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Enhanced Production and Secretion of Heterologous Proteins by the Filamentous Fungus *Aspergillus oryzae* via Disruption of Vacuolar Protein Sorting Receptor Gene *Aovps10*▽†

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Filamentous fungi have received attention as hosts for heterologous protein production because of their high secretion capability and eukaryotic posttranslational modifications. However, despite these positive attributes, a bottleneck in posttranscriptional processing limits protein yields. The vacuolar protein sorting gene *VPS10* encodes a sorting receptor for the recognition and delivery of several yeast vacuolar proteins. Although it can also target recombinant and aberrant proteins for vacuolar degradation, there is limited knowledge of the effect of its disruption on heterologous protein production. In this study, cDNA encoding AoVps10 from the filamentous fungus *Aspergillus oryzae* was cloned and sequenced. Microscopic observation of the transformant expressing AoVps10 fused with enhanced green fluorescent protein showed that the fusion protein localized at the Golgi and prevacuolar compartments. Moreover, disruption of the *Aovps10* gene resulted in missorting and secretion of vacuolar carboxypeptidase AoCpyA into the medium, indicating that AoVps10 is required for sorting of vacuolar proteins to vacuoles. To investigate the extracellular production levels of heterologous proteins, Δ*Aovps10* mutants expressing either bovine chymosin (CHY) or human lysozyme (HLY) were constructed. Interestingly, the Δ*Aovps10* mutation increased the maximum extracellular production levels of CHY and HLY by 3- and 2.2-fold, respectively. Western blot analysis of extracellular heterologous proteins also demonstrated an improvement in productivity. These results suggest that AoVps10 plays a role in the regulation of heterologous protein secretion in *A. oryzae* and may be involved in the vacuolar protein degradation through the Golgi apparatus.

The filamentous fungus *Aspergillus oryzae* is an important microorganism with a long history of usage in the Japanese food fermentation industry (19). Since it can secrete various and large amounts of enzymes into growth medium (4) and is a GRAS (generally regarded as safe) organism, *A. oryzae* is also an excellent host for homologous (fungal) and heterologous protein production (36). Moreover, unlike any previously described microbial production/secretion system, such as *Escherichia coli* (10), proper eukaryotic posttranslational modifications, including glycosylation and protein folding, are expected to occur in *A. oryzae*. For these reasons, this microorganism is considered one of the most adequate hosts to produce higher eukaryotic proteins. In an attempt to enhance heterologous protein production in *A. oryzae*, our group previously constructed multiple protease gene disruptants (16, 25, 45), which led to a significant improvement of recombinant bovine chymosin and human lysozyme yields (16, 45).

In general, the production levels of proteins from higher eukaryotes (animals and plants) by *A. oryzae* are much lower than those of homologous (fungal) proteins (15, 16, 31, 41). It has been shown that the bottleneck in the production of heterologous proteins is not caused by low expression of the heterologous gene but is due to posttranscriptional processes in the secretory pathway (42).

Several homologues of vacuolar protein sorting (*VPS*) genes, which play important roles in the secretory pathway, have been isolated and characterized (21, 39, 44). Among them, the *VPS10* gene codes for a type I transmembrane receptor protein, which is responsible for the recognition and sorting of soluble vacuolar hydrolases, such as carboxypeptidase Y (CPY) and proteinase A (PrA), to the vacuole by cycling between the late-Golgi and prevacuolar compartments (3, 5, 24). In *Saccharomyces cerevisiae*, however, a *vps10* mutant missorts and secretes CPY into the medium (24). The Vps10p receptor has also been implicated in the targeting and delivery of several recombinant proteins from the late-Golgi compartments to vacuoles for degradation by the vacuolar protease complex (11, 12). In our previous work, secreted protein RNase T_1 (RntA) fused with enhanced green fluorescent protein (EGFP), RntA-EGFP, was visualized in the vacuoles, as well as in the hyphal tips (27), indicating that a portion of the expressed heterologous proteins was presumably transported into the vacuoles for degradation. These observations suggest that for the improvement of heterologous protein production in *A. oryzae*, it is necessary to elucidate the machinery which mediates protein trafficking between the Golgi apparatus and vacuoles.

Although several studies have been performed with the aim of improving heterologous protein production in *A. oryzae*, heterologous protein degradation through the receptor protein Vps10p has not been investigated. Therefore, it was expected that the elucidation of the molecular mechanisms of intracellular protein trafficking to the vacuole could improve the uti-

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TABLE 1. Strains used in this study

Name	Parental strain	Genotype	Source or reference
RIB40		Wild-type	23
niaD300	RIB40	*niaD*$^-$	29
NSPlD1	NSR-ΔlD2	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA*	25
VTG1	niaD300	*niaD*$^-$ pnVTG [P*Aovps10*::*Aovps10* cDNA-*egfp*::T*amyB*::*niaD*]	This study
NSlDv10	NSPlD1	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* Δ*Aovps10*::*pyrG*	This study
NSlD1	NSPlD1	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* pgEpG [*pyrG*]	This study
SlD-CG	NSlD1	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* pgEpG [*pyrG*] pCGFP [P*AocpyA*::*AocpyA-egfp*::T*nos3′*::*niaD*]	This study
SlDv10-CG	NSlDv10	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* Δ*Aovps10*::*pyrG* pCGFP [P*AocpyA*::*AocpyA-egfp*::T*nos3′*::*niaD*]	This study
SlD1	NSlD1	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* pgEpG [*pyrG*] pgDN [*niaD*]	This study
SlDv10-1∼2	NSlDv10	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* Δ*Aovps10*::*pyrG* pgDN [*niaD*]	This study
SlDv10-VTG1∼2	NSlDv10	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* Δ*Aovps10*::*pyrG* pnVTG [P*Aovps10*::*Aovps10* cDNA-*egfp*::T*amyB*::*niaD*]	This study
SlD-AKC1	NSlD1	*niaD*$^-$::pgAKCN [P*amyB*::*amyB-kex2-CHY*::T*amyB*::*niaD*] *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* pgEpG [*pyrG*]	This study
SlDv10-AKC3∼4	NSlDv10	*niaD*$^-$::pgAKCN [P*amyB*::*amyB-kex2-CHY*::T*amyB*::*niaD*] *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* Δ*Aovps10*::*pyrG*	This study
SlD-HLY1	NSlD1	*niaD*$^-$::pgAFLN [P*amyB*::*amyB-kex2-HLY*::T*amyB*::*niaD*] *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* pgEpG [*pyrG*]	This study
SlDv10-HLY1∼2	NSlDv10	*niaD*$^-$::pgAFLN [P*amyB*::*amyB-kex2-HLY*::T*amyB*::*niaD*] *sC*$^-$ *adeA*$^-$ Δ*argB*::*adeA*$^-$ Δ*ligD*::*argB* Δ*pyrG*::*adeA* Δ*Aovps10*::*pyrG*	This study

lization of *A. oryzae* as a host for heterologous protein production. Here, we report the disruption of a vacuolar protein sorting gene and its effect on production levels of heterologous proteins in *A. oryzae*. This is the first report demonstrating that disruption of a vacuolar protein sorting gene in filamentous fungi leads to enhanced production levels of heterologous proteins.

