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Influence of α-1,3-glucan synthase gene *agsE* on protoplast formation for transformation of *Aspergillus luchuensis*

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Aspergillus luchuensis **NBRC4314 recently underwent genome sequencing. We have not used the frequently used protoplast–polyethylene glycol (PEG) method but have used agrobacterium-mediated transformation (AMT) to genetically engineer this strain because it was difficult to generate protoplasts using commercial cell wall lytic enzymes. In this study, we initially investigated the various conditions for protoplast formation in *A. luchuensis*. We found that *A. luchuensis* protoplasts could be generated using a minimal medium for the preculture medium, a static culture for the preculture condition, and Yatalase and α-1,3-glucanase as cell-wall lytic enzymes. These protoplasts could then be transformed with the protoplast–PEG method. Because α-1,3-glucanase was needed to form protoplasts in *A. luchuensis*, we investigated the role of the α-1,3-glucan synthase gene *agsE* in protoplast formation, one of five α-1,3-glucan synthase genes in *A. luchuensis* and a homolog of the major α-1,3-glucan synthase *agsB* in *Aspergillus nidulans*. We disrupted *agsE* in *A. luchuensis* (Δ*agsE*) with AMT and found that protoplast formation in Δ*agsE* was comparable with protoplast formation in *Aspergillus oryzae* with Yatalase. The Δ*agsE* protoplasts were also competent for transformation with the protoplast–PEG method. Hence, *agsE* appears to inhibit protoplast formation in *A. luchuensis*.**

[**Key words:** *Aspergillus luchuensis*; Protoplast; α-1,3-Glucan; *agsE*; Transformation]

Aspergillus luchuensis is a black koji mold that is used widely for brewing traditional Japanese distilled spirits, such as awamori in the Okinawa islands and shochu in the Kyushu district (1–3). Because the genome of *A. luchuensis* NBRC4314 has been sequenced and deposited in DDBJ/EMBL/GenBank (4), researchers now aim to study the molecular biology of *A. luchuensis*. In our previous study to develop a highly efficient gene-targeting system for *A. luchuensis*, we constructed a deletion of the *ligD* gene, encoding the human DNA ligase IV homolog, using an agrobacterium-mediated transformation (AMT) method (5). Notably, transformation of *A. luchuensis* with the protoplast–polyethylene glycol (PEG) method to construct the *ligD* disruptant did not produce candidate transformants (5). This is because it was especially difficult to generate *A. luchuensis* protoplasts using commercial cell-wall lytic enzymes.

Meanwhile, for marker recycling in *Aspergillus oryzae*, we developed a simple method to use the Cre-*loxP* recombination system by directly introducing Cre into protoplasts (6). Pohl et al. (7) also reported a simple method for using the CRISPR/Cas9 system for genome editing in *Penicillium chrysogenum* by directly introducing preassembled CRISPR-Cas9 ribonucleoproteins into protoplasts. The AMT method has merits, including the ability to construct multiple gene disruptants at once in a simple operation; however, it can be difficult to introduce ribonucleoproteins or enzymes, such as Cre recombinase. Therefore, developments in protoplast formation and the protoplast–PEG method with the protoplast in *A. luchuensis* are important for compensating the shortcomings of the AMT method.

In this study, we investigated various culture conditions and the role of cell-wall lytic enzymes for protoplast formation in *A. luchuensis*. Subsequently, we disrupted a gene that seemed to be responsible for inhibiting protoplast formation; in this *A. luchuensis* disruptant, we showed that protoplasts could be formed efficiently and transformed with the protoplast–PEG method.

MATERIALS AND METHODS

Strains, media, and molecular biology techniques Standard *Escherichia coli* manipulations were performed as described previously (8). *E. coli* strain DH5α (Nippon Gene Co., Ltd., Tokyo, Japan) was used to propagate plasmids. *A. luchuensis* genomic DNA was isolated as described previously (9). *A. luchuensis* NBRC4314 (RIB2604: National Research Institute of Brewing Stock Culture), which was used for the genome sequencing project (4), was used as our wild-type strain. The *A. luchuensis ligD* knockout mutant (Δ*ligD*) (5) derived from NBRC4314 was used as a recipient strain for constructing the *agsE*-knockout mutant. These strains were maintained on potato dextrose (PD) plates (BD Difco, Tokyo, Japan). Czapek-dox (CD) medium (2% glucose, 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05%

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$MgSO_4 \cdot 7H_2O$, and 0.002% $FeSO_4 \cdot 7H_2O$ [pH 5.5]) and YPD (1% yeast extract, 2% polypeptone, 2% glucose) were used as a pre-cultivation medium for generating protoplasts. For transformation experiments with the protoplast–PEG method, MMS medium (2% glucose, 0.2% NH_4Cl, 0.1% $[NH_4]_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.002% $FeSO_4 \cdot 7H_2O$ [pH 5.5]) supplemented with 1.2 M sorbitol was used.

