Original paper

Construction of Quintuple Protease and Double Amylase Gene Deletant for Heterologous Protein Production in *Aspergillus oryzae* KBN616

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We constructed a quintuple protease (*alp*, *npII*, *pepE*, *npI*, *pepA*) and double amylase (*taaG3*, *taaG1*) gene deletant, KO4, for the development of a heterologous gene expression system for an industrial *shoyu koji* mold, *Aspergillus oryzae* KBN616. Multiple gene deletion was performed using the Latour system, a simple and effective chromosome modification method developed in *Schizosaccharomyces pombe*. Gene deletion was confirmed by Southern blot analysis for protease genes and PCR amplification for amylase genes. Deletion of the five protease genes reduced the extracellular protease activity to approximately 1.0% of the level of the parent strain. Double deletion of the amylase genes completely eliminated all detectable α -amylase activity. These results indicate the construction of a host strain applicable to the efficient production and purification of heterologous proteins.

Keywords: *Aspergillus oryzae*, multiple gene deletion, protease, amylase

Introduction

The filamentous fungus *Aspergillus oryzae* has been employed for the production of traditional Japanese fermented foods and industrial enzymes for more than a thousand years. *A. oryzae* is considered an attractive microorganism for heterologous gene expression and foreign protein production due to its ability to secrete large amounts of enzymes such as amylases and proteases. Attempts have been made to improve the production and purification of heterologous proteins in *Aspergillus* at the (post)translational level (Gouka *et al.*, 1997).

Proteolytic degradation of recombinant gene products by host-specific proteases results in low production yields of heterologous proteins. To overcome this problem, double, quintuple, and decuple protease gene disrupted strains were constructed (Jin *et al.*, 2007, Maruyama and Kitamoto, 2008, Yoon *et al.*, 2009, Yoon *et al.*, 2011). The use of such strains resulted in a significant improvement

in the production of bovine chymosin or human lysozyme as a reporter heterologous protein because of the synergistic effects of multiple protease gene disruptions. However, culture conditions and distinct characteristics of the strain employed affect the expression profiles of protease genes (te Biesebeke *et al.*, 2005, Jin *et al.*, 2007). The information regarding *A. oryzae* RIB40 proteases cannot be applied completely to the heterologous protein production of other *A. oryzae* strains.

Coexpression of endogenous secretory proteins and heterologous proteins can lead to competition within the secretory pathway, resulting in reduced heterologous protein production. In addition, endogenous proteins secreted in the culture medium interfere with the purification of the produced heterologous proteins. To overcome this issue, Taka-amylase A (TAA) was suppressed by RNA interference with the *taa* genes, since *A. oryzae* secretes TAA abundantly in the culture medium and the *taa* gene promoter is

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frequently used for homologous or heterologous protein production (Nemoto *et al.*, 2009).

In our previous studies, a transformation system was developed for an industrial *shoyu koji* mold, *A. oryzae* KBN616, and several genes from this strain were overexpressed or repressed to elucidate their functions in *shoyu* production (Kitamoto *et al.*, 1995, Kitamoto *et al.*, 1996, Kitamoto *et al.*, 1998a, Kitamoto *et al.*, 1998b, Kitamoto *et al.*, 1999a, Kitamoto *et al.*, 1999b, Kitamoto *et al.*, 2001a, Kitamoto *et al.*, 2001b). Furthermore, heterologous genes from other fungi were successfully expressed and secreted in this strain in order to characterize the gene products (Yoshino-Yasuda *et al.*, 2011, Yoshino-Yasuda *et al.*, 2012, Chaya *et al.*, 2014).

In this paper, to develop an efficient heterologous gene expression system for *A. oryzae* KBN616, multiple gene deletion was performed using the Latour system, a simple and effective chromosome modification method developed in *Schizosaccharomyces pombe* (Hirashima *et al.*, 2006). As a result, we constructed a quintuple protease (*alp*, *npII*, *pepE*, *npl*, *pepA*) and double amylase (*taaG3*, *taaG1*) gene deletion with significantly low protease and no amylase activities.

Materials and Methods

Fungal strains, culture media and transformation *A. oryzae* wild-type strain, KBN616 (Kitamoto *et al.*, 1993), and *pyrG* gene disrupted strain, P4 (Mahmud *et al.*, 2013), were used as DNA donors and for transformations. The strains generated in this study are listed in Table 1. RB medium (3% rice bran, 1% polypeptone, 0.5% KH₂PO₄, 0.5% KCl, 0.1% NaNO₃, and 0.05% MgSO₄·7H₂O) was used for liquid cultivation of *A. oryzae*. Skim milk agar medium (1% skim milk, 1% glucose, 0.15% KH₂PO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.125% Triton X-100 and 1.5% agar) was used

for detection of protease activity. SP agar medium (2% soluble starch, 1% polypeptone, 0.5% KH₂PO₄, 0.5% KCl, 0.1% NaNO₃, and 0.05% MgSO₄·7H₂O) was used for detection of α -amylase activity. Czapek-Dox (CD) minimal medium plates containing 0.1% 5-fluoroorotic acid (5-FOA), 0.15% uridine and 0.07% uracil were used for the positive selection of *pyrG*-deficient strains. Protoplast transformation of *A. oryzae* was carried out according to a previously described method (Kitamoto *et al.*, 1995).