MATERIALS AND METHODS

Strains, media, and transformation. The *A. oryzae* wild-type strain, RIB40 (23) and a strain with a highly efficient gene-targeting background (*niaD*$^-$ *sC*$^-$ Δ*pyrG* Δ*ligD*), NSPlD1 (25), were used as a DNA donor and transformation host, respectively. The strains generated in the present study are listed in Table 1. DPY (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ [pH 5.5]) and potato dextrose agar (Nissui Pharmaceutical, Tokyo, Japan) media were used for the normal growth and maintenance of all strains. M medium [0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and 2% glucose (pH 5.5)] supplemented with 0.15% methionine (M+Met) was used as a selective medium for disrupting the *Aovps10* gene. 5× DPY (pH 5.5) (10% dextrin, 5% polypeptone, 2.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ [pH 5.5]) was used as a medium for production of CHY. 5× DPY (pH 8.0; 10% dextrin, 5% polypeptone, 2.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ [pH 8.0]) was used as a medium for production of HLY. Czapek-Dox (CD) medium (0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 2% glucose [pH 5.5]) supplemented with 0.0015% methionine was used for *niaD*-based plasmid integration and for microscopic observations. *Escherichia coli* DH5α [*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used for DNA manipulation. *A. oryzae* was transformed according to a previously reported method (19).

Molecular techniques for DNA manipulation. The BP and LR recombination reactions for all plasmid construction using the MultiSite Gateway system were performed as instructed by the manufacturer (Invitrogen, Carlsbad, CA) (22). The PCR for cloning was performed with a 50-μl mixture containing 1× PCR buffer with Mg^{2+}, deoxynucleoside triphosphate (dNTP) solution (0.2 mM each), primers (0.2 μM each), 1.25 U of PrimeSTAR HS DNA polymerase (TaKaRa, Otsu, Japan), and 100 ng of DNA. The thermocycling program consisted of an initial heating (98°C, 2 min), followed by 30 cycles of denaturation at (98°C, 10 s), annealing (58°C, 15 s), and extension at (72°C, 1 min/kb). The PCR for verification of the *Aovps10* gene disruption was performed with a 20-μl mixture containing 1× PCR buffer with Mg^{2+}, dNTP solution (0.2 mM each), primers (0.5 μM each), 0.5 U of ExTaq DNA polymerase (TaKaRa), and 10 ng of DNA. The thermal cycling parameters consisted of an initial heating (98°C, 2 min), followed by 30 cycles of denaturation at (98°C, 10 s), annealing (58°C, 30 s), and extension at (72°C, 6 min). All of the primers used in the present study are listed in Table 2.

Cloning of *Aovps10* cDNA and sequencing. For the cloning of *Aovps10* cDNA, a DNA fragment containing the *Aovps10* cDNA was amplified by reverse transcription-PCR. Total RNA was extracted by using an RNeasy midikit (Qiagen), which was then subjected to mRNA purification by using an Oligotex-dT30<Super> kit (TaKaRa). Total cDNA was synthesized by SuperScript reverse transcriptase (Invitrogen) and was used as a template for PCR with the two primers VPS10-cDNA-PF and VPS10-cDNA-RG and ligated to pT7Blue, generating pVPTcD. cDNA sequences of AoVps10-encoding genes were determined by ABI Prism 310NT genetic analyzer (Applied Biosystems, Foster City, CA).

Construction of the *Aovps10* gene disruptant. The 1.4-kb upstream flanking region of the *Aovps10* open reading frame (ORF) was amplified with the primers aB4-5v10_F and aB1r-5v10_R using genomic DNA as a template and then inserted into pDONRP4-P1R by the BP recombination reaction, generating the 5′ entry clone, pg5′v10. The 0.3-kb upstream and 1.4-kb downstream flanking regions of the gene were amplified with the primer pairs aB2r-v10_F/f-v10+p_R and f-v10+p_F/aB3-v10_R, respectively. The two fragments were fused with the primers aB2r-v10_F and aB3-v10_R and inserted into pDONRP2R-P3 by the BP recombination reaction, generating the 3′ entry clone, pg3′v10+p. The obtained 5′ and 3′ entry clones, together with the center entry clone plasmid, pgEpG, were combined for the LR recombination reaction with the destination vector, pDESTR4-R3, generating pgΔv10pG. The gene disruption fragment for *Aovps10* was amplified with the primers aB4-5v10_F and aB3-v10_R using pgΔv10pG as a template and was introduced into the NSPlD1 strain (*niaD*$^-$ *sC*$^-$ Δ*pyrG* Δ*ligD*). M+Met medium was used for selection of transformants. For verification of the *Aovps10* gene disruption, genomic DNA was used as a template for PCR analysis using the primers Aovps10up-1527bp-F and Aovps10down-4671bp-R.

Expression plasmid construction. The plasmid encoding AoVps10-EGFP was constructed as follows. The ORF of the *Aovps10* gene was PCR amplified with the primers VPS10-cDNA-PF and VPS10-cDNA-RG-I using pVPTcD as a template. The amplified *Aovps10* cDNA fragment was phosphorylated and inserted into SmaI-digested pgEHH (22), generating pgVTcD (Invitrogen; for generation of the center entry clone). The promoter region of the *Aovps10* gene was amplified with the primers VPS10up-F and VPS10up-R using genomic DNA as a template. The amplified *Aovps10* promoter fragment was phosphorylated and inserted into SmaI-digested pg5′Pp (22), generating 5′ entry clone, pgVTPr. The generated center entry clone (pgVTcD), 5′ entry clone (pgVTPr), and 3′ entry

TABLE 2. Primers used in this study

Function	Primer	Sequence (5′–3′)[a]
Amplification of *Aovps10* cDNA for cloning	VPS10-cDNA-PF	CGGTCATGATTTATCGATGGC
	VPS10-cDNA-RG	TCAAGCCTCGTCATCTTCATC
Amplification of *Aovps10* cDNA for expression plasmid construction	VPS10-cDNA-PF	CGGTCATGATTTATCGATGGC
	VPS10-cDNA-RG-I	AGCCTCGTCATCTTCATCCAA
Amplification of *Aovps10* native promoter region for expression of plasmid construction	VPS10up-F	GAACTTCTCATCAAACAGCACCC
	VPS10up-R	GACCGCTACTCTTGAAACATTTGAAAC
Amplification of *AocpyA* gene for expression of plasmid construction	CPY-1458F	GGGGATATCCGCTGGTGCTGCAGACTTGA
	CPY1678R	GGGGATATCGAACCATTCACCACCCAACCAG
Disruption of *Aovps10* gene	aB4-5v10_F	GGGGACAACTTTGTATAGAAAAGTTGCATGGACTACTCGCTTTGCGATA
	aB1r-5v10_R	GGGGACTGCTTTTTTGTACAAACTTGCAACGCACACAAAGGCTTACGTT
	aB2r-v10_F	GGGGACAGCTTTCTTGTACAAAGTGGGATCGATACTGGATGGTGTGCT
	f-v10+p_R	**CACTATAGATCAACGCACACAAAGGCTTACGTT**
	f-v10+p_F	**GTGTGCGTTGATCTATAGTGTACGGTTGTGGGC**
	aB3-v10_R	GGGGACAACTTTGTATAATAAAGTTGCTTCAGGCGCTGTTAAACATGTC
Verification of *Aovps10* gene disruption/ verification PCR	Aovps10up-1527bp-F	GGATTCGGACCTGAGTTCTGATA
	Aovps10down-4671bp-R	TAAAATCATGGATACGGCGGAGC
Southern blot probe	Aovps10up-1500bp-F	CATGGACTACTCGCTTTGCGATAA
	Aovps10up-413bp-R	ATCTCAACCATGGGGTGAACATC

[a] The *att*B sequences for cloning with MultiSite Gateway system are underlined. The nucleotide sequences for fusion PCR are indicated in boldface.

clone (pg3′E) (22) were mixed with the destination vector (pgDN) (22) for the LR recombination reaction. The resulting plasmid pnVTG contained the *Aovps10* cDNA-*egfp* fusion gene under the control of the *Aovps10* native promoter, along with the *niaD* marker.