Construction of *agsE* mutant The plasmid (Δ*agsE*::*hph*/pRIE) used for *agsE* disruption was generated as follows. All primers are described in Table S1. The 5′ and 3′ fragments of the *agsE* gene were obtained by PCR with primers 5agsEFw + 5agsERv and 3agsEFw + 3agsERv (Table S1), using *A. luchuensis* NBRC4314 genomic DNA as a template. The hygromycin B resistance gene (*hph*) cassette was generated from pBAl_hph (O. Yamada, unpublished data) digested with *Kpn*I. The pBAl_hph plasmid carried an *hph*-expression cassette, which consisted of a glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter and terminator. The binary vector, pRIE (5), was digested with *Eco*RI. These four DNA fragments were ligated using an In-Fusion Cloning kit (Takara Bio Inc., Shiga, Japan), resulting in Δ*agsE*::*hph*/pRIE. *A. luchuensis* Δ*ligD* was transformed with Δ*AlagsE*::*hph*/pRIE using the AMT method (10) (Fig. S1). *A. luchuensis* transformants were screened for hygromycin resistance (0.1 μg/mL) and subcultured at least once on CD agar plates containing hygromycin. These transformants were subjected to colony PCR as described previously (6) using primer sets 1 (hphFw and agsEconfRv) and 2 (agsEconfFw and agaEconfRv) (Table S1). When the Δ*agsE*::*hph* fragment was inserted into the targeted *agsE* locus, a 2.7 kb product was generated with primer set 1, whereas a 2.0 kb product was generated with set 2, indicating that the fragment was inserted into the ectopic locus or that the candidate was still heterokaryotic. A correct homologous integration resulting in the replacement of the resident *agsE* gene with the Δ*agsE*::*hph* construct was confirmed by Southern blot. The *agsE* probe used for hybridization was obtained by PCR with primers agsEp-Fw and agsEp-Rv (Table S1) using the *A. luchuensis* wild-type genome as the template.

Protoplast generation in *A. luchuensis* wild-type and Δ*agsE* strains Protoplasts of wild-type *A. luchuensis* were prepared using the method of modified protoplast generation for *Aspergillus kawachii* (11). The wild-type strain was cultivated at 30°C for 18 h in CD medium with shaking or as a stationary culture; then, the mycelia were harvested. Wild-type mycelia were treated with 20 mg/mL Yatalase (Takara Bio Inc.) and 2.5 μg/mL purified α-1,3-glucanase (kindly donated by Dr. Shigekazu Yano of Yamagata University) (12,13) in 0.6 M ammonium sulfate, 50 mM maleate buffer, pH 5.5 for 3 h. On the other hand, to compare protoplast formation between Δ*agsE* and the wild-type, these mycelia were treated under the same conditions as above, but without α-1,3-glucanase. The protoplast cells were separated from unreacted mycelia with a miracloth (Merck Millipore Corp., Darmstadt, Germany). Protoplasts were then counted with a Thoma cell-counting chamber under a microscope; each batch was counted more than 3 times.

Transformation of *A. luchuensis* with the protoplast–PEG method Wild-type *A. luchuensis* protoplasts were transformed via PEG according to a method described by Gomi et al. (14) with the pPTRII vector (Takara Bio Inc.) harboring the pyrithiamine resistance gene (*ptrA*). *A. luchuensis* transformants were selected on MMS plates with 0.1 μg/mL pyrithiamine and were subcultured at least once on MMS agar plates containing pyrithiamine. These candidates were subjected to colony PCR as described previously (6) using the primer set pPTRIIconf-Fw and pPTRIIconf-Rv (Table S1).

Construction of *oliC31* marker cassette and transformation into *A. luchuensis* Δ*agsE* with the protoplast–PEG method The pCR_Al_oliC31 plasmid harboring the *oliC31* marker was created as described previously (15). *A. luchuensis oliC* containing the promoter and terminator was obtained by PCR with primers AloliCf and AloliCr (Table S1) using *A. luchuensis* NBRC4314 genomic DNA as a template. The amplified fragment was cloned into the pCR-Blunt vector (Thermo Fisher Scientific Inc., Yokohama, Japan) and sequenced. The *oliC* gene was mutated with the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies Ltd., Tokyo, Japan) with the primers AloliCmut1 and AloliCmut2 (Table S1), resulting in pCR_Al_oliC31. *A. luchuensis* Δ*agsE* was transformed as described above with pCR_Al_oliC31 digested with *Not*I. Transformants were screened for oligomycin resistance (1.5 μg/mL) and subcultured at least once on MMS agar containing oligomycin (1.5 μg/mL). These transformants were subjected to colony PCR (6) with the primer set (oliC31conf-Fw and oliC31conf-Rv) (Table S1). The PCR products were digested with *Kpn*I to confirm integration of the *oliC31* fragment at the *oliC* locus, as the *Kpn*I site was vested to *oliC31* with the mutation.

