔ contained significantly less glucose ($p < 0.01$; Fig. 2(C)). The AI fraction derived from each strain contained glucose, glucosamine, and mannose. The glucose and glucosamine contents of the AI fraction derived from each strain were around 28 and 20% of the total dry weight of the AI fraction, respectively (Fig. 2(D)). Together, these results show that although the *ags* genes play a major

Table 1. Strains used in this study.

Aspergillus nidulans	Genotype
Wild-type (ABPU1)	*biA1*, *pyrG89*, *wA3*, *argB2*, *pyroA4, ligDΔ*::*ptrA*
agsAΔagsBΔ	*biA1*, *pyrG89*, *wA3*, *pyroA4, ligDΔ*::*ptrA, agsAΔ*::*pyrG*, *agsBΔ*::*argB*
Aspergillus oryzae	Genotype
Wild-type (NS4 *ligDΔ* :: *sC*, *adeAΔ* :: *ptrA)*	*ligDΔ* :: *sC*, *adeAΔ* :: *ptrA*, *niaD*$^{-}$, *adeA*$^{+}$
agsAΔagsBΔagsCΔ (TripleΔ)	*ligDΔ* :: *sC*, *adeAΔ* :: *ptrA*, *niaD*$^{-}$, *adeA*$^{+}$, *agsAΔ* :: *loxP*, *agsBΔ* :: *loxP*, *agsCΔ* :: *loxP*
agsAΔagsBΔagsCΔamyDΔamyGΔ (QuintupleΔ)	*ligDΔ* :: *sC*, *adeAΔ* :: *ptrA*, *niaD*$^{-}$, *agsAΔ* :: *loxP*, *agsBΔ* :: *loxP*, *agsCΔ* :: *loxP, amyDΔamyGΔ* :: *adeA*
Wild-type-cutL1	*ligDΔ* :: *sC*, *adeAΔ* :: *ptrA*, *adeA*$^{+}$, PglaA142-*cutL1*-TagdA :: *niaD*
TripleΔ-cutL1	*ligDΔ* :: *sC*, *adeAΔ* :: *ptrA*, *adeA*$^{+}$, *agsAΔ* :: *loxP*, *agsBΔ* :: *loxP*, *agsCΔ* :: *loxP*, PglaA142-*cutL1*-TagdA :: *niaD*
QuintupleΔ-cutL1	*ligDΔ* :: *sC*, *adeAΔ* :: *ptrA*, *agsAΔ* :: *loxP*, *agsBΔ* :: *loxP*, *agsCΔ* :: *loxP*, *amyDΔamyGΔ* :: *adeA*, PglaA142-*cutL1*-TagdA :: *niaD*