The AoCpyA-EGFP expression plasmid was constructed by amplifying the *AocpyA* gene, including the promoter region, using the primers CPY-1458F and CPY1678R and genomic DNA of *A. oryzae* RIB40 strain as a template. The amplified fragment was digested with EcoRI and EcoRV and then ligated with EcoRI/EcoRV-digested pBluescript II SK(+), creating pCG1. The fragment of the *egfp* gene with *nos3′* terminator (~1 kb) excised from pBEGFP-F (26) by the EcoRI was inserted into the EcoRI site of pCG1, generating pCG2. The 4.3-kb fragment containing *AocpyA-egfp* with *nos3′* terminator was excised by EcoRV and SmaI and then ligated with the HindII/SmaI-digested/blunted pNR10 containing the *niaD* marker gene to generate pCGFP.

The expression plasmids for bovine chymosin (CHY) and human lysozyme (HLY) used in the present study were constructed as described previously (32, 45).

Southern blot analysis. The *Aovps10* gene disruptants and strains expressing heterologous proteins were analyzed by Southern blot analysis. Briefly, after electrophoresis, the digested genomic DNAs were transferred onto the Hybond N+ membrane (GE Healthcare, Buckinghamshire, United Kingdom). The enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (GE Healthcare) and a LAS-4000miniEPUV luminescent image analyzer (Fuji Photo Film, Tokyo, Japan) were used for detection.

Western blot analysis. CHY-expressing transformants were cultured in 20 ml of 5× DPY (pH 5.5) medium at 30°C for 4 days. Portions (4 μl) of the culture supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a cellulose nitrate membrane Immobilon-NC (Millipore, Bedford, MA) by using a semidry blotting system (Nihon Eido, Tokyo, Japan). The membrane was immunostained using a polyclonal rabbit serum against CHY (Nordic Immunological Laboratories, Tilburg, Netherlands) and anti-rabbit immunoglobulin G labeled with horseradish peroxidase (Vector Laboratories, Peterborough, United Kingdom), and the bands were visualized by using an ECL Advance Western blotting detection kit (GE Healthcare).

Fluorescence microscopy. For fluorescence microscopy, we used an Olympus system microscope model BX52 (Olympus, Tokyo, Japan) equipped with an UPlanApo 100× objective lens (1.35 numerical aperture) (Olympus). A GFP filter (495/520-nm excitation, 510-nm dichroic, 530/535-nm emission; Chroma Technologies, Brattleboro, VT) was used for observing EGFP fluorescence. Confocal microscopy was performed with an IX71 inverted microscope (Olympus) equipped with a 100× Neofluor objective lens (1.40 numerical aperture); 488-nm (Furukawa Electric, Japan) and 561-nm (Melles Griot) semiconductor lasers; GFP, DsRed, and DualView filters (Nippon Roper, Chiba, Japan); a CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan); and an Andor iXon cooled digital charge-coupled device camera (Andor Technology PLC, Belfast, United Kingdom). Images were analyzed with the Andor iQ software (Andor Technology PLC). Approximately 10^5 conidia were inoculated in 100 μl of liquid medium, followed by incubation on cover glasses for fluorescence microscopy or in glass-based dishes (Asahi Techno Glass, Chiba, Japan) for confocal laser microscopy. FM4-64 (Molecular Probes, Eugene, OR) was prepared as a 1.6 mM solution in dimethyl sulfoxide. Approximately 18 h after inoculation, the cultures were transferred into a medium containing 8 μM FM4-64 and incubated for 2 min at room temperature. After incubation, FM4-64-containing medium was replaced with fresh dye-free medium, and the samples were examined.

Measurement of EGFP fluorescence in the medium. Extracellular EGFP fluorescence was measured by using a SAFIRE multifunctional microplate reader with excitation and emission wavelengths of 488 and 510 nm, respectively (Tecan Group Ltd., Mannedorf, Switzerland).

Protease activity assay. The total extracellular protease activity in the culture medium (DPY [pH 5.5]) of each *A. oryzae* strain was analyzed by using a Calbiochem protease assay kit (EMD Bioscience, San Diego, CA), in which a fluorescein thiocarbamoyl-labeled casein (FTC-casein) was used as the protease substrate. The total protease activity of each sample was then determined from the obtained absorbance at 492 nm of the proteolytically cleaved small FTC-peptides in the reaction supernatant, which were collected by precipitating the uncleaved FTC-casein with trichloroacetic acid (3.6% [wt/vol]) treatment.

Measurement of CHY production yield. Approximately 2×10^5 conidia of the CHY-expressing transformant were inoculated into 20 ml of 5× DPY (pH 5.5)

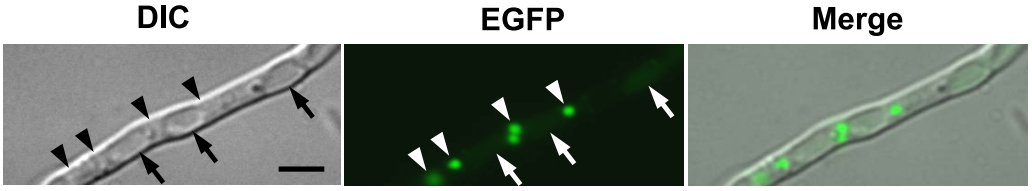

FIG. 1. Localization of AoVps10-EGFP. The strain expressing AoVps10-EGFP (VTG1) was cultivated in CD medium adjusted to pH 5.5 at 30°C for 18 h on a cover glass and observed by DIC and fluorescence microscopy. The arrows and arrowheads in the panel indicate vacuoles and dot-like structures, respectively. Bar, 5 μm.

medium and then cultured at 30°C for 3 to 6 days. The CHY activity was measured by the method described previously by Yoon et al. (45).

HLY activity assay. Approximately 10^6 conidia of the HLY-expressing transformant were inoculated and cultured in 100 ml of 5× DPY (pH 8.0) medium at 30°C. Lysozyme activity was measured as described by Morsky and Aine (30). The culture supernatant (20 μl) was mixed with 80 μl of a 150-μg/ml suspension of *Micrococcus lysodeikticus* ATCC 4698 lyophilized cells (Sigma) in 50 mM phosphate buffer (pH 6.2). The decrease in absorbance at 450 nm of the mixture, which was due to lysis of the bacterial cells, was monitored at room temperature. One unit was defined as the activity that reduced the absorbance value by 0.001 per min.

