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Cell wall α-1,3-glucan prevents α-amylase adsorption onto fungal cell in submerged culture of *Aspergillus oryzae*

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We have previously reported that α-amylase (Taka-amylase A, TAA) activity disappears in the later stage of submerged *Aspergillus oryzae* culture as a result of TAA adsorption onto the cell wall. Chitin, one of the major components of the cell wall, was identified as a potential factor that facilitates TAA adsorption. However, TAA adsorption only occurred in the later stage of cultivation, although chitin was assumed to be sufficiently abundant in the cell wall regardless of the submerged culture period. This suggested the presence a factor that inhibits TAA adsorption to the cell wall in the early stage of cultivation. In the current study, we identified α-1,3-glucan as a potential inhibiting factor for TAA adsorption. We constructed single, double, and triple disruption mutants of three α-1,3-glucan synthase genes (*agsA*, *agsB*, and *agsC*) in *A. oryzae*. Growth characteristics and cell wall component analysis of these disruption strains showed that AgsB plays a major role in α-1,3-glucan synthesis. In the *ΔagsB* mutant, TAA was adsorbed onto the mycelium in all stages of cultivation (early and later), and the *ΔagsB* mutant cell walls had a significantly high capacity for TAA adsorption. Moreover, the α-1,3-glucan content of the cell wall prepared from the wild-type strain in the later stage of cultivation was markedly reduced compared with that in the early stage. These results suggest that α-1,3-glucan is a potential inhibiting factor for TAA adsorption onto the cell wall component, chitin, in the early stage of submerged culture in *A. oryzae*.

[**Key words:** *Aspergillus oryzae*; α-Amylase adsorption; α-1,3-Glucan; Cell wall; Submerged culture]

For over a thousand years, the filamentous fungus *Aspergillus oryzae* has been used in the production of traditional fermented foods, such as sake, soy sauce, and soybean paste (miso) in Japan (1). *A. oryzae* can produce copious amounts of amylolytic enzymes, particularly α-amylase (Taka-amylase A; TAA). However, TAA secreted in the early stage of submerged culture is adsorbed onto the surface of the fungal cell wall in the later stage of cultivation (2–5).

The fungal cell wall is mostly composed of polysaccharides: α-glucans (α-1,3-glucan and α-1,4-glucan, which are polysaccharides composed of D-glucose); β-glucans (β-1,3-glucan and β-1,6-glucan); mannan; and chitin (6–9). The polysaccharides in the fungal cell wall are classified into two groups according to their solubility in alkaline solutions. Alkaline-soluble polysaccharides are composed of α-1,3-glucan and some galactomannan, and the main components of alkaline-insoluble polysaccharides are β-1,3-glucan and chitin, both of which constitute the structural skeleton of the cell wall (10,11). We previously reported that treatment of the cell wall fraction with an alkaline solution revealed a relatively high TAA adsorption capacity, suggesting that the alkaline-insoluble fraction is involved in TAA adsorption (5). However, treatment of the alkaline-insoluble fraction with Zymolyase-100T significantly increased TAA adsorption capacity, whereas treatment of the alkaline-insoluble faction with chitinase reduced TAA adsorption capacity (5). Moreover, a chitin preparation adsorbed TAA efficiently, whereas curdlan from *Aeromonas faecalis*, composed of β-1,3-glucan, showed no TAA adsorption (5). The treatment of the cell wall fraction with the alkaline solution and enzymes indicated that chitin is a cell wall component that allows TAA adsorption. We assumed that chitin was sufficiently abundant in the cell wall, regardless of the culture period. However, the ability to adsorb TAA appeared in the later stage of cultivation but not in the early stage (5). This suggests that a putative factor that inhibits TAA adsorption exists in the cell wall in the early stage of cultivation. Because TAA absorption capacity was conferred by treatment of the cell wall fraction with dilute alkaline in the early stage of cultivation, a potential candidate for the inhibiting factor is an alkaline-soluble polysaccharide, namely α-1,3-glucan (5).

