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Characterization of Cell Wall α-1,3-Glucan–Deficient Mutants in *Aspergillus oryzae* Isolated by a Screening Method Based on Their Sensitivities to Congo Red or Lysing Enzymes

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Abstract: We previously reported that sensitivity to Congo Red (CR) or Lysing Enzymes (LE) is affected by the loss of cell-wall α-1,3-glucan (AG) in *Aspergillus nidulans*. We found that the amount of CR adsorbed to AG was significantly less than the amount adsorbed to β-1,3-glucan (BG) or chitin, suggesting that loss of cell-wall AG would increase exposure of BG on the cell surface, and thereby increase the sensitivity to CR. Generally, fungal BGs are known as biological response modifiers because of their recognition by Dectin-1 receptors in human immune systems. Therefore, isolation of AG-deficient mutants in *Aspergillus oryzae* has been used in the Japanese fermentation industry to create strains with increased ability to promote immune responses. Here, we aimed to isolate AG-deficient strains by mutagenizing *A. oryzae* conidia with chemical mutagens. Based on the increased sensitivity to CR in AG-deficient strains of *A. nidulans* and *A. oryzae*, we established a screening method for isolation of AG-deficient strains. Several candidate AG-deficient mutants of *A. oryzae* were isolated using the screening method; these strains showed increased sensitivity to CR and/or LE. Cytokine production was increased in the dendritic cells co-incubated with germinated conidia of the AG-deficient mutants. Furthermore, according to a Dectin-1 NFAT (nuclear factor of activator T cells)-GFP (green fluorescent protein) reporter assay, Dectin-1 response levels in the AG-deficient mutants were higher than those in wild-type *A. oryzae*. These results suggest that we successfully isolated AG-deficient mutants of *A. oryzae* with immunostimulatory effects.

Key words: *Aspergillus oryzae*, α-1,3-glucan, Congo Red sensitivity, Lysing Enzymes sensitivity, immunostimulatory effect

INTRODUCTION

The cell wall of fungi not only plays a role in maintaining cell morphology, but also helps to protect the cells from various kinds of extracellular environmental stress. In addition, for both animals and plant pathogenic fungi, the cell wall is the first body part to make contact with the host cells. Thus, understanding the structure of the cell wall is crucial to develop diverse methods of controlling pathogens. The cell walls of filamentous fungi are complex structures that are mainly composed of polysaccharides, such as α-1,3-glucan (AG), β-1,3-glucan (BG) with β-1,6-branches, galactomannan, and chitin.[1)] The importance of cell wall α-1,3-glucan in relation to fungal virulence has been studied in several pathogenic fungi. In the opportunistic pathogen *Cryptococcus neoformans*, loss of cell wall

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Abbreviations: AG, α-1,3-glucan; AGS, α-1,3-glucan synthase; BG, β-1,3-glucan; CR, Congo Red; DCs, dendritic cells; GFP, green fluorescent protein; LE, Lysing Enzymes; NFAT, nuclear factor of activator T cells.

AG leads to a loss of the surface capsule.[2] The capsule appears to promote pathogenesis in the wild type: mutants that lack cell wall AG are unable to grow in a mouse model of infection.[3] A correlation between reduction in AG level and virulence has also been observed in several dimorphic fungal pathogens, such as *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Blastomyces dermatitidis*.[4][5][6] Rappleye *et al*.[7] reported that AG conceals BG in the cell wall of *H. capsulatum* from Dectin-1-mediated detection by immune cells, blocking recognition of fungal invasion by host immune cells. It is assumed that pathogenic fungi have developed such unique stealth mechanisms to conceal their immunostimulatory molecular patterns from recognition via leukocyte receptors; this concealment disturbs the host immune responses and establishes pathogenesis.[7] In the rice blast fungus *Magnaporthe grisea*, AG masks BG and chitin in the cell wall of infectious hyphae, indicating that AG protects the fungal cell wall from digestive enzymes produced by plant cells during infection and inhibits recognition by host cells.[8]

Generally, fungal β-glucans (BG with β-1,6-linked side chains) are known as biological response modifiers because of their immunostimulatory activity.[9][10] For example, the polysaccharide krestin (PSK) is one of the most commonly used mushroom extracts derived from *Trametes versicolor*.[11] The major component is β-glucan compounds ranging in size from 94 to 100 kDa.[11] PSK can act as an immune modulator, by inducing gene expression of IL-8 in peripheral blood mononuclear cells after oral administration,[12] stimulating T-cell proliferation,[13] and improving the function of $CD4^+$ T cells in gut-associated lymphoid tissue.[14] Furthermore, β-glucans are present in the cell wall of the koji mold *Aspergillus oryzae*,[15] which is an important filamentous fungus used in the traditional Japanese fermentation industry to produce *sake* (rice wine), *shoyu* (soy sauce), and *miso* (soybean paste).[16] However, the cell wall of the koji mold *A. oryzae* also includes AG,[15] which is predicted to be located at the cell surface and conceal the immunostimulatory β-glucans.[1] Thus, a cell wall AG–deficient strain of koji mold might present β-glucans to the cell surface, resulting in increased immunostimulatory activity. Therefore isolation of AG-deficient strains would be expected to lead to the development of high value-added production of functional foods that include the cells of the koji mold, such as *miso* and *amazake* (a sweet drink made from fermented rice).