RESULTS

Cloning and sequence analysis of cDNA encoding *A. oryzae* AoVps10. The *A. oryzae* genome database DOGAN (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao) was first searched for the gene homologous to the *S. cerevisiae* vacuolar sorting receptor gene (*VPS10*), and a gene (ID AO090010000769) was identified and designated *Aovps10*. The predicted amino acid sequence of *Aovps10* was aligned and compared to using the CLUSTAL W (http://align.genome.jp/) program. Comparative sequence analysis between the nucleotide sequence of the *Aovps10* gene and the cDNA revealed that the AoVps10 sequence encoded for 1,488 amino acids and included one intron of 63 bp in the coding region. Furthermore, a strong sequence similarity was found among Vps10p from other yeasts. For example, Vps10p from *S. cerevisiae* and *S. pombe* shared 29% identity (see Fig. S1 in the supplemental material).

AoVps10 was found to contain a typical N-terminal signal peptide of 23 amino acid residues and two 23-amino-acid transmembrane domains near the C-terminal region, as determined by the web-based search programs SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Moreover, Asp box motifs (Ser/Thr-X-Asp-X-Gly-X-Thr-Trp/Phe) (17, 37) and two highly conserved residues of a cysteine-rich motif (13, 35) were present in both luminal domains 1 and 2 (see Fig. S1 in the supplemental material). The characteristics of these conserved amino acid sequences of AoVps10 agreed with known properties of Vps10p from *S. cerevisiae* and *S. pombe*. Thus, based on the observed similarities in the DNA and protein sequences, AoVps10 likely shares the identical functional properties as other sorting receptors for vacuolar proteins in *A. oryzae*.

Localization analysis of AoVps10-EGFP in *A. oryzae*. To determine the localization of AoVps10 in *A. oryzae*, a strain ectopically expressing *Aovps10* cDNA-*egfp*, was constructed. Examination of the AoVps10-EGFP-expressing strain (VTG1) by fluorescence microscopy revealed that AoVps10 localized as small punctate structures in the vicinity of vacuoles (Fig. 1). This localization pattern was similar to those observed in yeasts, such as *S. cerevisiae*, suggesting that Vps10p is a receptor that sorts several different vacuolar proteins by cycling between late-Golgi and prevacuolar compartments (3, 5, 24). Thus, we assumed that the dot-like structures observed near the vacuoles were prevacuolar compartments, and the structures distant from these vacuoles were late-Golgi, which is in agreement with the systematic observation of SNARE localization in *A. oryzae* (20, 38).

Disruption of the *Aovps10* gene. To generate a strain of *A. oryzae* unable to express *Aovps10*, the 1.4-kb flanking regions of the *Aovps10* ORF were cloned and ligated with the *pyrG* gene (Fig. 2A). In this construct, the 3′ end of the upstream flanking region (~0.3 kb) was fused with the downstream flanking region in order to flank the *pyrG* marker gene with ~0.3-kb directed repeats (Fig. 2A, hatched box). After the disrupted fragment of the *Aovps10* gene was introduced into the NSPlD1 strain and successful homologous recombination was confirmed by PCR and Southern blot analyses, five of the six transformants, representing an efficiency of 83%, contained a disruption of the *Aovps10* gene (Fig. 2B). Examination of the growth and morphological characteristics did not reveal any significant abnormalities in the *Aovps10* gene disruptant (data not shown). As a result, it was confirmed that an *Aovps10* gene disruptant, called NSlDv10, was successfully generated.

Functional analysis of AoVps10 in *A. oryzae*. Using the *S. cerevisiae CPY* gene, which encodes vacuolar carboxypeptidase Y, to search the *A. oryzae* genome database DOGAN allowed the identification of one homologous gene designated *AocpyA* (ID AO090103000332). The predicted amino acid sequence of the *AocpyA* gene was compared to the sequences of vacuolar carboxypeptidases (CPYs) from a filamentous fungus and several yeast species and demonstrated high similarities (see Fig. S2 in the supplemental material). Mature AoCpyA showed 90, 65, and 58% identities to CPYs from *A. nidulans* (33), *S. cerevisiae* (43), and *S. pombe* (40), respectively.

To examine the effect of the *Aovps10* gene disruption on the localization of carboxypeptidase Y (AoCpyA) in *A. oryzae*, the SlDv10-CG strain, which expresses the *AocpyA-egfp* fusion gene in the NSlDv10 background, was generated. Hyphae of the Δ*Aovps10* mutant were observed by both differential interference contrast (DIC) and confocal laser microscopy (Fig. 3A to D). For wild-type strain SlD-CG, large, elongated, vacuole-like structures identified by DIC microscopy were found to contain high levels of EGFP fluorescence during confocal microscopic analysis (Fig. 3A), although EGFP fluorescence was not present in septa or cell walls (Fig. 3B). In contrast, the

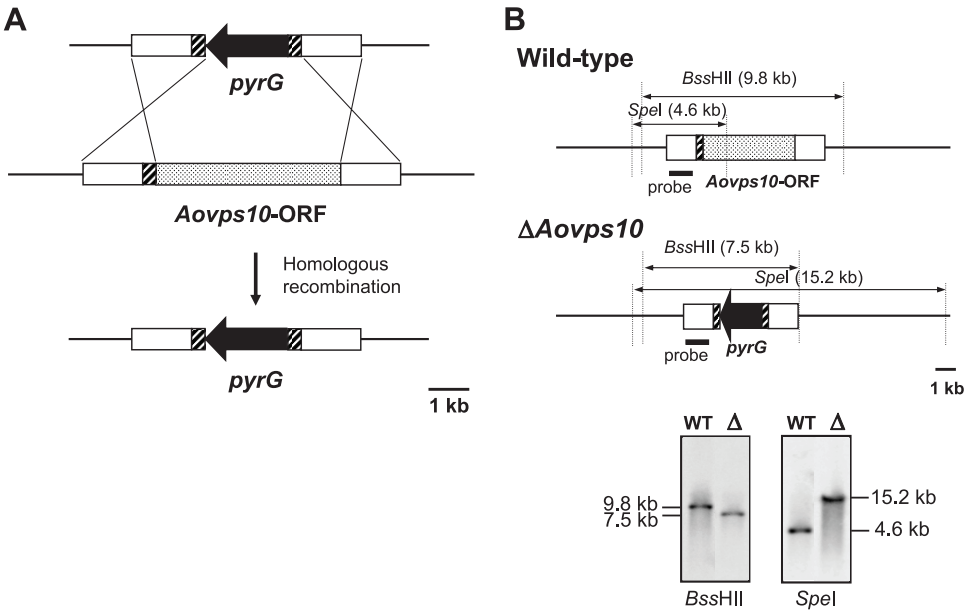

FIG. 2. Disruption of the *Aovps10* gene in the *A. oryzae* strain NSPlD1 (*niaD*$^{-}$ *sC*$^{-}$ Δ*pyrG* Δ*ligD*). (A) Schematic model of the approach used to disrupt the *Aovps10* gene using the *pyrG* marker gene. The open boxes (1.4 kb) represent the flanking regions of the *Aovps10* gene. The 0.3-kb upstream flanking region of the *Aovps10* gene (hatched boxes) was attached at the 5′ end of the downstream flanking region to introduce direct repeat sequences. (B) Southern blot analysis of the *Aovps10* gene disruptants. After isolated genomic DNA was digested with BssHII and SpeI, separated by gel electrophoresis, and subjected to Southern blot analysis, a clone was identified which exhibited the expected banding pattern for disruption of the *Aovps10* gene. "WT" and "Δ" represent the wild-type strain (NSPlD1) and gene disruptant (NSlDv10), respectively.