In the present study, we determined whether α-1,3-glucan in the cell wall is a potential inhibiting factor for TAA absorption. For this purpose, we used the single, double, and triple disruption strains of three α-1,3-glucan synthase genes in *A. oryzae*, some of which had been constructed previously (12). We found that disruption of one of the three α-1,3-glucan synthase genes, designated *agsB*, resulted in a significant decrease in the α-1,3-glucan

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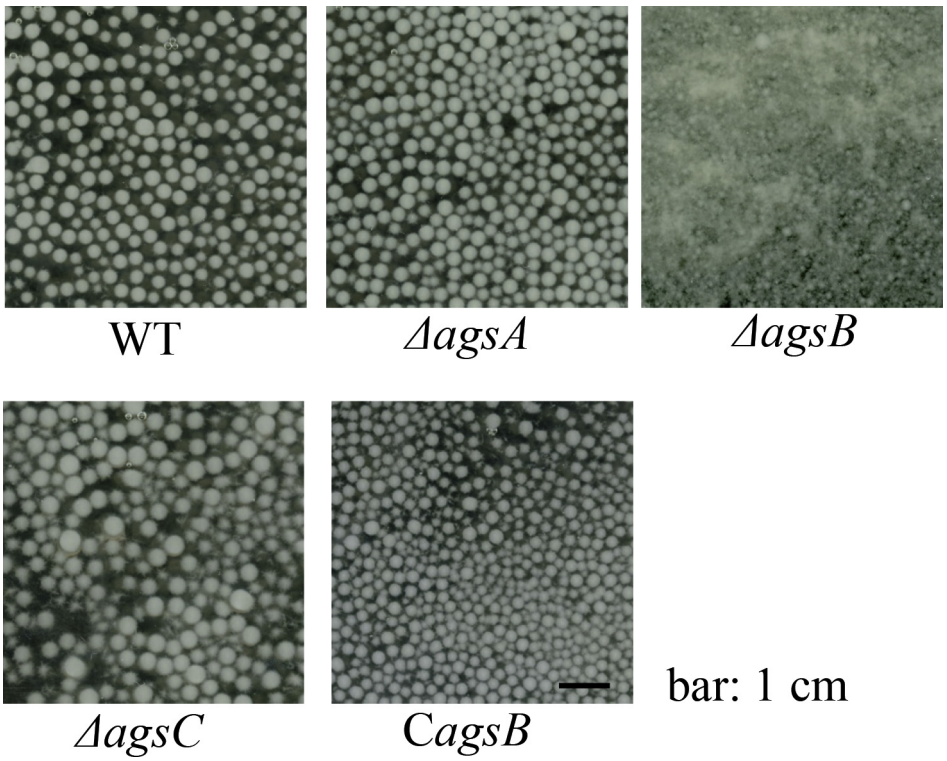

FIG. 1. Morphological phenotypes of α-1,3-glucan synthase gene disruptants in submerged culture. Approximately 1×10^6 conidiospores of each strain were grown in 50 mL YPM medium at 30°C for 24 h with shaking (120 rpm). Scale bar represents 1 cm.

content of the cell wall and a simultaneous increase in TAA absorption capability, even in the early stage of submerged culture. These results indicate the possibility that α-1,3-glucan in the cell wall is involved in preventing TAA adsorption onto the cell wall.

MATERIALS AND METHODS

Strains and media We used an *A. oryzae adeA*$^-$ strain (*niaD*$^-$, Δ*ligD*::*sC*, Δ*adeA*::*ptrA*) (13) constructed from the *ligD* disruptant (Δ*ligD*::*sC*, *niaD*$^-$) (14), which was derived from *A. oryzae* wild-type strain RIB40 (National Research Institute of Brewing Stock Culture) as the host strain for the disruption of α-1,3-glucan synthases genes. *Escherichia coli* DH5α (*endA1*, *hsdR17*, *supE44*, *recA1*, *gyrA96*, *thi-1*, *relA1*, *deoR*, Φ*80lacZ*ΔM15, Δ[*lacZYA*−*argF*]U169) was used for the construction and propagation of plasmid DNAs. *Saccharomyces cerevisiae* BY4741 (*MATa*, *his3*Δ, *leu2*Δ, *met15*Δ, *ura3*Δ) was used for *in vivo* plasmid construction for gene deletion. The minimal medium (MM) for *A. oryzae* culture was Czapek−Dox (CD) medium, which contained 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.2% KH_2PO_4, 0.05% $MgSO_4$, trace amounts of $FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $Na_2B_4O_7$, and $(NH_4)_6Mo_7O_{24}$, and 1% sugar. We added 0.01% adenine to the medium to culture of the *adeA*$^-$ strain, and used YPM medium containing 0.5% yeast extract, 1% Bacto Peptone, and 2% maltose for fungal growth and TAA production. Approximately 10^6 conidiospores per 50 mL of medium was inoculated and incubated at 30°C with shaking at 120 rpm.