Complementation analysis of *agsE* in *A. luchuensis* Δ*agsE* To construct the *agsE* expression plasmid pBAlagsE_blev2, the plasmid harboring the *Streptoalloteichus hindustanu ble* gene (phleomycin-resistance gene) was first generated as follows. A promoter of *gpdA* and a terminator of *gpdA* in *A. luchuensis* were amplified by PCR with primers Alble1 + Alble2 and Alble5 + Alble6 (Table S1) using *A. luchuensis* NBRC4314 genomic DNA as a template. The *ble* gene was amplified with primers Alble3 + Alble4 (Table S1), using a pPICZαA vector (Thermo Fisher Scientific) as a template. The cloning vector pBluescriptSK was then digested with *Kpn*I. These four DNA fragments were ligated using an In-Fusion Cloning kit (Takara Bio), resulting in pBAl_ble. Subsequently, the 1.4 kb fragment, consisting of the *gpdA* promoter and the *ble* gene, was obtained by digesting pBAl_ble with *Kpn*I and *Eco*RI. This fragment was ligated into the *Kpn*I and *Eco*RI sites of the pSK-T vector (Ogawa, unpublished data) containing the *A. oryzae gpdA* terminator, producing pBAl_blev2. Finally, the *A. luchuensis agsE* cassette containing a 3 kb homologous region including the *agsE* promoter was amplified with primers RagsE Fw + RagsE Rv (Table S1), using *A. luchuensis* NBRC4314 genomic DNA as a template. The pBAl_blev2 plasmid was then digested with *Kpn*I, and these two DNA fragments were ligated using an In-Fusion Cloning kit, resulting in pBAlagsE_blev2.

A. luchuensis ΔagsE was transformed with pBAlagsE_blev2 digested with *Bst*1107I as described above (Fig. S2). *A. luchuensis* transformants were screened for phleomycin resistance (100 μg/mL) (Nacalai Tesque Inc., Kyoto, Japan) and subcultured at least once on MMS agar containing phleomycin (100 μg/mL). These transformants were subjected to colony PCR with primers CagsEconf-Fw1 + CagsEconf-Rv1 and CagsEconf-Fw2 + CagsEconf-Rv2 (Table S1). When the digested pBAlagsE_blev2 plasmid was inserted into the target locus, 6.1 and 1.7 kb fragments were amplified with these primer sets, respectively.

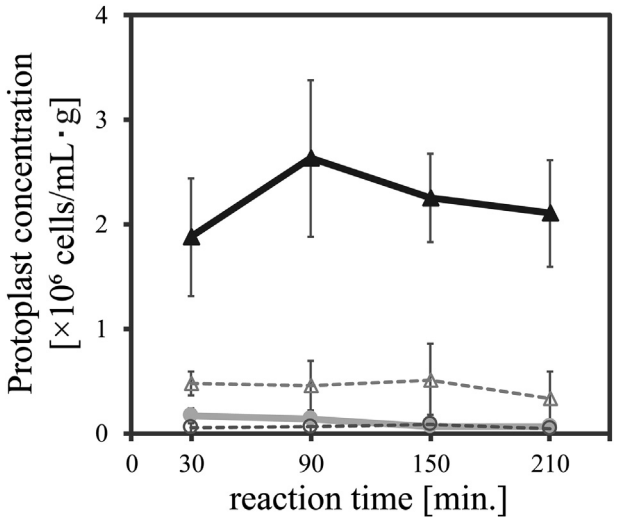

FIG. 1. Protoplast concentration under normal conditions (2% Yatalase, circles) or with α-1,3-glucanase (2% Yatalase + α-1,3-glucanase, triangles) in *A. luchuensis* NBRC4314. The solid line indicates statically cultured cells, and the broken line indicates shaking-cultured cells. Preculture conditions: CD liquid media at 30°C for 18 h.

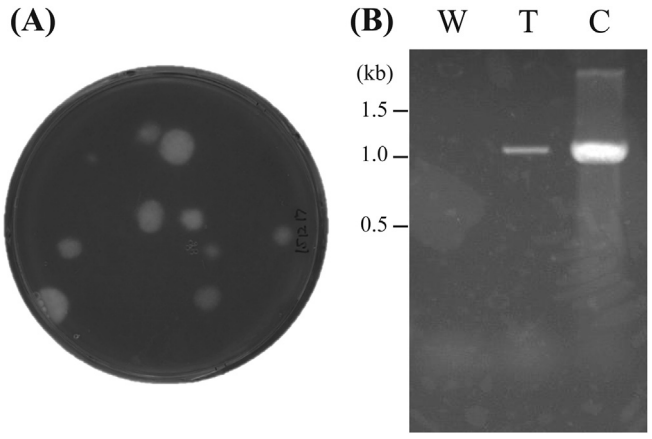

FIG. 2. Confirmation of transformation with pPTRII in *A. luchuensis* wild-type using the protoplast–PEG method. (A) Growth of transformants with pPTRII on MMS agar plates containing pyrithiamine at 30°C for 7 days. (B) Agarose gel electrophoresis of PCR-amplified fragments from pPTRII plasmid region in transformant. *A. luchuensis* wild-type is shown in lane W. Candidate transformed with pPTRII is shown in lane T. A positive control using pPTRII as a template is shown in lane C.