Δ*Aovps10* strain localized the AoCpyA-EGFP fusion protein to septa and cell walls as opposed to vacuoles (Fig. 3C and D). Moreover, these observations were consistent with the known septal localization patterns of secretory hydrolytic enzymes fused with (E)GFP, such as the RNase of *A. oryzae* (27) and the glucoamylase of *A. niger* (9). The dispersed, round structures observed in the Δ*Aovps10* strain were attributed to accumulation of the fusion protein at unknown organelles (Fig. 3C and D). To determine whether the small punctuate structures are endosomes, the Δ*Aovps10* strain was stained with FM4-64, a lipophilic styryl dye used as an endocytic marker. The dye labels cortical punctuate structures, as well as the membranes of circular, hollow organelles, and intermediate and large size vacuoles (34). The dispersed, round structures observed in the Δ*Aovps10* strain did not colocalize to endocytic organelles, suggesting that the unknown organelles were not involved in the endocytic pathway in *A. oryzae* (see Fig. S3 in the supplemental material). This result (Fig. 3A to D) indicates that in the *Aovps10* gene disruptant, intracellular trafficking of AoCpyA to vacuoles was reduced and, thus, its localization in vacuoles was not observed. The localization to septa and cell walls was attributed to AoCpyA, which was no longer transported to the vacuoles by disruption of the *Aovps10* gene and was instead missorted and secreted into the medium. Taken together, these results suggest that AoVps10 is a vacuolar protein sorting receptor that is required for targeting and delivery of vacuolar enzyme AoCpyA to the vacuole in *A. oryzae*.

To investigate the total amount of missorted AoCpyA caused by the *Aovps10* gene disruption, the culture medium for mutant strain SlDv10-CG, which expresses the *AocpyA-egfp* fusion gene, was measured for EGFP fluorescence. Since this measurement can be used to estimate the total amount of an EGFP-fused target protein, a microtiter plate assay was used as a fast, simple, and reliable method for the quantification of EGFP in the medium. After culturing for 24 h, no significant changes in the relative amount of EGFP fluorescence were observed between the Δ*Aovps10* and wild-type (SlD-CG) strains. However, after 48 h, the relative amount of EGFP fluorescence for strain SlDv10-CG was significantly higher than that of SlD-CG (Fig. 3E). This result is in agreement with the DIC and fluorescence microscopic analyses shown in Fig. 3A to D.

Complementation analysis by expressing the *Aovps10-egfp* fusion gene. To confirm whether AoVps10-EGFP is functional in Δ*Aovps10* mutant, the SlDv10-VTG strains, which express the *AocpyA-egfp* fusion gene in the NSlDv10 background, were generated. Western blot analysis with anti-AoCpyA antibody revealed the presence of proAoCpyA in the culture supernatant of the *Aovps10* gene disruptants (SlDv10-1~2). In contrast, the *Aovps10-egfp* fusion gene complemented strains SlDv10-VTG1 and SlDv10-VTG2 could not secrete proAoCpyA into the culture medium (Fig. 4A). Furthermore, total extracellular protease activity test also showed that missorting and secretion of proAoCpyA into the culture medium could be restored ca. 90% by the complementation of *Aovps10-egfp* fusion gene (Fig. 4B), suggesting that AoVps10-EGFP is functional in Δ*Aovps10* mutant.

Effect of the *Aovps10* gene disruption on heterologous protein production in *A. oryzae*. In order to investigate whether the production levels of heterologous proteins were improved in the *Aovps10* gene disruptant NSlDv10, we constructed strains expressing CHY, as a model protein. The initial translation product of CHY, prochymosin, contains a 42-amino-acid N-terminal sequence which is automatically cleaved at low pH to