Our previous work revealed that cell wall AG–deficient strains showed increased sensitivity to Congo Red (CR) in the model fungus *Aspergillus nidulans*.[17] Similarly, wild-type *A. oryzae* shows higher resistance to CR than do AG–deficient strains in which three AG synthase (AGS) genes are disrupted.[18][19] We also showed that cell wall AG–deficient strains of *A. nidulans* showed increased susceptibility to a commercial preparation of Lysing Enzymes from *Trichoderma harzianum* (LE), which contains β-1,3-glucanase and chitinase.[17] Our data revealed that AG–deficient strains of *A. nidulans* showed increased sensitivity to CR not only on plate culture,[17] but also under liquid culture conditions (whereas wild-type *A. nidulans* can grow in liquid medium containing 10 μg/mL CR, AG-deficient strains of *A. nidulans* scarcely grow in the same medium; Yoshimi *et al*. unpublished). Thus, it is possible to distinguish and separate AG-deficient strains from the wild-type strain of *A. nidulans* on the basis of differences in hyphal pellet size in CR-containing liquid medium. However, it is necessary to overcome the following two issues for the production of functional foods: 1) the isolation of an AG-deficient strain from industrial strains of the koji mold, *A. oryzae*; and 2) the breeding of the strain using mutagenesis because of the demand of non-genetically-modified organisms as functional foods in commercial markets.

Here, we developed a screening method to isolate AG–deficient mutants of *A. oryzae* based on CR sensitivity in liquid culture medium. Using this screening method, we succeeded in isolating several candidate AG–deficient mutants of *A. oryzae.* We explored the α-1,3-glucan content in the cell walls of the mutants, their sensitivity to CR and LE, and the levels of cytokine, tumor necrosis factor (TNF)-α, produced by dendritic cells (DCs) co-incubated with germinated-conidia of the mutants. In addition, a Dectin-1 NFAT (nuclear factor of activator T cells)-GFP (green fluorescent protein) reporter assay [22][23] was also performed to confirm immunostimulatory effects of the mutants. The results suggested that we had successfully isolated AG-deficient mutants of *A. oryzae* with immunostimulatory effects.

MATERIALS AND METHODS

Strains and media. Fungal strains used in this study are listed in Table 1. *Aspergillus nidulans* DagsA-DagsB, a strain in which the two genes encoding AGSs (*i.e., agsA*, *agsB*) are disrupted, was produced as previously described.[17] *A. nidulans* ABPU1 strain was used as the control strain for the DagsA-DagsB strain. All *A. nidulans* strains were cultivated in Czapek-Dox (CD) medium, which contains 6 g/L $NaNO_3$, 0.52 g/L KCl, 1.52 g/L KH_2PO_4, 0.59 g/L $MgSO_4{\cdot}7H_2O$, 1 mL/L trace elements solution [1 % g/L $FeSO_4{\cdot}7H_2O$, 8.8 g/L $ZnSO_4{\cdot}7H_2O$, 0.4 g/L $CuSO_4{\cdot}5H_2O$, 0.15 g/L $MnSO_4{\cdot}4H_2O$, 0.1 g/L $Na_2B_4O_7{\cdot}10H_2O$, 0.05 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$], and 20 g/L glucose. The CD medium was adjusted to pH 6.5 with KOH. 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.

Aspergillus oryzae tripleΔ strain, in which the three genes encoding AGSs (*agsA*, *agsB,* and *agsC*) were disrupted, was produced as previously described;[18][19] *A. oryzae* CNT (Δ*ligD*::*sC*, *adeA*$^+$) strain was used as the control for the tripleΔ strain; and the strains M302, M452, E602 were generated from the parental *A. oryzae* strains, Kaori and RIB161, by mutagenesis. All *A. oryzae* strains were cultivated in CDE medium, which is CD medium in which the nitrogen source is 70 mM sodium hydrogen L(+)-glutamate monohydrate instead of 70 mM $NaNO_3$. Conidia of *A. oryzae* were isolated from cultures grown on malt medium containing 90 g/L malt extract (Becton Dickinson and Company, Sparks, USA), 5 g/L yeast extract (Becton Dick-

Table 1. Strains used in this study.

Strain	Genotype	Source or reference
Aspergillus nidulans		
ABPU1	*biA1*, *pyrG89*, *wA3*, *argB2*, *pyroA4*, *ligD*Δ::*ptrA*	Yoshimi *et al*. 2013[17]
DagsA-DagsB	*biA1*, *pyrG89*, *wA3*, *argB2*, *pyroA4*, *ligD*Δ::*ptrA,* Δ*agsA*::*pyrG*, Δ*agsB*::*argB*	Yoshimi *et al*. 2013[17]
Aspergillus oryzae		
CNT (Δ*ligD*::*sC*, *adeA*$^+$)	*sC*$^-$, *niaD*$^-$, Δ*ligD*::*sC*, Δ*adeA*::*ptrA*, *adeA*$^+$	Miyazawa *et al*. 2016[18]
TripleΔ (Δ*agsA*Δ*agsB*Δ*agsC*)	*sC*$^-$, *niaD*$^-$, Δ*ligD*::*sC*, Δ*adeA*::*ptrA*, Δ*agsA*::*loxP*, Δ*agsB*::*loxP*, Δ*agsC*::*loxP*, *adeA*$^+$	Miyazawa *et al*. 2016[18]
Kaori	Wild type	Purchased[a]
RIB161	Wild type	From NRIB[b]
M302	Derived from Kaori	This study
M452	Derived from RIB161	This study
E602	Derived from RIB161	This study

[a] from Higuchi Matsunosuke Shoten Co., Ltd. [b] NRIB, National Research Institute of Brewing, Japan.

inson and Company), and 1 mL/L trace elements solution.

Mutagenesis. Fungal mutagenesis was performed as described previously[20] with slight modification. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and ethyl methanesulfonate (EMS, Sigma, St. Louis, USA) were used as mutagens. For treatment with MNNG, 1-mL aliquots of MNNG solution (20 mg/mL in distilled water) were added to 30-mL vials containing washed conidia (1×10^8 cells) suspended in 10 mL of 0.1 M sodium phosphate buffer (pH 6.4) and Tween 20 (0.02 %, v/v). For treatment with EMS, a 0.15-mL aliquot of EMS solution was mixed in 30-mL vials with washed conidia (1×10^8 cells) suspended in 10 mL of 0.1 M phosphate buffer (pH 8.0) and Tween 20 (0.02 %, v/v). Mutagenesis was timed from the addition of the chemical. During mutagen treatments, the conidial suspensions were maintained at 32 °C and stirred using magnetic stirrers in the vials. Treatments were terminated by centrifugation to submerge the conidia, the mutagenic solution was poured off, and the conidia were suspended in chilled water. The conidia were washed twice with water and then resuspended in water to the same density as in the mutagenesis step.