DNA techniques and PCR methods Standard DNA techniques were used in this study (Sambrook and Russell, 2001). Genomic DNA of *A. oryzae* was prepared according to a previously described method (Kitamoto *et al.*, 1993). PCR amplification was carried out with a GeneAmp9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PfuUltra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA) and TaKaRa Ex Taq DNA polymerase (Takara Bio, Otsu, Shiga) were used for PCR. Oligonucleotide primers used in this study are shown in Table 2. Essential cloning steps were confirmed by sequencing on a model 4000LS DNA sequencer (LI-COR, Lincoln, NE, USA).

Construction of protease gene deletion plasmids An *alp* gene deletion plasmid, pDelAlp-2, was constructed as follows. Both 1.0-kb 5'- and 3'-flanking regions of the *alp* gene were amplified from *A. oryzae* genomic DNA with the primer pairs delalp1/delalp2 and delalp5/delalp6. A 1.0-kb part of the coding region of the *alp* gene was amplified from *A. oryzae* genomic DNA with the primer pair delalp3/delalp4. The three PCR-products were mixed and used as a template in a fusion PCR that was carried out using the primer pair delalp1/delalp6. The fusion PCR product was subcloned into the *Sma*I site of pUC18, thereby generating pDelAlp-1. A 1.8-kb *Stu*I digested *pyrG* gene fragment, amplified from *A. oryzae* genomic DNA with the primer pair pyrGN2/pyrGC2, was inserted into the *Nru*I site of pDelAlp-1 to yield pDelAlp-2. Using the primers listed

Table 1. Strains used in this study

Name	Parental strain	Genotype	Reference
KBN616		Industrial <i>shoyu koji</i> mold	Kitamoto <i>et al.</i> , 1993
P4	KBN616	<i>pyrG</i>	Mahmud <i>et al.</i> , 2013
PDI1	P4	<i>pyrG</i> Δ <i>alp</i> :: <i>pyrG</i>	This study
PDE1	PDI1	<i>pyrG alp</i>	This study
PDI2	PDE1	<i>pyrG alp AnpII</i> :: <i>pyrG</i>	This study
PDE2	PDI2	<i>pyrG alp npII</i>	This study
PDI3	PDE2	<i>pyrG alp npII ApepE</i> :: <i>pyrG</i>	This study
PDE3	PDI3	<i>pyrG alp npII pepE</i>	This study
PDI4	PDE3	<i>pyrG alp npII pepE AnpI</i> :: <i>pyrG</i>	This study
PDE4	PDI4	<i>pyrG alp npII pepE npl</i>	This study
PDI5	PDE4	<i>pyrG alp npII pepE npl ApepA</i> :: <i>pyrG</i>	This study
PDE5	PDI5	<i>pyrG alp npII pepE npl pepA</i>	This study
KO1	PDE5	<i>pyrG alp npII pepE npl pepA ΔtaaG3</i> :: <i>pyrG</i>	This study
KO2	KO1	<i>pyrG alp npII pepE npl pepA taaG3</i>	This study
KO3	KO2	<i>pyrG alp npII pepE npl pepA taaG3 ΔtaaG1</i> :: <i>pyrG</i>	This study
KO4	KO3	<i>pyrG alp npII pepE npl pepA taaG3 taaG1</i>	This study