RESULTS AND DISCUSSION

Protoplast formation with α-1,3-glucanase in *A. luchuensis* wild-type and its transformation To prepare protoplasts of *A. luchuensis*, we first applied a method used to prepare protoplasts in *A. oryzae* with Yatalase, cellulase R-10 (Yakult, Tokyo, Japan) and lysing enzyme (Merck Millipore Corp.) (16). This method produced more than 10^8 *A. oryzae* protoplast cells per batch, whereas no *A. luchuensis* protoplasts were obtained, although the same amounts of mycelia were used for both strains. We compared protoplast formation between *A. luchuensis* and *A. kawachii*, referring to the method for *A. kawachii* with Yatalase and cellulase R-10 (11). This method produced 2.4×10^7 protoplast cells per batch in *A. kawachii* and 5.3×10^4 protoplast cells per batch in *A. luchuensis*; thus, we obtained approximately 450 times more *A. kawachii* protoplasts than *A. luchuensis* protoplasts.

Because protoplasts of *A. luchuensis* NBRC4314 were very difficult to make using these above methods (11,16), we investigated the use of various preculture methods and cell wall-degrading enzymes along with Yatalase (Fig. 1). We found that the most protoplasts were obtained when mycelia prepared from a static culture were treated by Yatalase and α-1,3-glucanase (Fig. 1). On the other hand, the use of zymolyase or cellulase in addition to Yatalase had no effect on protoplast formation (compared with Yatalase alone)

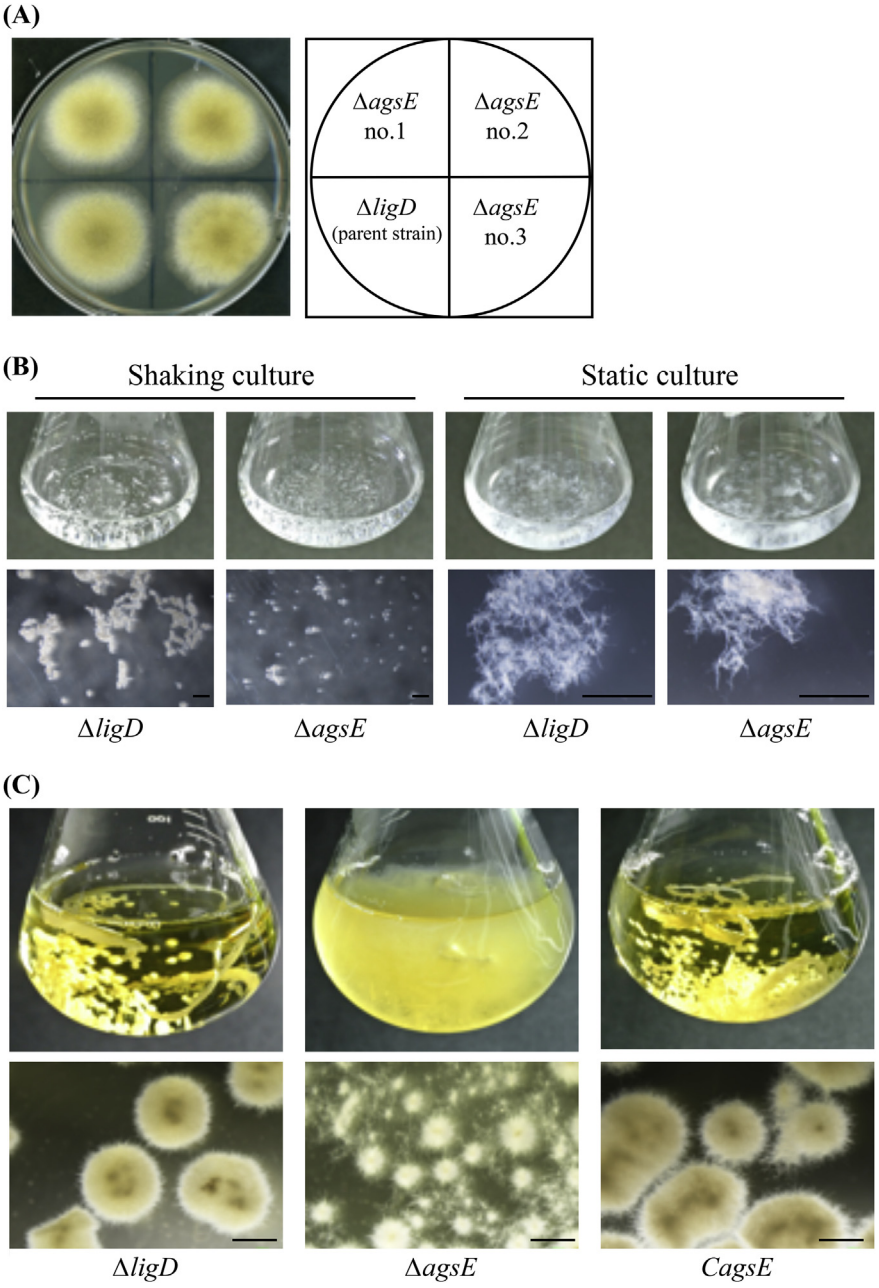

FIG. 3. Phenotypes of the *agsE* disruptants. (A) Left panel, *A. luchuensis* ΔligD (a parent strain of Δ*agsE*) and Δ*agsE* no. 1, 2, and 3 cells (1×10^4) were cultured on PD medium at 30°C for 5 days. The inoculated position of each cell type is indicated in the right panel. (B) Growth characteristics of the Δ*ligD* and Δ*agsE* strains. Conidia (final concentration, 2.5×10^5/mL) of each strain were inoculated into liquid CD medium and incubated at 160 rpm at 30°C for 24 h with shaking and static cultures. Upper panels, photographs of cultures in Erlenmeyer flasks. Bottom panels, representative hyphal pellets or hyphae of each strain under a stereomicroscope (bottom; bar: 1 mm). (C) Growth characteristics of the Δ*ligD*, Δ*agsE*, and C*agsE* strains. Conidia (final concentration, 2.5×10^5/mL) of each strain were inoculated into liquid YPD medium and incubated at 160 rpm at 30°C for 24 h. Upper panels, photographs of cultures in Erlenmeyer flasks. Bottom panels, representative hyphal pellets of each strain under a stereomicroscope (bottom; bar: 1 mm).