CR and LE sensitivity. The hyphal growth of the *A. nidulans* and *A. oryzae* strains in liquid culture containing CR were determined by measuring the mean diameters of the hyphal pellets. Conidia of *A. nidulans* and *A. oryzae* strains were suspended in CD or CDE liquid media (total of 1×10^5 cells/mL), respectively, containing various concentrations of CR (0, 1, 5, or 10 mg/mL). Conidia of each strain were incubated with rotation (160 rpm) at 37 °C (*A. nidulans*) or 30 °C (*A. oryzae*) for 16 or 20 h. The diameters of the hyphal pellets were then measured under a microscope (IX81, Olympus Corporation, Tokyo, Japan).

The sensitivity of *A. oryzae* strains to CR on plate media was evaluated by the method described previously.[17] Conidial suspensions of each strain (total of 1×10^4 cells) were spotted on the centers of plates containing various concentrations of CR (1, 5, 20, 40, or 80 mg/mL). The dose response was determined 4 days after incubation at 30 °C by plotting the mean diameters of the colonies on the treated media as a percentage of those on the control medium. Each experiment was performed in triplicate.

Susceptibility of the fungal cell wall to LE, a commercial preparation containing β-1,3-glucanase and chitinase (Sigma, St. Louis, USA), was assayed using the method described previously.[17] Washed 1-day old mycelia of *A. oryzae* (30 mg fresh weight) grown in CDE medium at 30 °C were suspended in 1 mL of 0.8 M NaCl in sodium phosphate buffer (10 mM, pH 6.0) containing 10 mg/mL LE. After 1, 2, or 4 h of incubation at 30 °C, the number of protoplasts generated from the mycelia was counted by using a hemocytometer (A106, SLGC, Tokyo, Japan). Mycelia incubated in 0.8 M NaCl in sodium phosphate buffer (10 mM, pH 6.0) containing no enzyme were used as controls.

Fractionation of cell walls by alkali treatment. The steps used for cell-wall fractionation have been described previously.[17] Mycelia cultivated in liquid CD medium at 37 °C for 24 h were collected and freeze-dried. According to their solubility in alkali, the freeze-dried mycelia were fractionated into four fractions: hot-water–soluble (HW); alkali-soluble 1 (AS1), which contained the water-soluble components after dialysis of the alkali-soluble fraction; alkali-soluble 2 (AS2), which contained the water-insoluble components after dialysis of the alkali-soluble fraction; and alkali-insoluble (AI). All fractions were freeze-dried, and each fraction was weighed.

Quantitative determination of the carbohydrate composition of the cell-wall fractions. The procedure for quantification of the carbohydrate composition of the cell-wall fractions has been described previously.[17] Each freeze-dried cell-wall fraction (10 mg dry weight) was hydrolyzed in sulfuric acid, and the carbohydrate composition was determined by using high-performance chromatography with a pulsed electrochemical detector and an anion-exchange column (Carbo PAC PA-1, 4 × 25 mm; Dionex Corporation, Sunnyvale, USA).

Assay of cytokine production. The conidia collected from colonies cultured for 1 week on CDE agar plates were used for assays of cytokine production. DCs were prepared from bone marrow cells as described previously.[21] When DCs were incubated with various amount of the conidia, the concentration of DCs was adjusted to 1×10^5 cells/mL and cultures were incubated for 24 h at 37 °C in a 5 % CO_2 incubator. The concentration of TNF-α in the culture superna-

Table 2. Diameter of fungal pellets in *A. nidulans* wild-type (ABPU1) and α-1,3-glucan–deficient (DagsA-DagsB) strains in medium containing Congo Red.

Concentration of CR (μg/mL)	Culture time (h)	Diameter of fungal pellet (μm) [a]	
		ABPU1	DagsA-DagsB
0	20	1360.7 ± 65.3	N.D.[b]
1	20	219.3 ± 71.4	218.6 ± 53.2
5	20	114.9 ± 69.2	N.D.[c]
10	20	58.9 ± 18.6	N.D.[c]

[a] Data are means ± S.D. (n = 20). [b] Not determined, because the hyphae of the DagsA-DagsB strain were completely dispersed. [c] Not determined, because only non-germinating conidia were detectable.

Table 3. Diameter of fungal pellets in *A. oryzae* wild-type (CNT) and α-1,3-glucan–deficient (tripleΔ) strains in medium containing Congo Red (CR).

Concentration of CR (μg/mL)	Culture time (h)	Diameter of fungal pellet (μm)[a]	
		CNT strain	TripleΔ strain
0	20	522.2 ± 45.6	315.6 ± 83.5[b]
1	20	575.0 ± 169.5	159.3 ± 90.6[b]
5	20	280.0 ± 190.7	73.3 ± 16.7[c]
10	20	115.6 ± 48.4	78.7 ± 36.5

[a] Data are means ± S.D. (n = 10). [b] $p < 0.0001$ *versus* CNT strain. [c] $p = 0.0075$ *versus* CNT strain.

tants was measured by ELISA (BD Biosciences, Franklin Lakes, USA).

Dectin-1–NFAT–GFP reporter assay. Dectin-1–NFAT–GFP reporter cells[22] were kindly provided by Professor Sho Yamasaki (Medical Institute of Bioregulation, Kyushu University, Japan). These cells were stimulated at 2.5 × 10^5/mL with various amounts of conidia of *A. oryzae* or heat-killed *Candida albicans* (ATCC18804) for 20 h, and the expression of GFP was analyzed using a BD FACS Canto II flow cytometer (BD Biosciences).