Table 2. Oligonucleotide primers used in this study

Name	Sequence (5' to 3')	Direction
Primers for protease and amylase gene deletion		
<i>alp</i> gene		
delalp1	CAGGATCCTGATACCATCAGAAGACC	Forward
delalp2	TACTCTGTACATGGACGAGGGATGCATGTGGTATT	Reverse
delalp3	GGTTACGAGTCGCGAGGCAAAGATTACAGGAGCATACC	Forward
delalp4	TTGGATCCGAAGACGTCAACGACCTTGCCA	Reverse
delalp5	GCATCCCTCGTCCATGTACAGAGTATACTTATGTT	Forward
delalp6	AATCTTTGCCTCGCGACTCGTAACCGAGGAAAAG	Reverse
<i>npII</i> gene		
delnpII1	CTGCATGCGAGAAGGACTTGGAGAGCTAC	Forward
delnpII2	TGAGGAGGATATTCCTTCTGGTCTGATGGCCTTCA	Reverse
delnpII3	TTATATAGAATCGCGAGGTCAAGCTGCCCAGATTG	Forward
delnpII4	GGGCATGCTTACCATTGGCATAACAAGGC	Reverse
delnpII5	CAGACCAGAAGGAATATCCTCCTCAAAATACTCC	Forward
delnpII6	CAGCTTGACCTCGCGATTCTATATAACGGTGCGTATGG	Reverse
delnpII7	TGGATGATGAACAGGCGGAACAGAC	Reverse
<i>pepE</i> gene		
delpepE1	TTGGATCCTGCTCTGCTGCGTCCAG	Forward
delpepE2	CAGTCAGATCTACGGCCCTTTTACAATACCGCCA	Reverse
delpepE3	GATATGGCATGTGCGGAGCAATTTGTGAGTAGACC	Forward
delpepE4	CAGGATCCACAGTACACTGGCTTGACTTAC	Reverse
delpepE5	GTAAAAGGGCCGTAGATCTGACTGTTGAGCAAGCA	Forward
delpepE6	CAAATTGCTCGCGACATGCCATATCTGACTACT	Reverse
delpepE8	CGAGCAATGTTAAGGTCATTATGGC	Forward
<i>npI</i> gene		
delnpI1	TGGTCGACTCTAGTGTTTCCTATCCT	Forward
delnpI2	AGTAGCACAACACTCCGTGAAGAGTATGCCAATAA	Reverse
delnpI3	CTGGAAGTTTCGCGACTGAGTCTTCGAGTGATGTTT	Forward
delnpI4	TGGTCGACGTGTTGGCAGTGTAGAAAAGCTG	Reverse
delnpI5	ACTCTTACGGAGTGTTGTGCTACTTACATCGCTG	Forward
delnpI6	GAAGACTCAGTCGCGAACTTTTCCAGCAATATGAGA	Reverse
delnpI7	GGAGCATTTGCGGAGTTATATTGGG	Forward
<i>pepA</i> gene		
delpepA1	ATGCATGCTAAGTGGAGAGCGACCCA	Forward
delpepA2	GTCTTTGTTTCGGAATGGTCATTGCTTGTAGG	Reverse
delpepA3	GTAGTTGTGGTCGCGAGCCCAATGACATTGAGTAC	Forward
delpepA4	GAGCATGCTCTGAATACCGCCAACGCAA	Reverse
delpepA5	CAATGACCATTTCGAAACAAAGACATACAAGGAG	Forward
delpepA6	CATTGGGCTCGCGACCACAACACTACTATGACCCGAATGC	Reverse
delpepA7	CCATTAGGGACTTTCCTGTCAGTCC	Forward
<i>taaG3</i> gene		
deltaaG31	GCAAGCTTAGCGCCTAAAACGCCTTAT	Forward
deltaaG32	CAGTAACGACTCCAAGCATGGCATCCCTTGCTAG	Reverse
deltaaG33	TCCGAGCGGATCGACGACTCGCGACTTGTAATAC	Forward
deltaaG34	GTAAGCTTCCATGACGTTCTGGTAGGGACAAG	Reverse
deltaaG35	CATGCCATGCTTGGAGTCGTTACTGCTGTCATCCC	Forward
deltaaG36	GTCGCGAGTCGTCGATCCGCTCGGAGTAATGAAAAG	Reverse
deltaaG37	GCGAATATGGCGGCAAATACACTAG	Reverse
<i>taaG1</i> gene		
deltaaG31	GCAAGCTTAGCGCCTAAAACGCCTTAT	Forward
deltaaG12	CAAAAGACCATACATCGCATCGACAAGGGACATA	Reverse
deltaaG13	TTCAGTCACAGGCCTTGTAATACTGCGGATCGGGTGTG	Forward
deltaaG34	GTAAGCTTCCATGACGTTCTGGTAGGGACAAG	Reverse

deltaaG15	TGTCGATGCGATGTATGGTCTTTTGTTCATAG	Forward
deltaaG16	GCAGTATTACAAGGCCTGTGACTGAAGCCATCCCTGA	Reverse
deltaaG17	CTGAGGTCATGCCACACTCATTTC	Reverse
Primers for <i>pyrG</i> amplification		
pyrGN2	CAAGGCCTGCTGGAATTGACATTATTATGG	Forward
pyrGC2	AAAGGCCTGATCAATACCGTACGGGAGATT	Reverse

Table 3. Deleted genes of *A. oryzae* assessed in this study

Gene	Gene ID	Genbank accession number
<i>alp</i>	AO090003001036	AP007155 / S79617
<i>npII</i>	AO090010000493	AP007175/S53810
<i>pepE</i>	AO090003000693	AP007155 / AB074195
<i>npI</i>	AO090011000036	AP007171/AF099904
<i>pepA</i>	AO090120000474	AP007166/ D13894
<i>taaG3</i>	AO090023000944	AP007157
<i>taaG1</i>	Not annotated	AP007155/M33218

in Table 2, the other four protease gene deletion plasmids, pDelNpII-2 to pDelPepA-2, were constructed using the same procedure as for the *alp* gene deletion plasmid, pDelAlp-2. The protease genes targeted in this study are listed in Table 3.