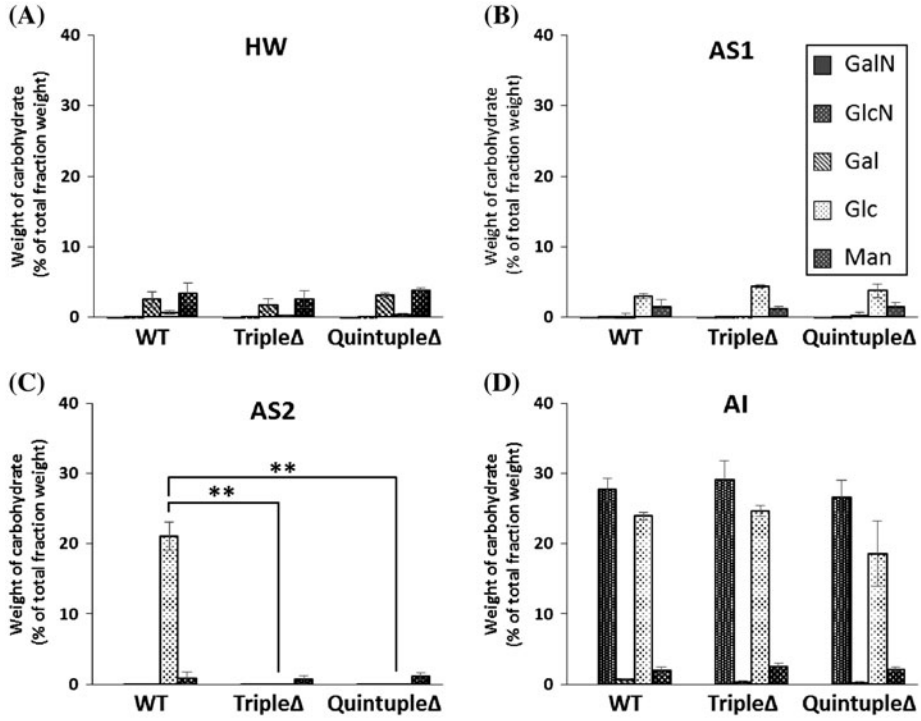

Fig. 2. Compositions of the monosaccharide fractions of the cell wall of *Aspergillus oryzae* wild-type (WT), *agsAΔagsBΔagsCΔ* triple mutant (tripleΔ), and *agsAΔagsBΔagsCΔamyDΔamyGΔ* quintuple mutant (quintupleΔ).

Notes: Wild-type, tripleΔ, and quintupleΔ strains were cultured in YPD medium and rotated at 160 rpm at 30 °C for 24 h. Monosaccharide compositions of (A) hot-water-soluble (HW), (B) water-soluble components after dialysis of the alkali-soluble fraction (AS1), (C) alkali-soluble (AS2), and (D) alkali-insoluble (AI) cell wall fractions as a percentage of total dry weight of each fraction are shown. Error bars represent the standard error of the mean calculated from three replicates (**$p < 0.01$). GalN, galactosamine; GlcN, glucosamine; Gal, galactose; Glc, glucose; Man, mannose.

role in α-1,3-glucan biosynthesis in *A. oryzae*, the *amyD* and *amyG* genes do not. The overall composition of the cell wall in the *amyDΔamyGΔ* strain was very similar to that of the wild-type strain, and the *amyDΔamyGΔ* disruptant formed hyphal pellets of similar size to those of the wild-type strain (data not shown).

Cutinase productivity in A. oryzae *tripleΔ-cutL1 and quintupleΔ-cutL1 under liquid culture conditions*

To examine the growth characteristics of *A. oryzae* tripleΔ and quintupleΔ in which the *cutL1* gene was overexpressed by the glaA142 promoter[29] (i.e. tripleΔ-cutL1 and quintupleΔ-cutL1 strains), *A. oryzae* wild-type-cutL1, tripleΔ-cutL1, and quintupleΔ-cutL1 were cultured for 24 h in YPM liquid medium containing maltose as an inducer of the *A. oryzae* glaA142 promoter. *A. oryzae* tripleΔ-cutL1 and quintupleΔ-cutL1 formed significantly smaller hyphal pellets than did the wild-type-cutL1 strain in YPM liquid medium ($p < 0.01$; Fig. 3(A) and (B)), which is consistent with the sizes of the pellets formed by *A. oryzae* wild-type, tripleΔ, and quintupleΔ in YPM liquid medium (data not shown). The mycelial dry weights of the tripleΔ-cutL1 and quintupleΔ-cutL1 strains were approximately twice that of wild-type-cutL1 ($p < 0.01$; Fig. 3(C)).