Statistical analysis. CR and LE sensitivity tests, monosaccharide composition, and cytokine production were analyzed using Welch's *t*-test (for comparison between two groups) or Dunnett's test (for multiple comparison); significance was defined as a *p*-value of less than 0.05.

RESULTS

Comparison of CR sensitivity in AG-deficient mutants of A. nidulans and A. oryzae in liquid culture.

To isolate AG-deficient strains on the basis of hyphal pellet size in CR-containing liquid medium, we first compared hyphal pellet sizes between the wild-type (ABPU1) and AG-deficient (DagsA-DagsB) strains of *A. nidulans* (Table 2). In the medium with 0 μg/mL CR, the hyphal pellet size of the DagsA-DagsB strain was not determined because of the complete dispersion of the hyphae in the liquid medium (Table 2). When the DagsA-DagsB strain was cultured in the medium containing 1 μg/mL CR, the hyphae of the DagsA-DagsB strain were loosely aggregated (Table 2); however, no differences in the size of the hyphal pellets were detected between the wild-type and DagsA-DagsB strains (Table 2). In contrast, whereas the wild-type strain could form hyphal pellets in the liquid medium with 5 μg/mL CR, the DagsA-DagsB strain scarcely germinated or grew in the same medium (Table 2).

Next, we carried out similar experiments with *A. oryzae* wild-type (CNT) and AG-deficient (tripleΔ) strains (Table 3). Although the hyphae of the *A. nidulans* DagsA-DagsB strain were completely dispersed in the medium with 0 μg/mL CR, under the same conditions the tripleΔ strains of *A. oryzae* formed hyphal pellets that were significantly smaller than those of the wild-type strain (Table 3);[18] the pellet size of the tripleΔ strain was 60 % of that of the wild-type strain (Table 3). In the 1 or 5 μg/mL CR-containing medium, the hyphal pellet size of the tripleΔ strain was significantly smaller than that of the wild-type strain; the size of the tripleΔ strain was less than 30 % of that of the wild-type strain (Table 3). No significant differences were detected in the pellet sizes between the wild-type and tripleΔ strains in the 10 μg/mL CR-containing medium (Table 3). These results suggested that AG-deficient strains of *A. nidulans* or *A. oryzae* could be isolated on the basis of the size of the hyphal pellets in CR-containing medium.

Establishment of screening methods for isolating AG-deficient mutants.

Based on the above CR sensitivity results, we established a screening method for isolating *A. oryzae* AG-deficient strains (Fig. 1). The steps were as follows: (1) Conidia of *A. oryzae* were mutagenized by chemical mutagens, such as MNNG and EMS, or by ultraviolet light exposure; (2) The mutagenized conidia were cultivated for 20 h in medium containing 5 μg/mL CR; (3) Germinated conidia were filtrated through a Falcon Cell Strainer (φ 70 μm, Corning, USA) to selectively isolate AG-deficient mutants, which were then grown on agar plates; and (4) Steps 1 to 3 were repeated at least 2 times. We also established an improved selection procedure incorporating a screen for susceptibility to LE (Fig. 1), because our previous study of *A. nidulans* [17] suggested that AG-deficient mutants are more susceptible to LE, resulting in a shorter time of liberation of protoplasts from the mycelia of the AG-deficient mutants than from those of the wild-type strain. Experimental trials using a mixture of conidia from the wild-type and tripleΔ strain of *A. oryzae* revealed that the AG-deficient strain was efficiently separated (>70 %) from the wild-type strain by the improved selection procedure (data not shown).

Practical applications and sensitivities to CR and LE of obtained strains.

For practical application of the improved selection procedure, we first attempted to isolate AG-deficient strains of *A. oryzae*. We treated conidia derived from the Kaori strain, which is the commercially available wild-type strain used for making *amazake*, with the mutagens MNNG and EMS. Then, we succeeded in isolating several candidates of AG-deficient mutants in *A. oryzae.* The test of sensitivity to CR revealed that one of the candidate strains, namely M302, was significantly sensitive to CR compared with the parental strain Kaori (Fig. 2B), as in the case of the tripleΔ strain