Construction of *taa* gene deletion plasmids A *taaG3* gene deletion plasmid, pDelTaaG3-2, was constructed as follows. Both 1.0-kb 5'- and 3'-flanking regions of the *taaG3* gene were amplified from *A. oryzae* genomic DNA with the primer pairs deltaaG31/deltaaG32 and deltaaG35/deltaaG36. A 1.0-kb part of the coding region of the *taaG3* gene was amplified from *A. oryzae* genomic DNA with the primer pair deltaaG33/deltaaG34. The three PCR-products were mixed and used as a template in a fusion PCR that was carried out using the primer pair deltaaG31/deltaaG34. The fusion PCR product was subcloned into the *Sma*I site of pUC18, thereby generating pDelTaaG3-1. A 1.8-kb *Stu*I digested *pyrG* gene fragment, amplified as described above, was inserted into the *Nru*I site of pDelTaaG3-1 to yield pDelTaaG3-2. Using the primers listed in Table 2, the *taaG1* gene deletion plasmid, pDelTaaG1-2, was constructed by the same procedure as for the *taaG3* gene deletion plasmid, pDelTaaG3-2, except for the use of *Stu*I instead of *Nru*I. The *taa* genes targeted in this study are listed in Table 3.

Southern blot analysis Genomic DNA was isolated from the *A. oryzae* strains as described previously (Kitamoto *et al.*, 1993). DNA was fractionated by electrophoresis on a 0.7% agarose gel and transferred to a Hybond-N⁺ membrane (GE Healthcare, Buckinghamshire, UK). Labeling of the probes and detection of the signals in Southern hybridization were carried out using the AlkPhos Direct Labelling and Detection System with CDP-Star (GE Healthcare). Hybridization was carried out at 55°C in the hybridization buffer, and the membrane was washed twice with the primary wash buffer at 55°C for 10 min and twice with the secondary wash buffer at room temperature for 5 min, according to the manufacturer's instructions.

Enzyme assay Approximately 5×10^5 conidia were inoculated into 100 mL of RB medium supplemented with 0.15% uridine and 0.07% uracil and then cultured at 30°C for 4 days. Total extracellular protease activity in the culture supernatant of *A. oryzae* strains was measured according to the method described in "Shoyu Shiken-Ho", with slight modifications (Nihon Shoyu Kenkyusho, 1985). Notably, the reaction mixture was incubated at pH7.0 for 60 min at 37°C instead of at pH7.0 for 10 min at 30°C.

The α -amylase activity of the culture supernatant was measured using an α -amylase assay kit (Kikkoman, Noda, Chiba).

Results and Discussion

Construction of an *A. oryzae* quintuple protease gene deletant Genome sequencing of *A. oryzae* RIB40 revealed the existence of 134 protease genes, consisting of 69 exopeptidases and 65 endopeptidases (Machida *et al.*, 2005). Of these, alkaline, neutral and acidic proteases contribute significantly to the digestion of soybean protein during soy sauce brewing (Nakadai *et al.*, 1973a, Nakadai *et al.*, 1973b, Nakadai *et al.*, 1973c, Nakadai and Nasuno, 1977).

Alp is the major alkaline protease secreted by *A. oryzae*. Firstly, the *alp* gene was deleted since the loss of *alp* gene function can be easily detected using the skim milk plate assay. pDelAlp-2, a plasmid carrying an *alp* deletion cassette, was used to construct the *alp* deletion mutant of *A. oryzae* P4 (Fig. 1A). *A. oryzae* P4 was transformed using a PCR-amplified fragment (primer pair, delalp1/delalp4; template, pDelAlp-2). Of the 99 transformants screened, 21 formed a smaller clear zone than *A. oryzae* KBN616 (parent strain of *A. oryzae* P4) on the skim milk agar plate after 48 h (Fig. 1B). Of these strains, *A. oryzae* PD11 was used for further experiments. Southern blot analysis of genomic DNA from *A. oryzae* PD11 and P4 digested with *Xho*I and *Bam*HI revealed that *A. oryzae* PD11 contained the predicted 8.9-kb fragment while *A. oryzae* P4 contained the 6.3-kb fragment (Fig. 1C). These results indicated that the PCR-amplified *alp* deletion cassette was integrated into the *alp* locus in *A. oryzae* PD11. Integration of the *alp* deletion cassette into the *alp* locus causes duplication of the 1.0-kb 3'-flanking region of the *alp* gene on both sides of the *pyrG* and *alp* genes as a direct repeat. Homologous recombination between these identical sequences results in deletion of both of the *pyrG* and *alp* genes with 5-FOA treatment. To delete the *pyrG* and *alp* genes, *A. oryzae* PD11 conidia (approximately 1×10^5) were spread on 5-FOA-, uridine- and uracil-containing CD plates, and 5-FOA-resistant colonies were selected after 5 days of cultivation

Fig. 1. Construction scheme and confirmation of the *alp* deletant.