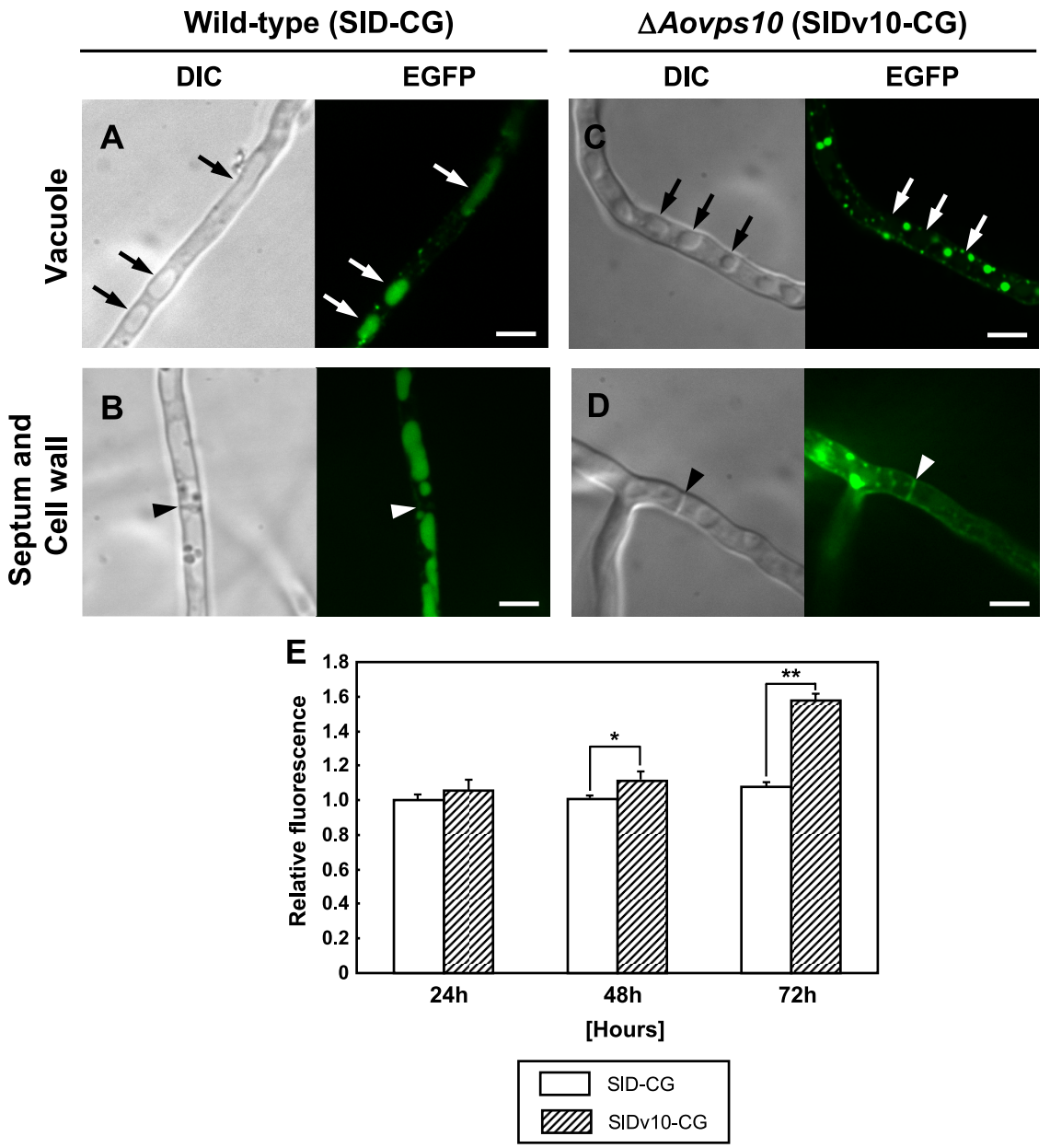

FIG. 3. Mislocalization of AoCpyA-EGFP in the *A. oryzae Aovps10* gene disruptant NSIDv10. The strains SID-CG (A and B) and SIDv10-CG (C and D), which both expressed AoCpyA-EGFP, were cultivated in CD medium adjusted to pH 5.5 at 30°C for 20 h and observed by DIC and confocal laser microscopy. The arrows and arrowheads indicate vacuoles (top panels) and septa (lower panels), respectively. Bars, 5 μm. (E) Total amount of extracellular EGFP fluorescence in the culture medium of strains SID-CG and SIDv10-CG expressing AoCpyA-EGFP. After culture in 20 ml of DPY medium (pH 5.5) at 30°C for 24 h to 72 h, the total relative amounts of each cell culture supernatant were measured and compared. Three replicate experiments were performed, and the average values and standard deviations were determined (*, $P < 0.05$; **, $P < 0.001$ [Student *t* test]).

yield active chymosin (7, 8). *A. oryzae* α-amylase (AmyB) was used as the carrier protein for fusion with CHY since such fusions have been shown to aid the successful production of heterologous proteins (15, 16, 31). A plasmid for CHY production was constructed in which the gene expression construct consisted of the *amyB* promoter and structural gene, followed by the region of the *CHY* gene that contains the prosequence. In addition, the Kex2 cleavage site (-Lys-Arg-) was included upstream of prochymosin (Fig. 5A, left). The plasmid for CHY expression was introduced into the *pyrG* marker gene-complemented strain (NSID1) and the *Aovps10* gene disruptant (NSIDv10) using the *niaD* gene as the selectable marker. The transformants possessing a single copy of the plasmid, which was integrated homologously at the *niaD* locus were identified by Southern blot analysis (data not shown).

The amount of CHY produced by each strain was then determined by the milk-clotting activity of the culture supernatant, as described above (Fig. 5B). Comparative time course

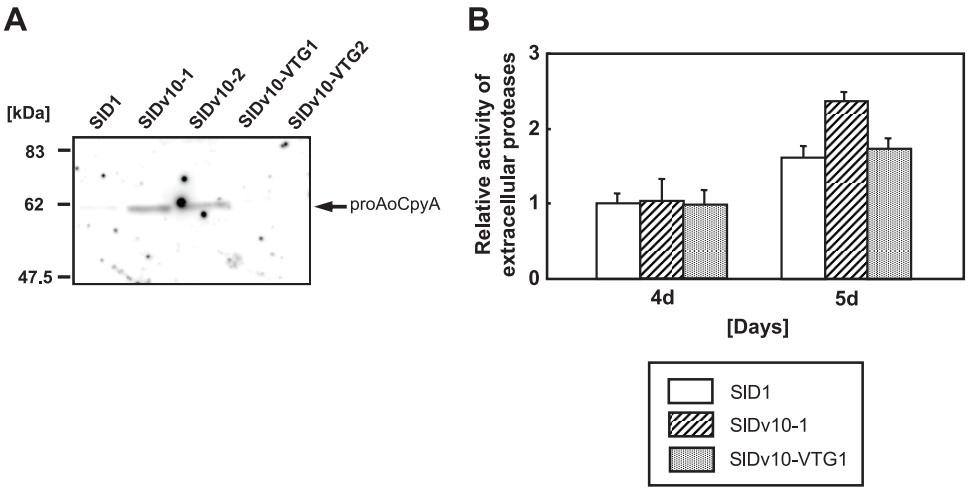

FIG. 4. Complementation analysis by expressing the *Aovps10-egfp* fusion gene. (A) Western blot analysis of the culture supernatant of the *Aovps10-egfp* fusion gene complemented strains. The control (SlD1), SlDv10, and SlDv10-VTG strains expressing AoVps10-EGFP were cultivated in 20 ml of DPY (pH 5.5) medium at 30°C for 2 days. Culture supernatants (20 μl) were subjected to Western blot analysis. The predicted bands of ~62 kDa in size were confirmed by using the anti-AoCpyA antibody. (B) Relative extracellular protease activity in the *Aovps10-egfp* fusion gene complemented strains. The control (SlD1), SlDv10, and SlDv10-VTG strains expressing AoVps10-EGFP were cultivated in 20 ml of DPY (pH 5.5) medium at 30°C for 4 to 5 days. The total extracellular protease activity was measured with each culture medium of the strain using FTC-casein as the protease substrate. Three replicate experiments were performed, and the values of the average and standard deviations are represented.

analysis revealed that the production yields by the *Aovps10* gene disruptants SlDv10-AKC3 and SlDv10-AKC4 were 83.1 and 70.3 mg/liter, respectively, representing 3- and 2.5-fold higher production levels than the control strain SlD-AKC1 (28.7 mg/liter) (Fig. 5B). The culture supernatant of the *Aovps10* gene disruptants was also examined by Western blot analysis with anti-CHY antibody, which confirmed the presence of mature CHY, as indicated by positively stained bands of ~35.4 kDa (Fig. 5C). Moreover, Western blot analysis indicated that the amount of CHY produced by the *Aovps10* gene disruptant was higher than that of the control strain.

To determine whether the extracellular production levels of other heterologous proteins were increased in the *Aovps10* gene disruptant, we next generated HLY-producing strains from NSlDv10 by introducing the HLY expression plasmid (Fig. 5A, right). Transformation was performed with the *A. oryzae niaD* gene as the selectable marker. Southern blot analysis demonstrated that a single copy of the plasmid was homologously integrated at the *niaD* locus in the obtained transformant (data not shown).

The disruptant of *Aovps10* gene was analyzed for extracellular HLY production (Fig. 5D). Disruption of the *Aovps10* gene positively affected the production of HLY. Comparative time course analysis showed that the production of HLY peaked on the 4 days when the strains were cultivated in the 5× DPY medium (pH 8.0). The maximum extracellular HLY production yields from SlDv10-HLY1 and SlDv10-HLY2 were 22.6 and 24.6 mg/liter, respectively, which were 2- and 2.2-fold higher than the levels in the control strain (SlD-HLY1; 11.1 mg/liter) (Fig. 5D). These results demonstrate that disruption of the vacuolar protein sorting receptor gene *Aovps10* in *A. oryzae* is very effective for increasing the extracellular production levels of not only CHY but HLY.

To date, the relationships between the vacuolar protein sorting gene disruption and homologous protein production are still unknown in the filamentous fungi. Thus, in order to investigate the effect of *Aovps10* gene disruption on homologous protein production in *A. oryzae*, we measured the activities of α-amylase, an endogenous protein secreted in large amounts. As a result, the activities of the *Aovps10* gene disruptant NSlDv10 were comparable to those of the control strain NSlD1 (data not shown), suggesting that AoVps10 receptor did not significantly affect the homologous protein production in *A. oryzae*.