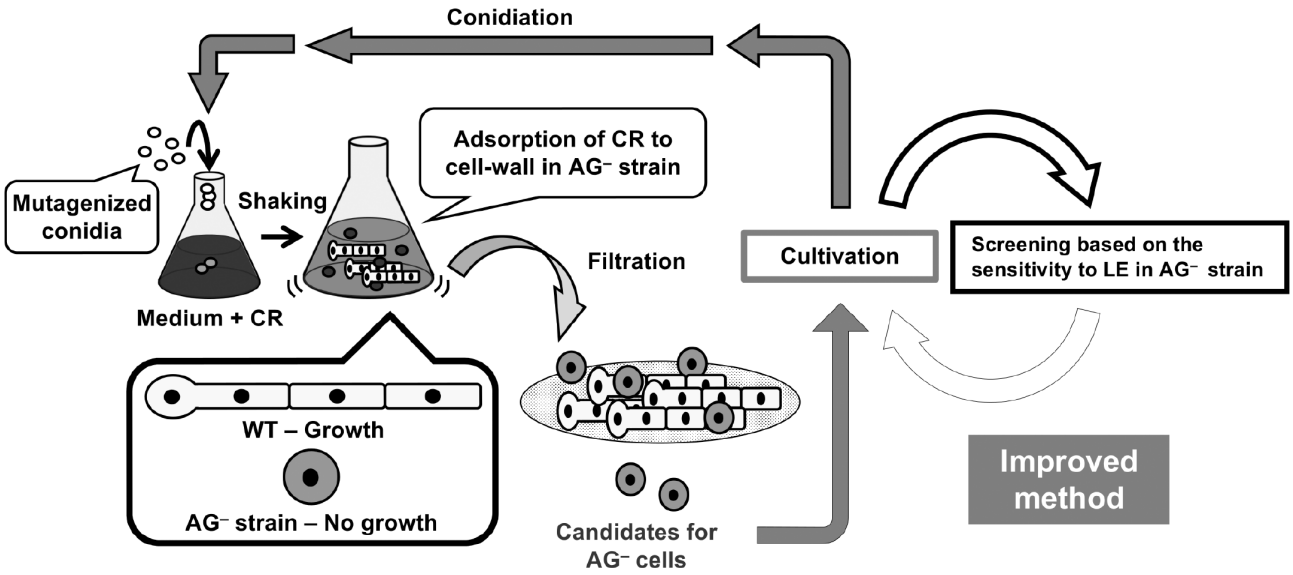

Fig. 1. Schematic representation of the method for isolation of AG-deficient strains based on their sensitivity to Congo Red and Lysing Enzymes.

in *A. oryzae* (Fig. 2A). In addition, the improved method was used to isolate candidate strains from another wild-type strain of *A. oryzae*, RIB161. Two candidate AG-deficient strains, namely M452 and E602, were isolated from the parental RIB161 strain. The M452 strain tended to be sensitive to 40 and 80 μg/mL of CR ($0.05 < p < 0.1$), but not significant (Fig. 2C). In contrast, the E602 strain was significantly sensitive to CR (Fig. 2C). Next, we assessed the susceptibility to LE of the above three candidate strains. Protoplasts formed from hyphal cells of *A. oryzae* tripleΔ strain were significantly higher in concentration compared with those from the wild-type strain after 4 h-treatment with 10 mg/mL LE (Fig. 3A). Similarly, the concentrations of protoplasts formed from hyphae of M302, M452, and E602 strains were significantly higher compared with those generated from hyphae of each parental strain, Kaori or RIB161, after 2-or 4-h treatment with LE (Figs. 3B and 3C). These data reveal that the methods established in this study were effective for isolating strains sensitive to CR and LE.

Analysis of the monosaccharide composition of the cell wall in the AG-deficient strains of A. oryzae.

To examine the composition of the cell wall in the three novel AG-deficient strains of *A. oryzae*, the 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. nidulans*, *A. oryzae*, and *A. fumigates*, the alkali-soluble (AS) fraction of the cell wall contains mainly AG with some galactomannan,[17)18)23)] and that the alkali-insoluble fraction is composed of chitin, BG with β-1,6-branches, and galactomannan.[17)18)24)] Here, the components of the cell wall from each strain were separated into four fractions: HW, AS1, AS2, and AI. The compositions of monosaccharides in each fraction were then calculated by weight (% of total of the four fractions) (Fig. 4, Table 4, and data not shown). In each strain, no significant differences were observed in the monosaccharide compositions of the HW and AS1 fractions (data not shown). The AS2 fraction derived from *A. oryzae* wild-type mainly contained glucose (approximately 7.6 % of the total fraction); however, that derived from the tripleΔ strain contained significantly less glucose than that in the wild-type ($p < 0.01$; Fig. 4A and Table 4). Similarly, the AS2 fraction derived from the parental strain Kaori also contained glucose (approximately 8 % of the total fraction); however, that derived from the candidate M302 strain contained significantly less glucose (approximately 5.2 % of the total fraction) than that in the parental strain ($p < 0.01$; Fig. 4B and Table 4). In addition, the AS2 fraction derived from the parental RIB161 strain contained glucose (approximately 6 % of the total fraction); however, those derived from the candidate M452 and E602 strains contained significantly less glucose (approximately 4 % of the total fraction) than that in the parental strain ($p < 0.05$; Fig. 4C and Table 4). The AI fraction derived from each strain contained glucosamine, galactose, glucose, and mannose (Table 4). The galactose and mannose were minor contents in the AI fraction derived from each strain (Table 4). No significant differences were detected in the glucosamine content of the AI fractions derived from each strain (Table 4). The glucose content of the AI fraction derived from the tripleΔ strain was significantly higher than that derived from the wild-type strain (Table 4). In contrast, no significant differences were detected in the glucose content of the AI fractions derived from the M302, M452, E602, and parental strains (Table 4). These results suggest that the candidate strains, M302, M452, and E602, contained AG in their cell wall, but that the AG contents were about 30 % to 35 % less than in each parental strain.

Cell wall–induced cytokine production.

It has been known for many years that fungal cell wall polysaccharides are recognized by many mammalian membrane-bound receptors such as Dectin-1. To confirm whether cytokine production by DCs was altered by the levels of