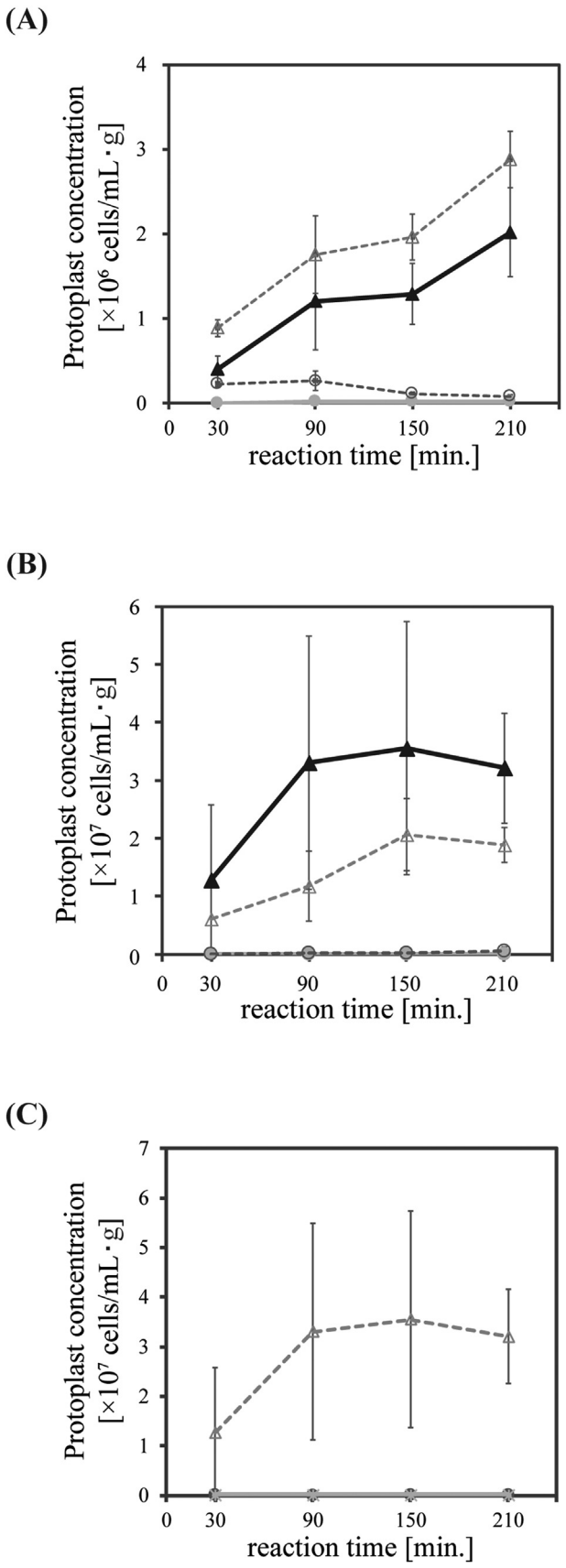

FIG. 4. Protoplast formation in Δ*agsE* and C*agsE* under normal conditions. (A) Protoplast concentration of *agsE* disruptant (triangles) and its parent strain (Δ*ligD*; circles). Solid line, results of the shaking-cultured cells; broken line, results of the statically cultured cells. Each strain was cultured in CD medium at 30°C for 18 h. (B) The legend symbols are the same as panel A. Each strain was cultured in YPD medium at 30°C for 18 h. (C) Protoplast concentration for Δ*agsE* (open triangle and broken line), its parent strain (Δ*ligD*; circle and solid line), and C*agsE* (star and solid line) under condition where Δ*agsE* protoplasts could be obtained (YPD medium, shaking-cultured cells, 18 h, 30°C) are indicated.