Despite the improvement in heterologous protein yields, a slight decrease in secretion was observed in the late growth phase of the Δ*Aovps10* mutant after 5 days of culture (Fig. 5B and D). In order to examine whether endopeptidase or exopeptidase activity influenced the yields of recombinant protein produced in the *Aovps10* gene disruptant, an assay to measure total extracellular protease activity was conducted (Fig. 6). This analysis confirmed that the total protease activity in the late growth phase (5 to 6 days) was greater than in the middle growth phase (3 to 4 days). Furthermore, the relative activity of extracellular proteases in the *Aovps10* gene disruptant (NSlDv10) was slightly higher than that of the wild-type strain (NSlD1), which may have been due to the missorting and accumulation of vacuolar proteases in the medium caused by the *Aovps10* gene disruption. Taken together, these results suggest that in order to achieve high total yields of heterologous protein in *A. oryzae*, it is also necessary to limit the total protease activity.

DISCUSSION

In the present study, we isolated a cDNA encoding AoVps10 from the filamentous fungus *A. oryzae* for the first time. The predicted amino acid sequence analysis of the *Aovps10* ORF showed a high similarity to vacuolar protein sorting receptors from several microorganisms (see Fig. S1 in the supplemental

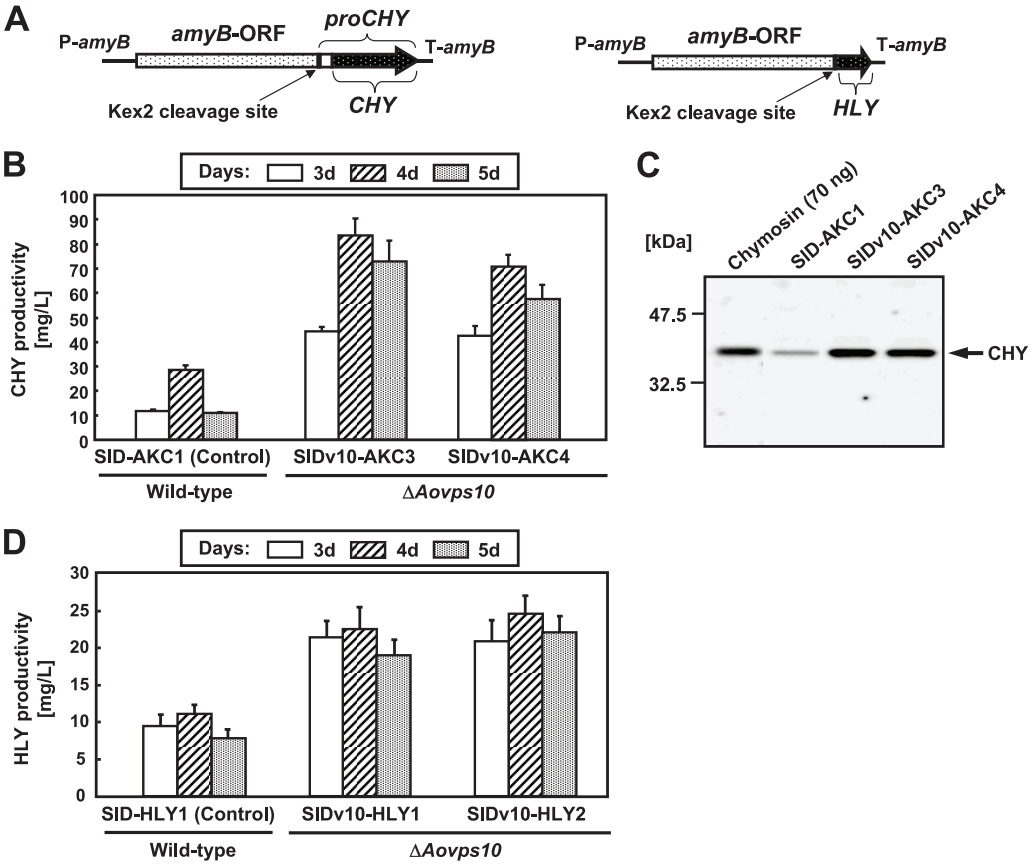

FIG. 5. Extracellular production of heterologous proteins by the *Aovps10* gene disruptant. (A) Schematic structures of either the CHY- or HLY-expressing cassette. The *proCHY* gene was fused with the *A. oryzae* α-amylase gene (*amyB*) and the Kex2 cleavage site (-Lys-Arg-) was inserted upstream of proCHY (left). The *HLY* gene was fused with the *amyB* gene. The Kex2 cleavage site was inserted at the boundary of two genes (right). (B) Time course analysis for extracellular production of CHY by the *Aovps10* gene disruptant. Approximately 2×10^5 conidia of the control (SlD-AKC1), SlDv10-AKC3, and SlDv10-AKC4 strains expressing CHY were cultivated in 20 ml of 5× DPY (pH 5.5) medium at 30°C for 3 to 5 days. The amount of CHY produced by each strain was calculated by determining the time required for a skimmed milk solution to clot using culture supernatant and comparing this value to a standard curve generated with purified bovine CHY (Sigma). (C) Western blot analysis of the culture supernatant of the CHY-expressing strains. The control (SlD-AKC1), SlDv10-AKC3, and SlDv10-AKC4 strains expressing CHY were cultivated in 20 ml of 5× DPY (pH 5.5) medium at 30°C for 4 days. Culture supernatants (4 μl) were subjected to Western blot analysis. Bands of ~35.4 kDa were detected using the anti-CHY antibody. "Chymosin" refers to purified bovine chymosin (~70 ng; Sigma). (D) Extracellular production of HLY by the *Aovps10* gene disruptant. The HLY-expressing transformants were cultivated in 100 ml of 5× DPY (pH 8.0) liquid medium at 30°C for 3 to 5 days. The production yields of HLY were calculated on the basis of lysozyme activities.

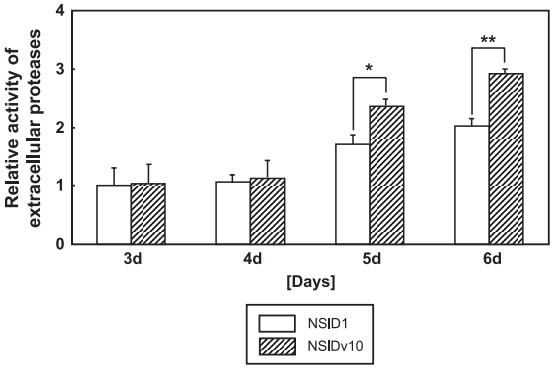

FIG. 6. Time course analysis for the relative extracellular protease activity in the *Aovps10* gene disruptant. The control (NSlD1) and the NSlDv10 strains were cultivated in 20 ml of 5× DPY (pH 5.5) medium at 30°C for 3 to 6 days. The total extracellular protease activity was measured with each culture medium of the strain using FTC-casein as the protease substrate. Three replicate experiments were performed, and the values of the average and standard deviations are represented (*, $P < 0.05$; **, $P < 0.001$ [Student *t* test]).

material), suggesting that the vacuolar protein sorting pathway is highly conserved in the filamentous fungi.