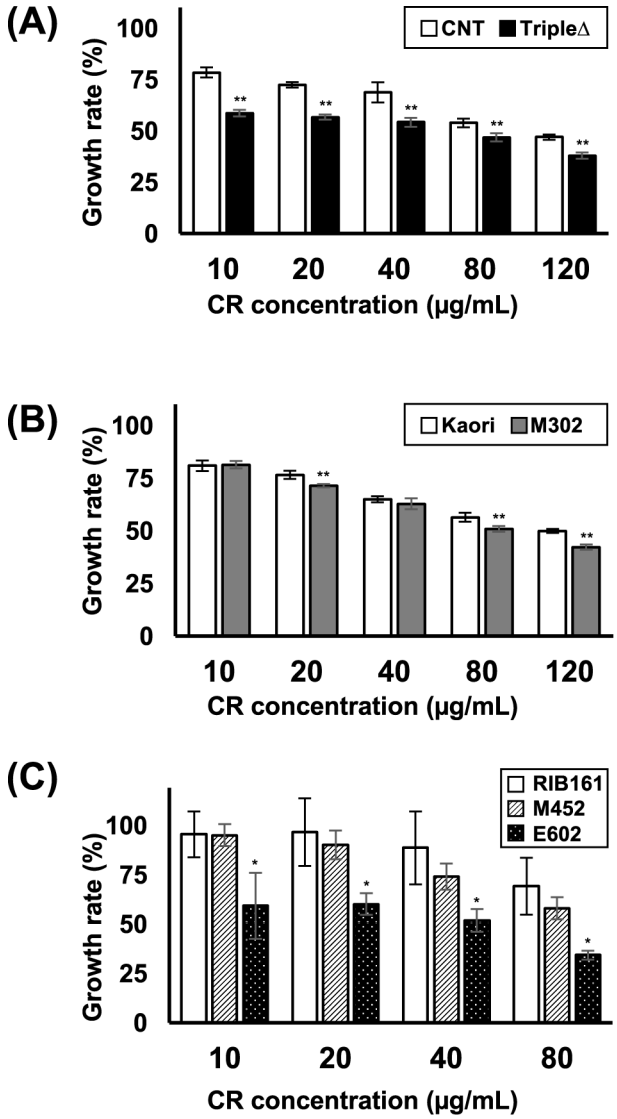

Fig. 2. Sensitivities of the *A. oryzae* AG-deficient and their parental strains to Congo Red (CR).

Growth rate after 4 d on CDE medium containing CR was calculated as a percentage of that of each strain on CR-free CDE medium. Error bars represent the standard deviation (n = 3). (A) CNT and TripleΔ strains. ** $p < 0.01$ *versus* parental strain; Welch's *t*-test. (B) Kaori and M302 strains. ** $p < 0.01$ *versus* parental strain; Welch's *t*-test. (C) RIB161, M452, and E602 strains. * $p < 0.05$ *versus* parental strain; Dunnett's test.

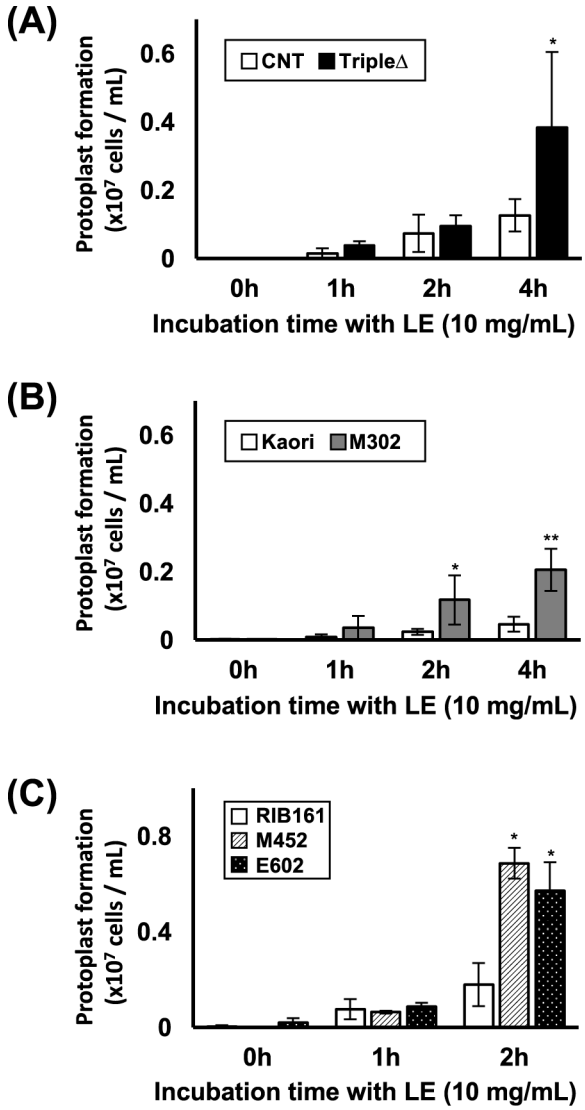

Fig. 3. Cell wall susceptibility of the *A. oryzae* AG-deficient strains and their parental strains to Lysing Enzyme (LE).

Susceptibility of mycelia cultured in CDE liquid medium. Mycelia cultured in CDE medium for 24 h (30 mg fresh weight) were digested in reaction buffer (10 mM phosphate buffer, pH 6.0) containing 10 mg/mL LE. After 1, 2, or 4 h of incubation at 30 C, the number of protoplasts in each sample was determined using a hemocytometer. Error bars represent the standard deviation (n = 3). (A) CNT and TripleΔ strains. * $p < 0.05$ *versus* parental strain; Welch's *t*-test. (B) Kaori and M302 strains. * $p < 0.05$, ** $p < 0.01$ *versus* parental strain; Welch's *t*-test. (C) RIB161, M452, and E602 strains. * $p < 0.05$ *versus* parental strain; Dunnett's test.

fungal cell wall AG, we assessed TNF-α production by DCs that were stimulated with the germinated conidia of *A. oryzae* AG-deficient strains or their parental strains. When the DCs were stimulated with the germinated conidia from the tripleΔ strain of *A. oryzae*, the TNF-α production was significantly higher than in cells treated with the germinated-conidia from the wild type (Fig. 5A). Similarly, when the DCs were stimulated with the germinated conidia from the newly isolated AG-deficient strains, M302, M452, or E602 strains, the TNF-α production was significantly higher than in cells treated with the germinated conidia from the parental strains (Figs. 5B and 5C). In addition, the results from the Dectin-1 NFAT-GFP reporter assay revealed that the conidia derived from the tripleΔ and the M302 strain could strongly stimulate the DCs compared with those from their parental strains (Supplementary material Fig. S1, the conidia from M452 and E602 were not tested). These data suggest that the cytokine production by DCs was increased in *A. oryzae* cells in which cell wall AG levels were reduced.