A. Scheme of the *alp* deletion. PCR-amplified DNA fragments from an *alp* deletion plasmid, pDelAlp-2, were transformed to *A. oryzae* P4 and 5-FOA resistant transformants were selected. An *alp* deletion mutant, *A. oryzae* PDE1, was obtained. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *alp* gene, respectively. The open and black arrows indicate the *pyrG* and *alp* genes, respectively. The direction of the arrow indicates the orientation of the *pyrG* and *alp* genes. **B.** Skim milk agar plate assay for detection of protease activity. *A. oryzae* P4 and transformants were grown on skim milk agar medium at 30°C for 48 hours and the formation of zones of clearance around colonies was assessed. The zone was made clearer by flooding the plates with a solution of 5% trichloroacetic acid. **C.** Southern blot analysis of genomic DNA isolated from *A. oryzae* P4, PDI1 and PDE1. Genomic DNA (about 5 µg) was digested with *XhoI* and *BamHI*, and processed for Southern blot hybridization. Hybridization was performed using the AlkPhos Direct system with the 1.0-kb PCR fragment amplified with the primers delalp5/delalp6 used as a probe.

at 30°C. 5-FOA-resistant colonies were obtained at a frequency of approximately 1×10^{-3} . Southern blot analysis of genomic DNA from *A. oryzae* PDE1, a 5-FOA-resistant strain, and PDI1 digested with *XhoI* and *BamHI* revealed that *A. oryzae* PDE1 contained the predicted 4.7-kb fragment while *A. oryzae* PDI1 contained the 8.9-kb fragment (Fig. 1C). These results indicated that the *alp* gene was excised from *A. oryzae* P4.

Secondly, pDelNpII-2, a plasmid carrying a *npII* deletion cassette, was used to construct the *npII* deletion mutant of *A. oryzae* PDE1 (Fig. 2A). *A. oryzae* PDE1 was transformed using a PCR-amplified fragment (primer pair, delnpII1/delnpII4; template, pDelNpII-2). The loss of NpII activity is not easily detected using the skim milk agar plate assay because NpII has low activity on milk casein (Sekine, 1973). Therefore, transformants were examined by detection of a DNA fragment that was PCR-amplified from the transformants using the primer pair delnpII1/delnpII7. For 3 out of the 57 transformants examined, a 6.3-kb fragment was detected, while a 3.7-kb fragment was detected from *A. oryzae*

PDE1 (Fig. 2A). Of these strains, *A. oryzae* PDI2 was used for further experiments. Southern blot analysis of genomic DNA from *A. oryzae* PDI2 and PDE1 digested with *SphI* revealed that *A. oryzae* PDI2 contained the predicted 9.7-kb fragment while *A. oryzae* PDE1 contained the 7.1-kb fragment (Fig. 2A). These results indicated that the PCR-amplified *npII* deletion cassette was integrated into the *npII* locus in *A. oryzae* PDE1. Deletion of both the *pyrG* and *npII* genes from *A. oryzae* PDI2 was performed using the same procedure as for the *pyrG* and *alp* genes from *A. oryzae* PDI1. Southern blot analysis of genomic DNA from *A. oryzae* PDE2, a 5-FOA-resistant strain, and PDI2 digested with *SphI* revealed that *A. oryzae* PDE2 contained the predicted 5.5-kb fragment while *A. oryzae* PDI2 contained the 9.7-kb fragment (Fig. 2A). These results indicated that the *npII* gene was excised from *A. oryzae* PDE1.

Thirdly, pDelPepE-2, a plasmid carrying a *pepE* deletion cassette, was used to construct the *pepE* deletion mutant of *A. oryzae* PDE2 (Fig. 2B). *A. oryzae* PDE2 was transformed using a

Fig. 2. Southern blot analysis of the multiple protease gene deletant.