(Table S2). Further, the use of minimal medium instead of YPD as the preculture medium most enhanced protoplast formation in *A. luchuensis* (data not shown). These results demonstrated that the efficient preparation of protoplast cells from *A. luchuensis* NBRC4314 required the following three components: (i) a minimal medium as the preculture medium, (ii) static (versus shaking) culture, and (iii) Yatalase and α-1,3-glucanase. As a result of the observation of hyphae in shaking and static cultures of CD liquid medium in *A. luchuensis*, the hyphae in static culture were shown to be less aggregated than that of the shaking culture (Fig. S3). This phenomenon was also observed in the Δ*ligD* strain derived from *A. luchuensis* wild-type (Fig. 3B). These results suggested that the decreased aggregation by the static cultivation caused the response of cell wall lytic enzymes to the hyphae in an easier way. Meanwhile, we reported that more amount of *Aspergillus nidulans* protoplasts can be obtained in a shaking culture of liquid CD medium by adding α-1,3-glucanase and a lysing enzyme, compared with using a lysing enzyme alone (17). In the case of *A. luchuensis*, we found the static culture to be more effective than the shaking culture for obtaining protoplasts. This finding suggests that *A. luchuensis* hyphae obtained from shaking cultivation show stronger aggregation than *A. nidulans* hyphae. Therefore, *A. luchuensis* protoplasts derived from shaking cultivation were less compared with those derived from static cultivation, even when α-1,3-glucanase was used.

We performed transformation experiments (protoplast−PEG method) with the *A. luchuensis* NBRC4314 protoplast cells (2×10^6 cells) prepared with the method described above, although the number of the protoplast cells remained than that of *A. kawachii*. The plasmid for the transformation was the pPTRII vector harboring *ptrA*, the pyrithiamine resistance gene, and AMA1, the replication origin in *A. nidulans*. As a result, some transformants were observed onto MMS agar plates containing pyrithiamine (Fig. 2A). To confirm whether these transformants carried the pPTRII plasmid, a PCR was conducted with primers pPTRIIconf-Fw and pPTRIIconf-Rv (Table S1), and we observed an approximately 1.0 kb fragment amplified in these transformants (Fig. 2B). The transformation efficiency in *A. luchuensis* NBRC4314 was 1.2 transformants/μg pPTRII plasmid DNA ($n = 3$), indicating that the *A. luchuensis* NBRC4314 protoplast cells were capable of undergoing transformation with the protoplast−PEG method.

Protoplast formation in *agsE* disruptants Because α-1,3-glucanase was needed to form protoplasts in the *A. luchuensis* wild-type strain, we examined the role of the α-1,3-glucan synthase gene *agsE* in protoplast formation; *agsE* is homologous to *agsB* in *A. nidulans* and shares 74% identity. (17). We used the ATM method to construct an *agsE* disruptant via homologous recombination with the Δ*agsE*::*hph* fragment from the Δ*agsE*::*hph*/pRIE plasmid (Fig. S1). The integration of the fragment and successful gene disruption were confirmed by colony PCR and Southern blot (Fig. S1). When we examined the growth characteristics of the Δ*agsE* strain, we found that the colonial growth rate and conidiation of Δ*agsE* on PD and CD plates were similar to those of the parent strain (Fig. 3A). In contrast, the hyphae of Δ*agsE* were dispersed in liquid CD medium with shaking culture, whereas the hyphae of the parent strain were aggregated (Fig. 3B). In static culture of liquid CD medium, the phenotype of the Δ*agsE* hypae were similar to that of the parent strain (Fig. 3B). In addition, the hyphae of the Δ*agsE*

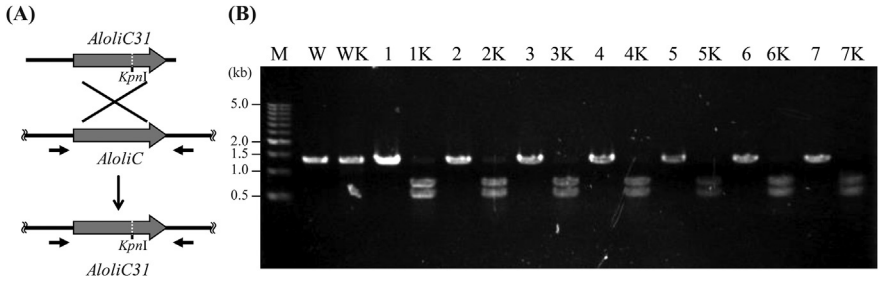

FIG. 5. Confirmation of transformant derived from *A. luchuensis* Δ*agsE* using the protoplast–PEG method. (A) Strategy for homologous recombination of *A. luchuensis* Δ*agsE* for replacing the native *oliC* with the *oliC31* marker gene. (B) Agarose gel electrophoresis of amplified DNA products obtained from PCR (numbered) and amplified DNA products digested with *Kpn*I from Δ*agsE* transformants (with capital K).

strain in liquid YPD medium with shaking culture were more dispersed than that in liquid CD shaking culture (Fig. 3C). Although the mycelia of the *A. nidulans agsB* disruptant were well dispersed under liquid culture conditions (17), those of *A. luchuensis ΔagsE* were not, and small pellets formed as in *A. oryzae ΔagsB* and Δtriple (18,19) (Δtriple indicates that all *ags* genes were disrupted in *A. oryzae*). In *A. oryzae*, because the Δtriple is not observed the completely dispersion, it has been predicted that other cell wall components in addition to α-1,3-glucan were involved in the dispersion (19). Meanwhile, we did not observe any differences in hyphae branching or the morphology of the hyphal tip between the Δ*agsE* and parent strains (data not shown).