The enzyme productivity of *A. oryzae* wild-type-cutL1, tripleΔ-cutL1, and quintupleΔ-cutL1 was measured by means of SDS-PAGE analysis of CutL1 (21.6 kDa) secreted into culture broth (Fig. 3(D) and

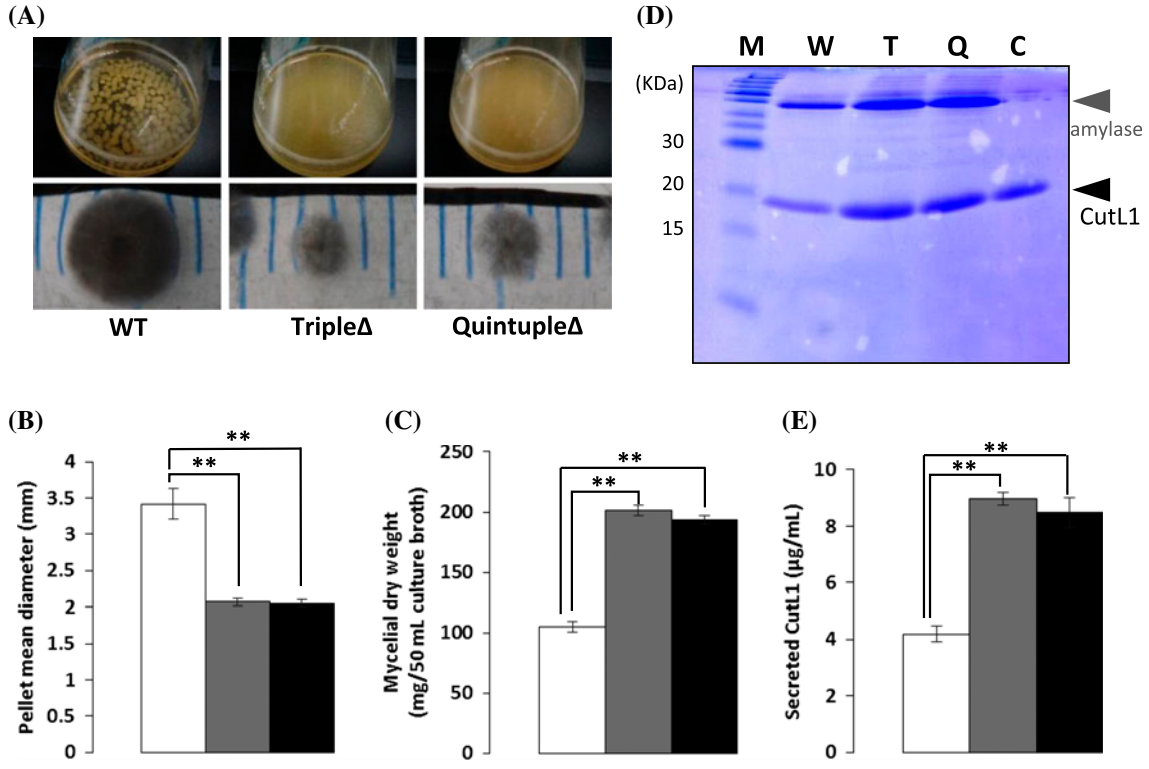

Fig. 3. CutL1 productivity of *Aspergillus oryzae* wild-type (WT), *agsAΔagsBΔagsCΔ* triple mutant (tripleΔ), and *agsAΔagsBΔagsCΔamyDΔamyGΔ* quintuple mutant (quintupleΔ) under liquid culture conditions.