A. Deletion of the *npII* gene. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *npII* gene, respectively. The open and black arrows indicate the *pyrG* and *npII* genes, respectively. The direction of the arrow indicates the orientation of the oligonucleotide primers delnpII6 and delnpII7. Genomic DNA was digested with *SphI*. The 1.0-kb PCR fragment amplified with the primers delnpII5/delnpII6 was used as a probe. **B.** Deletion of the *pyrG* gene. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *pepE* gene, respectively. The open and black arrows indicate the orientation of the *pyrG* and *pepE* genes. Small arrows indicate the position of the oligonucleotide primers delpepE8 and delpepE6. Genomic DNA was digested with *SmaI*. The 1.0-kb PCR fragment amplified with the primers delpepE5/delpepE6 was used as a probe. **C.** Deletion of the *npI* gene. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *npI* gene, respectively. The open and black arrows indicate the orientation of the oligonucleotide primers delnpI7 and delnpI6. Genomic DNA was digested with *SacI*. The 1.0-kb PCR fragment amplified with the primers delnpI5/delnpI6 was used as a probe. **D.** Deletion of the *pepA* gene. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *pepA* gene, respectively. The open and black arrows indicate the orientation of the *pyrG* and *pepA* genes. Small arrows indicate the position of the oligonucleotide primers delpepA7 and delpepA6. Genomic DNA was digested with *SacI*. The 1.0-kb PCR fragment amplified with the primers delpepA5/delpepA6 was used as a probe.

PCR-amplified fragment (primer pair, delpepE1/delpepE4; template, pDelPepE-2). The loss of PepE activity could not be detected using the skim milk agar plate assay because PepE is an intracellular acid protease (van den Hombergh *et al.*, 1997). Therefore, transformants were examined by detection of a DNA fragment that was PCR-amplified from the transformants using the primer pair delpepE8/delpepE6. For 5 out of the 100 transformants examined, a 2.1-kb fragment was detected, while a 4.4-kb fragment was detected from *A. oryzae* PDE2 (Fig. 2B). Of these strains, *A. oryzae* PDI3 was used for further experiments. Southern blot analysis of genomic DNA from *A. oryzae* PDI3 and PDE2 digested with *Sma*I revealed that *A. oryzae* PDI3 contained the predicted 9.3-kb fragment while *A. oryzae* PDE2 contained the 6.9-kb fragment (Fig. 2B). These results indicated that the PCR-amplified *pepE* deletion cassette was integrated into the *pepE* locus in *A. oryzae* PDE2. Deletion of both of the *pyrG* and *pepE* genes from *A. oryzae* PDI3 was performed using the same procedure as described above. Southern blot analysis of genomic DNA from *A. oryzae* PDE3, a 5-FOA-resistant strain, and PDI3 digested with *Sma*I revealed that *A. oryzae* PDE3 contained the predicted 4.6-kb fragment while *A. oryzae* PDI3 contained the 9.3-kb fragment (Fig. 2B). These results indicated that the *pepE* gene was excised from *A. oryzae* PDE2.

Fourthly, pDelNpI-2, a plasmid carrying a *npI* deletion cassette, was used to construct the *npI* deletion mutant of *A. oryzae* PDE3 (Fig. 2C). *A. oryzae* PDE3 was transformed using a PCR-amplified fragment (primer pair, delnpI1/delnpI4; template, pDelNpI-2). Disruption of three protease genes, *alp*, *pepE* and *npII*, caused significantly decreased protease activity in *A. oryzae* PDE3. Therefore, transformants were examined by detection of a DNA fragment that was PCR-amplified from the transformants using the primer pair delnpI7/delnpI6. For 12 out of the 99 transformants examined, a 2.1-kb fragment was detected, while a 5.1-kb fragment was detected from *A. oryzae* PDE3 (Fig. 2C). Of these strains, *A. oryzae* PDI4 was used for further experiments. Southern blot analysis of genomic DNA from *A. oryzae* PDI4 and PDE3 digested with *Sac*I revealed that *A. oryzae* PDI4 contained the predicted 8.0-kb fragment while *A. oryzae* PDE3 contained the 5.6-kb fragment (Fig. 2C). These results indicated that the PCR-amplified *npI* deletion cassette was integrated into the *npI* locus in *A. oryzae* PDE3. Deletion of both the *pyrG* and *npI* genes from *A. oryzae* PDI4 was performed using the same procedure as described above. Southern blot analysis of genomic DNA from *A. oryzae* PDE4, a 5-FOA-resistant strain, and PDI4 digested with *Sac*I revealed that *A. oryzae* PDE4 contained the predicted 2.6-kb fragment while *A. oryzae* PDI4 contained the 8.0-kb fragment (Fig. 2C). These results indicated that the *npI* gene was excised from *A. oryzae* PDE3.

Finally, pDelPepA-2, a plasmid carrying a *pepA* deletion cassette, was used to construct the *pepA* deletion mutant of *A. oryzae* PDE4 (Fig. 2D). *A. oryzae* PDE4 was transformed using a PCR-amplified fragment (primer pair, delpepA1/delpepA4;