DISCUSSION

Here, we established a novel screening method for isolation of cell wall AG-deficient mutants on the basis of their sensitivities to CR and LE. Using this method, we succeeded in isolating several *A. oryzae* mutant strains in which the levels of cell wall AG were 30 % to 35 % lower than in

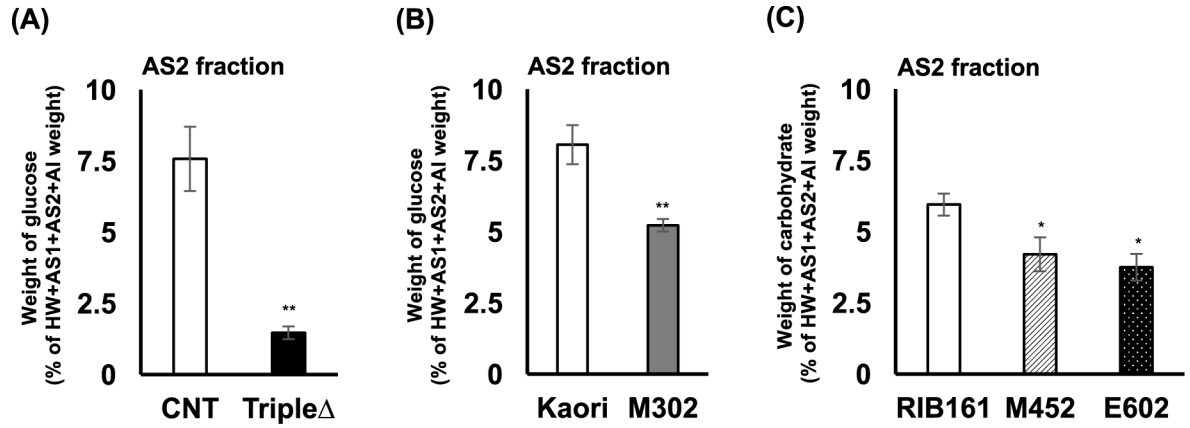

Fig. 4. Comparison of the glucose of cell-wall AS2 fractions derived from the *A. oryzae* AG-deficient strains and their parental strains.

The *A. oryzae* strains were cultured in CDE medium and rotated at 160 rpm at 30 C for 24 h. Glucose composition of alkali-soluble (AS2, water-insoluble components of the alkali-soluble fraction) cell wall fractions as a percentage of total fraction weight (HW [hot-water-soluble] + AS1 [water-soluble components of alkali-soluble fraction] + AS2 + AI) are shown. (A) CNT and TripleΔ strains. ** $p < 0.01$ *versus* parental strain; Welch's *t*-test. (B) Kaori and M302 strains. ** $p < 0.01$ *versus* parental strain; Welch's *t*-test. (C) RIB161, M452, and E602 strains. * $p < 0.05$, *versus* parental strain; Dunnett's test. Error bars represent standard deviation ($n = 3$).

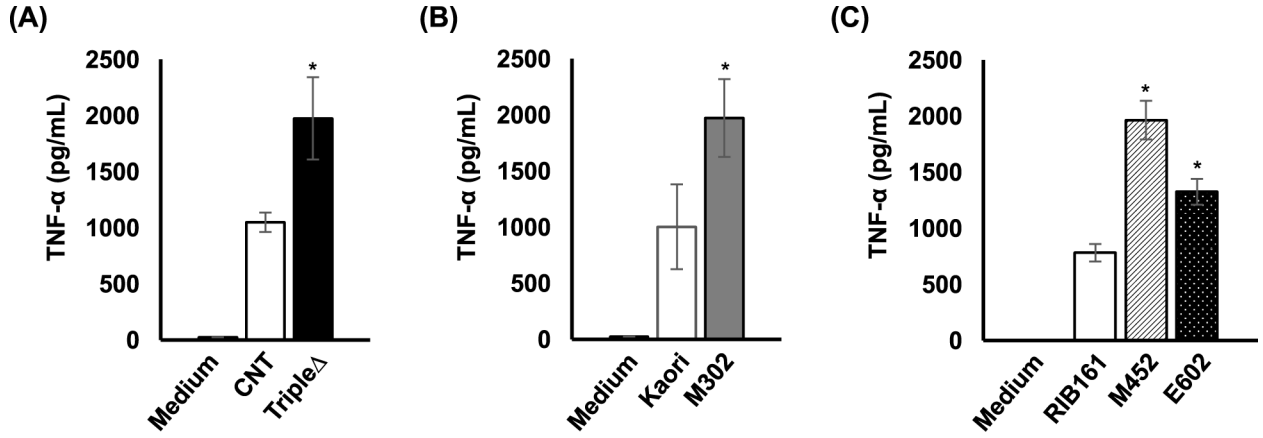

Fig. 5. Comparison of TNF-α production induced by the conidia of the *A. oryzae* AG-deficient strains and their parental strains.

(A) Dendritic cells (DCs) were treated with the conidia derived from the CNT and tripleΔ strains collected from malt medium at an MOI (multiplicity of infection) of 0.01 for 24 h. (B) DCs were treated with the conidia derived from Kaori or M302 strain collected from malt medium at an MOI of 0.01 for 24 h. (C) DCs were treated with the conidia derived from RIB161, M452, or E602 strain collected from malt medium at an MOI of 0.01 for 24 h. (A–C) TNF-α levels in the culture supernatants were then determined by ELISA. Medium, DCs were incubated in medium only. Error bars represent the standard deviation of triplicate cultures. Similar results were obtained in three independent experiments. * $p < 0.05$ *versus* parental strain.