Notes: (A) Growth characteristics of the wild-type-cutL1, tripleΔ-cutL1, and quintupleΔ-cutL1 strains. Photographs of cultures in Erlenmeyer flasks (upper) and of representative hyphal pellets of each strain as seen under a stereomicroscope (bottom; scale = 1 mm) at 24 h of culture. Conidia (final concentration, 1.0×10^5/mL) of each strain were inoculated into 50 mL of YPM medium and rotated at 100 rpm at 30 °C for 24 h. (B) The mean diameter of hyphal pellets for each strain was determined by measuring 10 randomly selected pellets under a stereomicroscope. Wild-type-cutL1, white bar; tripleΔ-cutL1, grey bar; quintupleΔ-cutL1, black bar. Error bars represent the standard deviation of the mean calculated from three replicates (**$p < 0.01$). (C) Mycelial dry weight of each strain. Mycelia grown for 24 h were filtered through Miracloth; collected mycelia were dried at 70 °C. Wild-type-cutL1, white bar; tripleΔ-cutL1, grey bar; quintupleΔ-cutL1, black bar. Error bars represent the standard error of the mean calculated from three replicates (**$p < 0.01$). (D) Proteins in 200 μL of the culture supernatant of each stain were precipitated by addition of 100 μl of 100% (w/v) trichloroacetic acid (TCA). Precipitated proteins were solubilized in 10 μL of sample buffer, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue. Lane M, Clearly Stained Protein Ladder (Takara; molecular weight marker); lanes W, T, and Q, culture supernatant of wild-type-cutL1, tripleΔ-cutL1, and quintupleΔ-cutL1 strains, respectively; lane C, 1 μg of purified CutL1 (control). (E) Amount of CutL1 secreted by each strain. CutL1 was quantified by using ImageJ software. Wild-type-cutL1, white bar; tripleΔ-cutL1, grey bar; quintupleΔ-cutL1, black bar. Error bars represent the standard error of the mean calculated from five replicates (**$p < 0.01$).

(E)). After 24 h of culture in YPM, the amount of CutL1 secreted by the tripleΔ-cutL1 and quintupleΔ-cutL1 strains was approximately twice that produced by the wild-type-cutL1 strain ($p < 0.01$; Fig. 3(E)).

To confirm that the improvement in protein productivity was attributable to the lack of α-1,3-glucan in the cell wall and the resulting small pellet size, we assessed the relationship between protein productivity and pellet size, controlled for inoculum size, under liquid culture conditions (Fig. S5). Conidia (final concentration, 1×10^3, 10^5, or 10^7/mL) of *A. oryzae* wild-type-cutL1 and tripleΔ-cutL1 strains were inoculated into 50 mL of YPM medium and rotated at 100 rpm at 30 °C for 24 h. In the cultures inoculated with 1×10^7/mL conidia, the wild-type-cutL1 strain produced pellets with a mean diameter of 1.9 mm, whereas in the culture inoculated with 1×10^5/mL, the wild-type-cutL1 strain produced pellets with a mean diameter of 3.4 mm ($p < 0.05$). The mean diameter of the pellets produced by the tripleΔ-cutL1 strain in the culture inoculated with 1×10^5/mL conidia (2.5 mm) was smaller than that of the pellets produced by the wild-type-cutL1 strain in the culture inoculated with 1×10^5/mL conidia (3.4 mm) ($p < 0.05$). The tripleΔ-cutL1 strain formed few hyphal pellets in the culture inoculated with 1×10^7/mL conidia. The amount of CutL1 secreted by the wild-type-cutL1 strain in the culture inoculated with 1×10^7/mL conidia was similar to that secreted by the tripleΔ-cutL1 strain in the culture inoculated with 1×10^5/mL conidia, but larger than the amount secreted by the wild-type strain in the culture inoculated with 1×10^5/mL conidia (Fig. S5).

Because the phenotype of *A. oryzae* tripleΔ-cutL1, including CutL1 productivity, was very similar to that of the quintupleΔ-cutL1 strain, we further compared the CutL1 productivity of the tripleΔ-cutL1 strain with that of the wild-type-cutL1 strain by using liquid medium containing 6% glucose to mimic industrial culture conditions in which culture media often contain high concentrations of glucose or fructose. Samples of culture broth were withdrawn from the flasks every 12 h and the concentration of CutL1, mycelial dry weight, pH, and the amount of glucose remaining in the culture broth were determined. The amount of CutL1 secreted by the tripleΔ-cutL1 strain was approximately 150% of that secreted by the wild-type-cutL1 at 60 h ($p < 0.05$)