template, pDelPepA-2). Transformants were examined by detection of a DNA fragment that was PCR-amplified from the transformants using the primer pair delpepA7/delpepA6. For 5 out of the 100 transformants examined, a 2.1-kb fragment was detected, while a 4.4-kb fragment was detected from *A. oryzae* PDE4 (Fig. 2D). Of these strains, *A. oryzae* PDI5 was used for further experiments. Southern blot analysis of genomic DNA from *A. oryzae* PDI5 and PDE4 digested with *Sal*I revealed that *A. oryzae* PDI5 contained the predicted 8.1-kb fragment while *A. oryzae* PDE4 contained the 6.0-kb fragment (Fig. 2D). These results indicated that the PCR-amplified *pepA* deletion cassette was integrated into the *pepA* locus in *A. oryzae* PDE4. Deletion of both the *pyrG* and *pepA* genes was performed using the same procedure as described above. Southern blot analysis of genomic DNA from *A. oryzae* PDE5, a 5-FOA-resistant strain, and PDI5 digested with *Sal*I revealed that *A. oryzae* PDE5 contained the predicted 3.6-kb fragment while *A. oryzae* PDI5 contained the 8.1-kb fragment (Fig. 2D). These results indicated that the *pepA* gene was excised from *A. oryzae* PDE4.

As a result, a quintuple protease gene (*alp*, *npII*, *pepE*, *npI*, *pepA*) deletant designated as *A. oryzae* PDE5 was successfully generated.

Construction of an A. oryzae quintuple protease and double amylase gene deletant *A. oryzae* KBN616, the parent strain of *A. oryzae* P4, contains two amylase genes, *taaG1* and *taaG3* (Kitamoto *et al.*, 2006). *taaG3* gene expression is approximately 4-fold higher than that of the *taaG1* gene due to the substitution of the CCAAT sequence to CCAAA in the *taaG1* gene promoter (Nemoto *et al.*, 2012, Yoshino-Yasuda *et al.*, 2013). The *taaG3* gene was deleted first since the loss of *taaG3* gene function can be detected easily using the starch plate assay. pDelTaaG3-2, a plasmid carrying a *taaG3* deletion cassette, was used to construct the *taaG3* deletion mutant of *A. oryzae* PDE5 (Fig. 3A). *A. oryzae* PDE5 was transformed using a PCR-amplified fragment (primer pair, deltaaG31/deltaaG34; template, pDelTaaG3-2). Of the 99 transformants screened, 13 transformants formed a smaller clear zone than *A. oryzae* PDE5 on the starch agar plate with KI-I₂ staining after 48 h (Fig. 3B), while the remainder formed similarly sized clear zones. Appropriate restriction enzyme sites for Southern blot analysis could not be found in the surrounding region of the *taaG3* gene. Therefore, integration of the *taaG3* deletion cassette into the *taaG3* locus was detected by PCR using the primer pair deltaaG31/deltaaG37. For these 13 transformants, a 6.8-kb fragment was detected, while a 4.2-kb fragment was detected from *A. oryzae* PDE5 (Fig. 3A). Of these strains, *A. oryzae* KO1 was used for further experiments. Deletion of both the *pyrG* and *taaG3* genes from *A. oryzae* KO1 was performed using the same procedure as described above and was detected by PCR using the primer pair deltaaG31/deltaaG37. A 6.8-kb fragment was amplified from the genomic DNA of *A. oryzae* KO1, whereas a 2.0-kb fragment was amplified from the genomic DNA of a 5-FOA-resistant strain, *A. oryzae* KO2 (Fig. 3A). These results indicated

Fig. 3. Construction scheme and confirmation of the *taa* gene deletion.
A. Deletion of the *taaG3* gene. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *taaG3* gene, respectively. The open and black arrows indicate the position of the oligonucleotide primers *deltaG31* and *deltaG37*. Agarose gel electrophoresis of amplified DNA fragments in the *taaG3* gene region of the *taaG3* disruption and deletion strains using the primer pair *deltaG31/deltaG37*. **B.** Starch agar plate assay for detection of amylase activity. The parent strain and transformants were grown on the starch agar medium at 30°C for 48 hours. Then, they were examined for halo formation around the colony by flooding the plates with a solution of KI-I₂. **C.** Deletion of the *taaG1* gene. Dark and light gray boxes indicate the 5'- and 3'-flanking regions of the *taaG1* gene, respectively. The open and black arrows indicate the position of the oligonucleotide primers *pyrG* and *taaG1* genes, respectively. The direction of the arrow indicates the orientation of the *pyrG* and *taaG1* genes. Small arrows indicate the position of the oligonucleotide primers *deltaG31* and *deltaG17*. Agarose gel electrophoresis of amplified DNA fragments in the *taaG1* gene region of the *taaG1* disruption and deletion strains using the primer pair *deltaG31/deltaG17*.

that the *taaG3* gene was successfully excised from *A. oryzae* KO1.