Construction of plasmid DNAs for α-1,3-glucan synthase gene disruption The plasmids for *agsA*, *agsB*, and *agsC* disruption were constructed as described previously (12). Briefly, to generate a plasmid for *agsA* disruption, the 5′- and 3′-flanking regions of *agsA* were amplified by polymerase chain reaction (PCR) with primer sets agsA-LU + agsA-LL-loxP and agsA-RU-loxP + agsA-RL using the *A. oryzae* RIB40 genomic DNA as the template. A self-excising marker cassette containing both the Cre recombinase gene (*cre*) and the *Aspergillus nidulans adeA* gene (*AnadeA*) flanked by mutant *lox* sequences *lox66* and *lox71* was prepared from the plasmid pAAAXP-Cre, as reported previously (13), by PCR with the primer set adeA-Cre-loxP-Fw + adeA-Cre-loxP-Rv. The yeast vector pYES2 (Life Technologies, Carlsbad, CA, USA) was digested using *Eco*RI and *Bam*HI. These four DNA fragments were assembled in *S. cerevisiae* through endogenous homologous recombination, which resulted in *ΔagsA*/pYES2 (Supplementary Fig. S1A). Similarly, we generated plasmids for *agsB* and *agsC* disruption. The nucleotide sequences of all the primers used for plasmid construction are shown in Supplementary Table S1.

Construction of disruption mutants for α-1,3-glucan synthase genes and complemented strain of *agsB* disruption We constructed single, double, and triple disruption mutants for *agsA*, *agsB*, and *agsC* using the Cre/*lox* marker recycling system developed previously (13). The construction of a single disruptant (*ΔagsA*), a double disruptant (*ΔagsAΔagsB*), and a triple disruptant (*ΔagsAΔagsBΔagsC*) have been described elsewhere (12). In this study, further disruptants including single *ΔagsB* and *ΔagsC* disruptants, double *ΔagsAΔagsC*, and *ΔagsBΔagsC* were constructed according to the methods previously reported (12). For selectable marker recycling, the Cre recombinase gene (*cre*) was regulated by the *Penicillium chrysogenum xylP* promoter, and the resulting transformants were cultivated under *cre*-inducing conditions, i.e., grown on a xylose plate that allowed removal of selectable markers together with a Cre expression construct (Supplementary Fig. S1B). Gene disruption was confirmed by genome PCR (Supplementary Fig. S2).

To construct the *agsB* complementation cassette for introduction into the *ΔagsB* strain, the DNA fragment containing the *agsB* promoter coding region and the 3′-flanking region was amplified with PCR using primer set agsB-comp + agsB-RL, using *A. oryzae* genomic DNA. Furthermore, the 5′-flanking region of the *agsB* gene and a self-excising marker cassette were also amplified using primer sets agsB-LU + agsB-LL-loxP and adeA-Cre-loxP-Fw + adeA-Cre-loxP-Rv, respectively. These three PCR-amplified DNA fragments were then assembled with pYES2 digested by *Eco*RI and *Bam*HI in the yeast (Supplementary Fig. S1C).

Fungal transformation The transformation of *A. oryzae* was performed according to the method described by Gomi et al. (15). YPD liquid medium was used to cultivate *A. oryzae* strains for protoplast preparation.

Sensitivity to cell wall-perturbing agents For each strain, approximately 10^5−10 conidiospores were inoculated on CD plates containing 30 μg/mL of Calcofluor-white (CFW) or 200 μg/mL of Congo red (CR), and cultivated at 30°C for 72 h.

Fractionation of the cell wall components by alkali treatment and determination of the carbohydrate composition of the cell wall fractions Cell wall component fractionation was carried out by the hot water and alkaline treatment method (16). The carbohydrate composition of the cell wall fractions was quantitatively determined as described previously (16).

Measurement of α-amylase activity We used the iodine−starch method to determine α-amylase activity (5,17). The mycelium was harvested by filtration through Miracloth (EMD Chemicals Inc., San Diego, CA, USA), and was incubated in 0.1 M phosphate buffer (pH 7.0) for 60 min to liberate the α-amylase that had been adsorbed on the fungal cell wall. The α-amylase activity levels in the phosphate buffer and the culture supernatant were added to calculate the total α-amylase activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis The culture supernatant and 0.1 M phosphate buffer containing TAA released from the cell wall were concentrated by precipitation with 10% trichloroacetic acid (TCA) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

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