the parental strains. *Aspergillus* species generally possess several genes encoding AGSs. *A. oryzae* contains three AGS genes, namely *agsA*, *agsB*, and *agsC*.[1)18)] The human pathogen *A. fumigatus* also has three such genes, *AGS1*, *AGS2*, and *AGS3*.[1)25)26)] *A. fumigatus AGS1* (*AfAGS1*), *AGS2* (*AfAGS2*), and *AGS3* (*AfAGS3*) are orthologs of *A. oryzae agsB*, *agsA*, and *agsC*, respectively.[1)] Disruption of *AfAGS1* leads to a 50 % decrease in the level of cell wall AG, whereas disruption of *AfAGS2* has no detectable effect on AG levels.[25)] Disruption of *AfAGS3* results in overexpression of *AfAGS1*, which may compensate for the loss of *AfAGS3* to maintain normal cell wall composition.[26)] In addition, the lack of cell wall AG in *A. fumigatus* triple AGS mutant is accompanied by an increase in the BG and chitin content of the mycelia.[27)] Although such compensation mechanisms were not detected in *A. nidulans agsA* and *agsB*,[17)] the loss of AG might be counterbalanced by altered expression of another AGS in *A. oryzae*. The reason for the remaining cell wall AG in the M302, M452, and E602 strains might be such a compensation mechanisms.

Sequencing analyses of the open reading frame of the three AGS genes in each of the M302, M452, and E602 strains revealed that these strains did not have any mutations in the open reading frame of AGS genes (data not shown). It is therefore possible that the decreased levels of AG in the three mutant strains are due to abnormal transcriptional regulation of AGS genes, irregular processing of AGSs, or genetic alterations in unknown mechanisms involved in biosynthesis of AG. In *A. nidulans*, the transcriptional regulation of AGSs, *agsA* and *agsB*, is regulated by the MAP kinase MpkA signaling pathway.[28)] Although the relationships between the three AGSs and MpkA remain unclear in *A. oryzae*, the control mechanisms for the cell wall signaling might become abnormal in the M302, M452, and E602 strains. To confirm this possibility, we assessed the levels of transcription of the three AGSs in the M302, M452, E602, and their parental strains; unexpectedly, no significant differences were detected in the levels of tran-

Table 4. The compositions of monosaccharides in AS2 and AI fractions.

Fraction	Strain	Weight of carbohydrate (% of HW+AS1+AS2+AI weight) [a]			
		GlcN	Gal	Glc	Man
AS2	CNT	trace[b]	0.16 ± 0.02	7.57 ± 1.13	1.27 ± 0.19
	TripleΔ	trace	0.11 ± 0.02*	1.47 ± 0.22**	0.18 ± 0.03**
	Kaori	trace	0.13 ± 0.01	8.01 ± 0.62	0.82 ± 0.06
	M302	trace	0.13 ± 0.01	5.22 ± 0.22**	0.43 ± 0.02**
	RIB161	trace	0.28 ± 0.02	5.95 ± 0.39	0.02 ± 0.002
	M452	trace	0.18 ± 0.03*	4.21 ± 0.60*	0.06 ± 0.009*
	E602	trace	0.14 ± 0.02*	3.75 ± 0.47*	0.04 ± 0.005
AI	CNT	2.73 ± 0.40	0.40 ± 0.06	4.56 ± 0.67	0.82 ± 0.12
	TripleΔ	3.20 ± 0.19	0.40 ± 0.02	6.01 ± 0.35*	1.23 ± 0.07*
	Kaori	3.37 ± 0.62	0.44 ± 0.08	5.33 ± 0.98	1.20 ± 0.22
	M302	3.93 ± 0.43	0.47 ± 0.05	6.81 ± 0.75	0.77 ± 0.09
	RIB161	2.61 ± 0.35	0.64 ± 0.09	7.41 ± 1.00	0.79 ± 0.11
	M452	3.14 ± 0.29	0.50 ± 0.05*	8.11 ± 0.75	0.58 ± 0.05
	E602	3.00 ± 0.19	0.20 ± 0.01*	6.68 ± 0.43	0.52 ±0.03

[a] Data are means ± S.D. (n = 3). * $p < 0.05$ *versus* parental strain. ** $p < 0.01$ *versus* parental strain. GlcN, glucosamine; Gal, galactose; Glc, glucose; Man, mannose. [b] Less than 0.01 % of the total fraction.

scription of the three AGSs under our experimental conditions (cultured in CDE medium, 24 h, data not shown). In *A. oryzae*, the cell wall integrity signaling pathway has been studied by analyzing the functions of the subtilisin-like processing protease KexB (orthologous to *S. cerevisiae* Kex2p).[29] The KexB recognition sequences (KR and RR) are present in the putative amino acid sequences of all three AGSs in *A. oryzae*, raising the possibility that alteration(s) in the processing mechanisms of AGSs via KexB protein might occur in the AG-deficient strains. Further analysis of the AG-deficient strains of *A. oryzae* will be required to clarify these points.

Aspergillus oryzae form multinucleated conidia (homokaryotic and heterokaryotic conidia).[30][31][32] It is generally thought that conidia of industrial strains contain large numbers of nuclei because of long-term selective advantage for the effective production of enzymes.[31][32] Because of this heterokaryosis, the AG-deficient phenotype of the mutated nuclei might be masked by the normal phenotype derived from the coexisting non-mutated nuclei. Therefore, stabilizing the properties by isolating homokaryotic conidia might be required for the practical use of the AG-deficient strains of *A. oryzae*. Despite this potential limitation, the production of the cytokine TNF-α by DCs was increased when the DCs were treated with germinated conidia derived from the AG-deficient strains of *A. oryzae* (Figs. 5B and 5C). In addition, the Dectin-1 NFAT-GFP reporter assay showed stronger stimulation of DCs by the conidia derived from the AG-deficient strains than that by the parental strains (Supplementary material Fig. S1). These data suggest that the AG-deficient strains of *A. oryzae* isolated in this study might be practically applicable to industrial use. Further analyses including mouse *in vivo* assays are required to assess the effectiveness of AG-deficient strains of *A. oryzae* for the production of immunostimulatory processed foods.

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