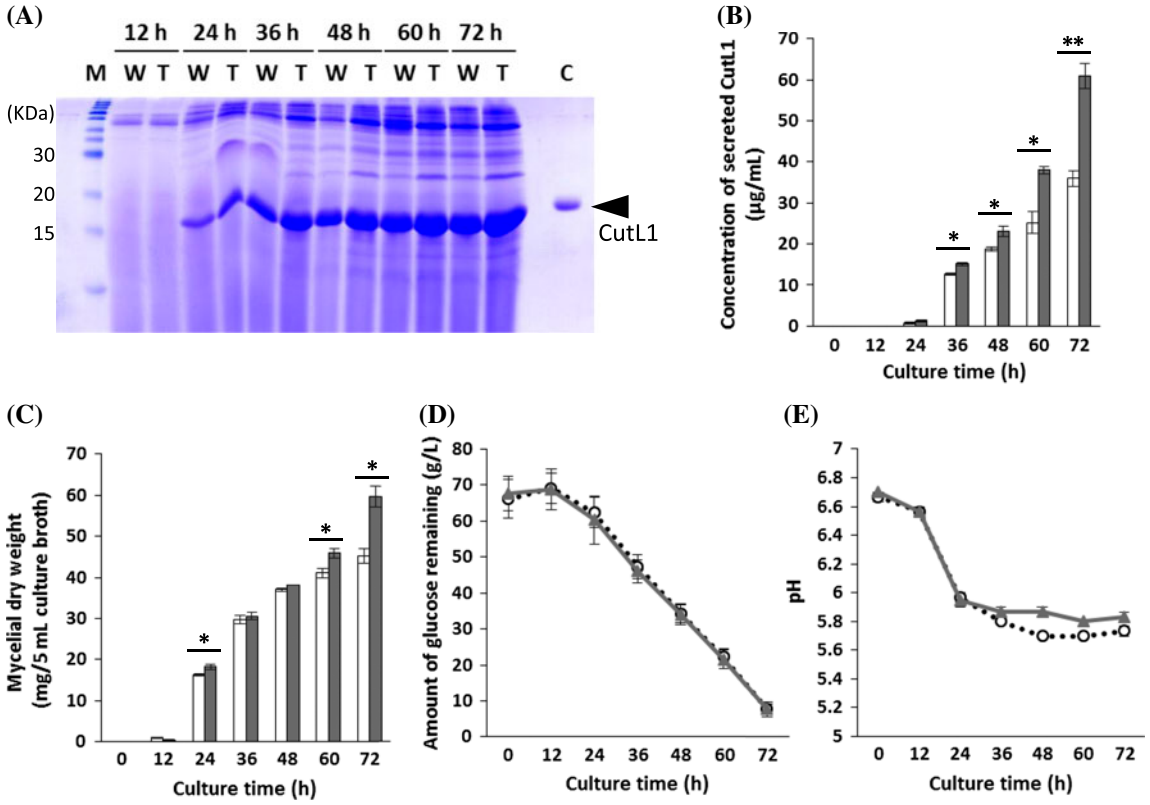

Fig. 4. CutL1 productivity of *Aspergillus oryzae* wild-type-cutL1 and tripleΔ-cutL1 strains grown in the presence of 6% glucose.

Notes: (A) Proteins in 200 μL of the culture supernatants of wild-type-cutL1 and tripleΔ-cutL1 stains were precipitated with trichloroacetic acid. Precipitated proteins were solubilized, subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue. Lane M, Clearly Stained Protein Ladder (Takara; molecular weight marker); lanes W and T, culture supernatant of wild-type and tripleΔ strains, respectively; lane C, 1 μg of purified CutL1 (control). Supernatants were sampled every 12 h. (B) The amount of CutL1 secreted (μg/mL) was quantified by using ImageJ software. Wild-type-cutL1, white bar; tripleΔ-cutL1, grey bar. Error bars represent the standard error of the mean calculated from three replicates (*$p < 0.05$, **$p < 0.01$). (C) Mycelial dry weight. Samples were withdrawn every 12 h and filtered through Miracloth. The mycelia were washed with water and dried at 70 °C. Wild-type-cutL1, white bar; tripleΔ-cutL1, grey bar. Error bars represent the standard error of the mean calculated from three replicates (*$p < 0.05$). (D) The amount of glucose remaining in the supernatant was measured by means of the phenol/H_2SO_4 method. Wild-type-cutL1, dotted line/circles; tripleΔ-cutL1, grey line/triangles. Error bars represent the standard error of the mean calculated from three replicates. None of the differences were statistically significant. (E) pH of the culture supernatants. Wild-type-cutL1, dotted line/circles; tripleΔ-cutL1, grey line/triangles. Error bars represent the standard error of the mean calculated from three replicates. None of the differences were statistically significant.