To examine the involvement of the *A. luchuensis agsE* gene in protoplast formation, we prepared Δ*agsE* protoplasts. Mycelia from a shaking and static cultures of Δ*agsE* and Δ*ligD* (a parent strain of Δ*agsE*) in liquid CD and YPD medium were treated with Yatalase for 180 min. Compared with the Δ*ligD*, we obtained a large number of Δ*agsE* protoplasts under even the above conditions (Fig. 4A,B). In addition, there were no significance of protoplast concentrations between static and shaking cultures with YPD or CD medium. On the other hand, in the Δ*agsE*, the number of the protoplast obtained from YPD culture was higher than that of CD culture (Fig. 4A, B). It seemed that the difference of the protoplast formation was due to the earlier growth rate of Δ*agsE* in YPD culture compared to that in CD culture. Finally, we obtained approximately 15 times more Δ*agsE* protoplasts that were prepared under the YPD medium with shaking culture condition than wild-type protoplasts that were prepared in static culture with liquid CD medium and using Yatalase and α-1,3-glucanase. On the other hand, we did not obtain any more Δ*agsE* protoplasts when both Yatalase and α-1,3-glucanase were used (data not shown). These findings suggest that AgsE inhibited protoplast formation in *A. luchuensis* by synthesizing α-1,3-glucan in the liquid culture condition. Meanwhile, it was reported that the *agsA* gene in *Aspergillus niger* was induced in the presence of the cell wall stress-inducing compounds such as Calcofluor White, SDS, and caspofungin (20). Therefore, we predict that each Ags enzyme has a role in responding to different environmental conditions encountered by *A. luchuensis*.

Transformation of the Δ*agsE* strain with the protoplast–PEG method To determine whether the *A. luchuensis* Δ*agsE* protoplasts were transformation competent, we transformed the protoplasts using the protoplast–PEG method. The transforming plasmid was pCR_Al_oliC31 harboring *oliC31*, the oligomycin-resistance gene. Next, pCR_Al_oliC31 was digested with *Not*I and inserted into the *oliC* locus of *A. luchuensis* Δ*agsE* (Fig. 5A). We obtained more than 100 transformants on MMS agar plates containing oligomycin. Seven transformants were randomly selected and verified by colony PCR with the primers oliC31conf-Fw and oliC31conf-Rv. To distinguish between the native *oliC* gene and the *oliC31* marker gene, we digested the amplified bands with *Kpn*I (Fig. 5B), since the *oliC31* marker gene was added the *Kpn*I site when it was made from the native *oliC* in *A. luchuensis* with QuikChange Site-Directed Mutagenesis Kit. *Kpn*I digestion confirmed that homologous recombination was successful in all seven transformants. Thus, these results also demonstrate that *A. luchuensis* Δ*agsE* protoplasts were competent for transformation via the protoplast–PEG method. Furthermore, it would be much more efficient to use the Δ*agsE* for transformation on a regular basis because we obtained more protoplasts with this strain than the wild-type and because we did not have to prepare and use the purified α-1,3-glucanase.

Complementation analysis of *agsE* in *A. luchuensis* Δ*agsE* To verify that the loss of *agsE* allowed us to generate and transform *A. luchuensis* protoplasts, we constructed the complementation strain of *agsE* (C*agsE*) to perform complementation experiments (Fig. S2). The pBAlagsE_blev2 plasmid harboring the *ble* marker cassette was digested with *Bst*1071I and inserted into the Δ*agsE* strain using the protoplast–PEG method. The obtained transformants were confirmed by colony PCR with primers CagsEconf-Fw1 + CagsEconf-Rv1 and CagsEconf-Fw2 + CagsEconf-Rv2 (Fig. S2). The hyphae of C*agsE* were aggregated in liquid YPD medium with shaking culture similar to that of Δ*ligD* (a parent strain of Δ*agsE*) (Fig. 3C). To confirm the involvement of *A. luchuensis agsE* in protoplast formation, we prepared C*agsE* protoplasts. The protoplast cells prepared from the hyphae of the C*agsE* strain and the Δ*ligD* strain in YPD liquid culture using only Yatalase were not formed, whereas Δ*agsE* protoplasts were well formed under the same condition (Fig. 4B). Hence, it was evident that *agsE* inhibited protoplast formation in *A. luchuensis*. Notably, the Δ*agsE* mutation can be removed from transformants by using the pBAlagsE_blev2 plasmid; this will allow us to use the Δ*agsE* strain as a host for various genetic experiments. We are confident that this new strain will allow researchers to probe the molecular biology of *A. luchuensis*.

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References

1. **Yamada, O., Takara, R., Hamada, R., Hayashi, R., Tsukahara, M., and Mikami, S.:** Molecular biological researches of Kuro-Koji molds, their classification and safety, J. Biosci. Bioeng., **112**, 233–237 (2011).
2. **Futagami, T., Mori, K., Wada, S., Ida, H., Kajiwara, Y., Takashita, H., Tashiro, K., Yamada, O., Omori, T., Kuhara, S., and Goto, M.:** Transcriptomic