Next, pDelTaaG1-2, a plasmid carrying a *taaG1* deletion cassette, was used to construct the *taaG1* deletion mutant of *A. oryzae* KO2 (Fig. 3C). *A. oryzae* KO2 was transformed using a PCR-amplified fragment (primer pair, deltaaaG31/deltaaaG34; template, pDelTaaG1-2). Of the 100 transformants screened, 28 transformants did not form a clear zone on the starch agar plate with KI-I₂ staining after 48 h (Fig. 3B), while the remainder did. Integration of the *taaG1* deletion cassette into the *taaG1* locus was detected by PCR using the primer pair deltaaaG31/deltaaaG17. For these 28 transformants, a 6.8-kb fragment was detected, while a 4.2-kb fragment was detected from *A. oryzae* KO2 (Fig. 3C). Of these strains, *A. oryzae* KO3 was used for further experiments. Deletion of both of the *pyrG* and *taaG1* genes from *A. oryzae* KO3 was performed using the same procedure as described above and was detected by PCR using the primer pair deltaaaG31/deltaaaG17. A 6.8-kb fragment was amplified from the genomic DNA of *A. oryzae* KO3, whereas a 2.0-kb fragment was amplified from the genomic DNA of the 5-FOA-resistant strain, *A. oryzae* KO4 (Fig. 3C). These results indicated that the *taaG1* gene was excised from *A. oryzae* KO2.

As a result, a quintuple protease (*alp*, *npII*, *pepE*, *npl*, *pepA*) and double amylase (*taaG3*, *taaG1*) gene deletant designated as *A. oryzae* KO4 was successfully generated. This is the first report on the combined deletion of protease and amylase genes in a filamentous fungus.

Effect of quintuple protease and double amylase gene deletion on protease and amylase activities To assess the effect of quintuple protease and double amylase gene deletion on protease and amylase activities, *A. oryzae* P4, PDE1, PDE2, PDE3, PDE4, PDE5, KO2 and KO4 were cultivated for 4 days at 30°C in RB medium supplemented with 0.15% uridine and 0.07% uracil. Total protease activity was reduced to 6.0% compared to *A. oryzae* P4 with *alp* gene deletion (Fig. 4). For further deletion of the *npII* and *pepE* genes, no significant changes in total protease activity were observed due to the low activity on milk casein or the intracellular acid protease, whereas additional deletion of the *npl* and *pepA* genes reduced the activity to approximately 1.0% of the level of *A. oryzae* P4 (Fig. 4). In the case of *A. oryzae* RIB40, disruption of the *alp* gene and ten protease genes including *alp* gene showed approximately 35% and 50% lower activity as compared to that of the control strain, respectively (Jin *et al.*, 2007, Yoon *et al.*, 2011), results which differ from our data. Alp is a major proteolytic enzyme secreted by the *shoyu koji* mold, *A. oryzae*, and is thought to be vital in producing the palatable taste of soy sauce via hydrolysis of raw materials. *A. oryzae* KBN616, from which *A. oryzae* P4 was derived, is an industrial *shoyu koji* mold. Therefore, *A. oryzae* P4 secretes large amounts of Alp in the culture medium as described above. As a result, extracellular total protease activity is presumed to be severely reduced in *A. oryzae* PDE1 due to the *alp* deletion.

Fig. 4. Effect of multiple gene deletion on the production of extracellular protease and α -amylase.

The *A. oryzae* strains were cultivated as described in the Materials and Methods. Total protease (gray box, left y-axis) and α -amylase (white box, right y-axis) activities were measured in the culture supernatant of each strain. The activity of two independent experiments is presented as the average $\pm$ standard deviation.

Fig. 5. SDS-polyacrylamide gel electrophoresis of culture supernatants for the multiple gene deletants.

A. oryzae strains were cultivated as described in the Materials and Methods. Culture supernatants (9 μ L) were subjected to SDS-PAGE on a 12.5% gel and stained with Coomassie Brilliant Blue. Arrowheads indicate bands of TAA and Alp.

Amylase activity was reduced to approximately 20% compared to *A. oryzae* P4 with *taaG3* gene deletion (Fig. 4), which was in accordance with the previously described results (Nemoto *et al.*, 2012, Yoshino-Yasuda *et al.*, 2013). Additional deletion of the *taaG1* gene completely eliminated all detectable α -amylase activity.

The culture supernatants of the protease and amylase gene deletants were subjected to SDS-PAGE analysis (Fig. 5). Deletion of the *alp* gene resulted in the disappearance of the 34-kDa Alp protein, and deletion of the other protease genes had little effect on the secreted protein profiles. Furthermore, the intensities of the TAA band was lower in the *taaG3* gene deletant and no band was

observed in the double deletant, which is in good agreement with the α -amylase activity data.

In this study, we constructed an *A. oryzae* quintuple protease gene (*alp*, *npII*, *pepE*, *npI*, *pepA*) and double amylase gene (*taaG3*, *taaG1*) deletant. The significant reduction of extracellular protease activity and suppression of TAA secretion in *A. oryzae* should result in the efficient production and purification of heterologous proteins. To evaluate the effects of the resultant multiple deletions, we are currently working to produce heterologous proteins using *A. oryzae* KO4.

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