and 170% of that secreted by the wild-type-cutL1 at 72 h ($p < 0.01$) (Fig. 4(A) and (B)). Although the growth rate (mean 8.2 mg/24 h) of the wild-type-cutL1 strain was decreased at 48 h, that (mean 21.7 mg/24 h) of tripleΔ-cutL1 was not changed even at 72 h ($p < 0.01$) (Fig. 4(C)). These results suggest that *A. oryzae* mutants lacking α-1,3-glucan in their cell wall can be cultured at a high cell-density. The rate of glucose consumption and pH of the culture broth were comparable for the tripleΔ-cutL1 and wild-type-cutL1 strains (Fig. 4(D) and (E)).

Discussion

Filamentous fungi such as aspergilli are used in industrial fermentation processes to produce commercially valuable enzymes and low molecular weight compounds such as organic acids and drug leads.[30] However, under liquid culture conditions filamentous fungi form hyphal pellets, which lowers cell density and prevents high fermentation productivity. *A. nidulans* mutants in which the *agsB* gene encoding the major α-1,3-glucan synthase was disrupted (*agsB*Δ) lost most of the α-1,3-glucan from their cell wall and formed dispersed hyphae in liquid culture rather than pellets[4]; we hypothesized that the same might be true in the industrial fungus *A. oryzae*. We disrupted the three genes encoding α-1,3-glucan synthases in *A. oryzae* (*agsA*Δ*agsB*Δ*agsC*Δ), and we found that the hyphae of the tripleΔ strain lost most of α-1,3-glucan in the cell wall (Fig. 2(C)) but still formed small pellets under liquid culture conditions. The pellets formed by the tripleΔ strain were smaller than those formed by the wild-type strain under liquid culture conditions (Fig. 1(A) and (B)).

Whereas the wild-type strain of the human pathogen *A. fumigatus*, which possesses three α-1,3-glucan synthase genes, forms aggregated, germinating conidia, the mutant in which three α-1,3-glucan synthase genes are disrupted does not.[31,32] Furthermore, in the industrial fungus *Aspergillus niger*, culture conditions such as glucose concentration, temperature, pH, and ionic strength of the culture broth have been shown to affect the aggregation of conidia and hyphae.[33,34] Also in *A. niger*, melanin located on the surface of conidia

contribute to the aggregation of conidia into pellets.[35] It is noteworthy that an *A. nidulans* mutant lacking melanin did not produce cell-wall α-1,3-glucan.[36] Together, these data suggest that melanin, in combination with α-1,3-glucan, is involved in the aggregation of conidia and hyphae. However, in the present study, *A. oryzae* wild-type did not produce melanin under liquid culture conditions and yet still formed aggregated hyphal pellets (Figs. 1, 3, 4).