analysis of temperature responses of *Aspergillus kawachii* during barley koji production, Appl. Environ. Microbiol., **81**, 1353–1363 (2015).
3. **Futagami, T., Mori, K., Yamashita, A., Wada, S., Kajiwara, Y., Takashita, H., Omori, T., Takegawa, K., Tashiro, K., Kuhara, S., and Goto, M.:** Genome sequence of the white koji mold *Aspergillus kawachii* IFO 4308, used for brewing the Japanese distilled spirit shochu, Eukaryot. Cell, **10**, 1586–1587 (2011).
4. **Yamada, O., Machida, M., Hosoyama, A., Goto, M., Takahashi, T., Futagami, T., Yamagata, Y., Takeuchi, M., Kobayashi, T., Koike, H., and other 17 authors:** Genome sequence of *Aspergillus luchuensis* NBRC 4314, DNA Res., **23**, 507–515 (2016).
5. **Takahashi, T., Mizutani, O., Shiraishi, Y., and Yamada, O.:** Development of an efficient gene-targeting system in *Aspergillus luchuensis* by deletion of the non-homologous end joining system, J. Biosci. Bioeng., **112**, 529–534 (2011).
6. **Mizutani, O., Masaki, K., Gomi, K., and Iefuji, H.:** Modified Cre-*loxP* recombination in *Aspergillus oryzae* by direct introduction of Cre recombinase for marker gene rescue, Appl. Environ. Microbiol., **78**, 4126–4133 (2012).
7. **Pohl, C., Kiel, J. A., Driessen, A. J., Bovenberg, R. A., and Nygard, Y.:** CRISPR/Cas9 based genome editing of *Penicillium chrysogenum*, ACS Synth. Biol., **5**, 754–764 (2016).
8. **Sambrook, J. and Russell, D. W.:** Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001).
9. **Chigira, Y., Abe, K., Gomi, K., and Nakajima, T.:** *chsZ*, a gene for a novel class of chitin synthase from *Aspergillus oryzae*, Curr. Genet., **41**, 261–267 (2002).
10. **Michielse, C. B., Hooykaas, P. J., van den Hondel, C. A., and Ram, A. F.:** Agrobacterium-mediated transformation of the filamentous fungus *Aspergillus awamori*, Nat. Protoc., **3**, 1671–1678 (2008).
11. **Tashiro, S., Futagami, T., Wada, S., Kajiwara, Y., Takashita, H., Omori, T., Takahashi, T., Yamada, O., Takegawa, K., and Goto, M.:** Construction of a *ligD* disruptant for efficient gene targeting in white koji mold, *Aspergillus kawachii*, J. Gen. Appl. Microbiol., **59**, 257–260 (2013).
12. **Yano, S., Yamamoto, S., Toge, T., Wakayama, M., and Tachiki, T.:** Occurrence of a specific protein in basidiomycete-lytic enzyme preparation produced by *Bacillus circulans* KA-304 inductively with a cell-wall preparation of *Schizophyllum commune*, Biosci. Biotechnol. Biochem., **67**, 1976–1982 (2003).
13. **Yano, S., Rattanakit, N., Wakayama, M., and Tachiki, T.:** Cloning and expression of a *Bacillus circulans* KA-304 gene encoding chitinase I, which participates in protoplast formation of *Schizophyllum commune*, Biosci. Biotechnol. Biochem., **69**, 602–609 (2005).
14. **Gomi, K., Iimura, Y., and Hara, S.:** Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene, Agric. Biol. Chem., **51**, 2549–2555 (1987).
15. **Ward, M. and Turner, G.:** The ATP synthase subunit 9 gene of *Aspergillus nidulans*: sequence and transcription, Mol. Gen. Genet., **205**, 331–338 (1986).
16. **Mizutani, O., Nojima, A., Yamamoto, M., Furukawa, K., Fujioka, T., Yamagata, Y., Abe, K., and Nakajima, T.:** Disordered cell integrity signaling caused by disruption of the *kexB* gene in *Aspergillus oryzae*, Eukaryot. Cell, **3**, 1036–1048 (2004).
17. **Yoshimi, A., Sano, M., Inaba, A., Kokubun, Y., Fujioka, T., Mizutani, O., Hagiwara, D., Fujikawa, T., Nishimura, M., Yano, S., and other 5 authors:** Functional analysis of the α-1,3-glucan synthase genes *agsA* and *agsB* in *Aspergillus nidulans: agsB* is the major α-1,3-glucan synthase in this fungus, PLoS One, **8**, e54893 (2013).
18. **Zhang, S., Sato, H., Ichinose, S., Tanaka, M., Miyazawa, K., Yoshimi, A., Abe, K., Shintani, T., and Gomi, K.:** Cell wall α-1,3-glucan prevents α-amylase adsorption onto fungal cell in submerged culture of *Aspergillus oryzae*, J. Biosci. Bioeng., **124**, 47–53 (2017).
19. **Miyazawa, K., Yoshimi, A., Zhang, S., Sano, M., Nakayama, M., Gomi, K., and Abe, K.:** 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, Biosci. Biotechnol. Biochem., **80**, 1853–1863 (2016).
20. **Damveld, R. A., vanKuyk, P. A., Arentshorst, M., Klis, F. M., van den Hondel, C. A., and Ram, A. F.:** Expression of *agsA*, one of five 1,3-α-D-glucan synthase-encoding genes in *Aspergillus niger*, is induced in response to cell wall stress, Fungal Genet. Biol., **42**, 165–177 (2005).