Contrary to the completely dispersed hyphae of *A. nidulans agsB*Δ and *agsA*Δ*agsB*Δ,[4] the small hyphal pellets produced by *A. oryzae* tripleΔ suggest that other genes are involved in hyphal aggregation besides the three *ags* genes. He et al. recently reported that *amyD* and *amyG* are involved both in α-1,3-glucan biosynthesis and hyphal aggregation in *A. nidulans*.[18] Therefore, we hypothesized that orthologs of *amyD* and *amyG* may be involved in hyphal aggregation in *A. oryzae* tripleΔ. We disrupted the *amyD* and *amyG* genes in the *A. oryzae* tripleΔ strain to construct a quintupleΔ strain; however, *A. oryzae* quintupleΔ still formed small hyphal pellets, suggesting that neither AmyD nor AmyG was involved in hyphal aggregation in *A. oryzae* tripleΔ. Analysis of the composition of the hyphal cell wall showed that most of the α-1,3-glucan was lost in *A. oryzae* tripleΔ and quintupleΔ (Fig. 2(C)). Therefore, besides α-1,3-glucan, other cell-wall components must be responsible for the remaining hyphal aggregation in *A. oryzae* tripleΔ under liquid culture conditions. We are now searching for these additional factors.

Although we did not achieve complete hyphal dispersion in *A. oryzae* under liquid culture conditions, the tripleΔ and quintupleΔ strains of *A. oryzae* did form smaller hyphal pellets than did the wild-type strain. We hypothesized that the larger surface area provided by the smaller pellets formed by *A. oryzae* tripleΔ and quintupleΔ might result in improved fermentation productivity. *A. oryzae* tripleΔ and quintupleΔ, whether or not also overexpressing CutL1, formed hyphal pellets that were approximately 30% smaller than those of the wild-type strain when cultured in YPD/YPDS (Figs. 1 and 4) and YPM (Fig. 3) liquid media. Furthermore, the pH of the culture broth in which tripleΔ or quintupleΔ strain was grown was slightly higher than that of the culture broth in which the wild-type strain was grown at 24–48 h of culture. Together, these results suggest that the small mycelial pellets of the tripleΔ and quintupleΔ strains, whether or not also overexpressing CutL1, meant that fewer cells inside the pellets were subject to anaerobic conditions, leading to an increase in the total energy yield by respiration and consequently an increase in biomass (Figs. 1(B) and 3(C)). In addition, the sizes of the mycelial pellets produced by the wild-type strain were larger than those produced by the tripleΔ and quintupleΔ strains (Figs. 1(B) and 3(B)). Because the cells inside the pellets are considered to be under anaerobic conditions and therefore cannot fully metabolize sugars via the tricarboxylic cycle, the cells inside the larger pellets of the wild-type strain are thought to produce organic acids. The total number of cells under anaerobic conditions in the hyphal pellets of the wild-type strain was considerably larger than those in the hyphal pellets of the tripleΔ and quintupleΔ strains, but the pH of the culture broth in which the wild-type strain was grown was only slightly lower than that of the culture broth in which the tripleΔ and quintupleΔ strains were grown. We therefore hypothesized that the increased biomass of the tripleΔ and quintupleΔ strains under liquid culture conditions may improve fermentation productivity.

Because the *A. oryzae* secretory enzyme CutL1 is used to degrade polyesters such as polybutylene succinate-co-adipate,[19] we selected CutL1 as a model enzyme for comparing enzyme productivity among the *A. oryzae* wild-type, tripleΔ, and quintupleΔ strains. In YPM medium, tripleΔ-cutL1 and quintupleΔ-cutL1 strains formed smaller hyphal pellets than wild-type-cutL1 (Fig. 3(B)). As expected, the CutL1 productivity of the tripleΔ-cutL1 and quintupleΔ-cutL1 strains were significantly higher than those of the wild-type strain (Fig. 3(E)). Based on the data presented in Fig. 3(B) and (C), no significant differences were observed in CutL1 secretion among the wild-type-cutL1 (37.6 ± 0.6 ng CutL1/mg mycelial dry weight), tripleΔ-cutL1 (44.6 ± 2.8 ng/mg), and quintupleΔ-cutL1 (43.9 ± 5.8 ng/mg) strains. This suggests that the increase in the production of CutL1 per batch in the *A. oryzae* tripleΔ strain was attributable to the increased biomass of tripleΔ-cutL1, and therefore this strain may be highly beneficial for use in industrial fermentation processes.

In the tripleΔ and quintupleΔ strains, the production of endogenous amylase (AmyB) was also increased compared to that in the wild-type strain (Fig. 3(D)), suggesting that the protein secretion machinery in the α-1,3-glucan-deficient mutants remains genetically unaltered. This result suggests that α-1,3-glucan-deficient mutants may be used to produce not only CutL1 but also other secretory proteins under liquid culture conditions.

Small hyphal pellets similar to those produced by the tripleΔ strain can be achieved by increasing the inoculum size of wild-type conidia (1×10^5/mL), which leads to an improvement in CutL1 productivity (Fig. S5). Therefore, regardless of the presence or absence of α-1,3-glucan in the cell wall, when small pellets can be formed, enzyme productivity can be improved. However, it is not feasible to prepare and inoculate a larger number of conidia (e.g. 1×10^7/mL conidia) on an industrial scale. Since higher productivity was achieved by the tripleΔ strain even with a small inoculum size (e.g. 1×10^5/mL conidia) (Fig. S5), α-1,3-glucan-deficient strains are still likely to have beneficial industrial uses.

Cheap carbon sources such as sugar cane and corn steep liquor are commonly used in industrial fermentation processes. Such carbon sources generally contain high concentrations of sucrose, glucose, fructose, or glucose oligomers, which induce carbon catabolite repression and limit fermentation productivity. Therefore, we examined the fermentation productivity of *A. oryzae* wild-type-cutL1 and tripleΔ-cutL1 strains in the presence of a high concentration of glucose (6% w/v) (Fig. 4(A) and (B)). Interestingly, the tripleΔ-cutL1 strain produced a significantly higher amount of CutL1 (Fig. 4(B)) and a greater amount of biomass at 48 h of culture compared with the wild-type-cutL1 strain (Fig. 4(C)). After 48 h of culture, the growth rate of

the wild-type strain was decreased, but that of the tripleΔ-cutL1 strain was not. This decrease in the growth rate of the wild-type strain might be due to the formation of larger pellets, leading to an increase in the number of cells under anaerobic conditions inside the pellets and to the observed decrease in protein productivity after 48 h of culture. In contrast, the cells of the tripleΔ-cutL1 strain were kept under aerobic conditions until the end of the culture period and a higher protein productivity compared with the wild-type strain was observed. Consequently, the constructed *A. oryzae* tripleΔ strain may be useful for improving the productivity of industrial fermentation processes.

In the present study, we succeeded in improving the enzyme productivity of the industrial fungus *A. oryzae* by constructing mutants lacking α-1,3-glucan in their cell wall. Removal of α-1,3-glucan from the cell wall of *A. oryzae* hyphal cells led to the formation of smaller hyphal pellets. The smaller hyphal pellets formed by the *A. oryzae* tripleΔ strain meant that more hyphal cells were subject to aerobic conditions, which increased the efficiency of the respiratory energy generation that is required for enzyme production. Contrary to the complete hyphal dispersion of the *A. nidulans agsB*Δ and *agsA*Δ*agsB*Δ mutants developed previously, the *A. oryzae* tripleΔ and quintupleΔ strains still formed small hyphal pellets, suggesting that other factors affect hyphal aggregation in *A. oryzae*. Our group is now examining the remaining factors that affect hyphal aggregation, and complete hyphal dispersion in *A. oryzae* would lead to better fermentation productivity. Our approach should be applicable to other industrial fungi that have α-1,3-glucan in their cell walls.

Author contributions

KM, AY, and KA conceived and designed the experiments. KM, ZS, MS, and KG carried out the construction of the fungal mutants. KM performed the essential experiments and analyzed the data. KM, AY, and KA wrote the paper. All authors discussed the results and commented on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental material

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