We investigated the effect of the *Aovps10* gene disruption in *A. oryzae* on heterologous protein production and also elucidated the molecular mechanism of intracellular protein trafficking to vacuoles by AoVps10. As shown in Fig. 1, the localization pattern of AoVps10 suggests that Vps10p is a receptor that sorts several different vacuolar proteins by cycling between late-Golgi and prevacuolar compartments. Moreover, functional analysis of AoVps10 revealed that it is a vacuolar protein sorting receptor required for targeting and delivery of AoCpyA to vacuoles in *A. oryzae* (Fig. 3). Interestingly, it was also confirmed that either recombinant CHY or HLY production in a single *Aovps10* gene disruptant resulted in 3- and 2.2-fold higher yields than the wild-type strain, respectively (Fig. 5), which suggests that increased protein production levels are due to defects in the vacuolar protein trafficking pathway between the Golgi apparatus and vacuoles. The remarkable increase in

CHY production by only a single gene disruption is very intriguing since it was comparable to the yield of a quintuple protease gene disruptant (84.4 mg/liter) (45). Although very recently the *vps10* gene deletion was found to be effective for the production of recombinant human growth hormone (r-hGH) in the fission yeast *S. pombe* (14), this is the first report of targeted disruption of a vacuolar protein sorting gene in a filamentous fungus which could dramatically enhance the production levels of heterologous proteins.

Previously, we used DNA microarrays to monitor the expression of 134 protease genes for screening of the disruption target (18). In the course of monitoring global gene expression, several protease genes, including *tppA*, *pepE*, *nptB*, *dppIV*, and *dppV*, were identified which were elevated in the late growth phase (5 to 6 days) (18) and were subsequently disrupted to examine their effect on heterologous protein degradation (45). Based on these studies, it is expected that disruption of the vacuolar protein sorting gene *Aovps10*, in addition to these protease genes, might synergistically improve heterologous protein productivity and yields in *A. oryzae*. Meanwhile, alternatively, unexpected proteolytic degradation by the activities of certain kinds of missorted and secreted vacuolar proteases into the culture medium might also occur, since AoVps10 receptor is responsible for the recognition and sorting of some vacuolar proteases to the vacuole. These gene disruption combinations should provide new insights into molecular breeding approaches for filamentous fungi.

We measured the activities of α-amylase and could not observe any significant differences between the wild-type strain and the Δ*Aovps10* mutants. α-Amylase is not a vacuolar hydrolase but one of the most efficiently and abundantly secreted endogenous enzyme in *A. oryzae*. Therefore, it is suggested that α-amylase is independent to AoVps10 receptor.

The protein quality control process is a mechanism by which newly synthesized proteins in the endoplasmic reticulum (ER) are monitored and assisted to ensure that proper folding and assembly occur (6). This process also detects misfolded or aberrant proteins and removes them from the secretory pathway. Another mechanism for removing abnormal proteins from the secretory pathway has been identified that involves routing targeted proteins from the Golgi to the vacuolar system for degradation (2). Although delivery of incorrectly assembled membrane complexes for lysosomal degradation has been described in mammalian cells (1, 28), it seems likely that a Golgi-based quality control process might play a more prominent role in yeast (12, 46) and the filamentous fungus *A. oryzae* for removing both misfolded and aberrantly assembled proteins.

In conclusion, we have demonstrated in the present study that the vacuolar protein sorting pathway is highly conserved in the filamentous fungus *A. oryzae*, with AoVps10 playing a role in the regulation of the heterologous protein secretory pathway by its involvement in vacuolar protein degradation through the Golgi apparatus. In future studies, transcriptomic analysis by DNA microarrays to identify genes affecting heterologous protein production, together with the determination of ligand-binding domains for recombinant protein interactions, will reveal important details about the specific role of AoVps10 in heterologous protein production.

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REFERENCES

1. **Armstrong, J., S. Patel, and P. Riddle.** 1990. Lysosomal sorting mutants of coronavirus E1 protein, a Golgi membrane protein. J. Cell Sci. **95:**191–197.
2. **Arvan, P., X. Zhao, J. Ramos-Castaneda, and A. Chang.** 2002. Secretory pathway quality control in the Golgi, plasmalemmal, and endosomal systems. Traffic **3:**771–780.
3. **Cereghino, J. L., E. G. Marcusson, and S. D. Emr.** 1995. The cytoplasmic tail domain of the vacuolar protein sorting receptor Vps10p and a subset of *VPS* gene products regulate receptor stability, function, and localization. Mol. Biol. Cell **6:**1089–1102.
4. **Christensen, T., H. Woeldike, F. Boel, S. B. Mortensen, K. Hjortshoej, L. Thim, and M. Hansen.** 1988. High-level expression of recombinant genes in *Aspergillus oryzae*. Biotechnology **6:**1419–1422.
5. **Cooper, A. A., and T. H. Stevens.** 1996. Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases. J. Cell Biol. **133:**529–541.
6. **Ellgard, L., M. Molinari, and A. Helenius.** 1999. Setting the standards: quality control in the secretory pathway. Science **286:**1882–1888.
7. **Foltmann, B.** 1970. Prochymosin and chymosin (prorennin and rennin). Methods Enzymol. **19:**421–436.
8. **Foltmann, B.** 1971. The biochemistry of prorennin (prochymosin) and rennin (chymosin), p. 217–254. *In* H. A. McKenzie (ed.), The milk proteins, vol. 2. Academic Press, Inc., New York, NY.
9. **Gordon, C. L., V. Khalaj, A. F. Ram, D. B. Archer, J. L. Brookman, A. P. Trinci, D. J. Jeenes, J. H. Doonan, B. Wells, P. J. Punt, C. A. van den Hondel, and G. D. Robson.** 2000. Glucoamylase::green fluorescent protein fusions to monitor protein secretion in *Aspergillus niger*. Microbiology **146:**415–426.
10. **Gottesman, S.** 1990. Minimizing proteolysis in *Escherichia coli*: genetic solutions. Methods Enzymol. **185:**119–129.
11. **Holkeri, H., and M. Makarow.** 1998. Different degradation pathways for heterologous glycoproteins in yeast. FEBS Lett. **429:**162–166.
12. **Hong, E., A. R. Davidson, and C. A. Kaiser.** 1996. A pathway for targeting soluble misfolded proteins to the yeast vacuole. J. Cell Biol. **135:**623–633.
13. **Horazdovsky, B. F., D. B. DeWald, and S. D. Emr.** 1995. Protein transport to the yeast vacuole. Curr. Opin. Cell Biol. **7:**544–551.
14. **Idiris, A., H. Tohda, M. Sasaki, K. Okada, H. Kumagai, Y. Giga-Hama, and K. Takegawa.** 2009. Enhanced protein secretion from multiprotease-deficient fission yeast by modification of its vacuolar protein sorting pathway. Appl. Microbiol. Biotechnol. **85:**667–677.
15. **Ito, K., T. Asakura, Y. Morita, K. Nakajima, A. Koizumi, A. Shimizu-Ibuka, K. Masuda, M. Ishiguro, T. Terada, J. Maruyama, K. Kitamoto, T. Misaka, and K. Abe.** 2007. Microbial production of sensory-active miraculin. Biochem. Biophys. Res. Commun. **360:**407–411.
16. **Jin, F. J., T. Watanabe, P. R. Juvvadi, J. Maruyama, M. Arioka, and K. Kitamoto.** 2007. Double disruption of the proteinase genes, *tppA* and *pepE*, increases the production level of human lysozyme by *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. **76:**1059–1068.
17. **Jørgensen, M. U., S. D. Emr, and J. R. Winther.** 1999. Ligand recognition and domain structure of Vps10p, a vacuolar protein sorting receptor in *Saccharomyces cerevisiae*. Eur. J. Biochem. **260:**461–469.
18. **Kimura, S., J. Maruyama, M. Takeuchi, and K. Kitamoto.** 2008. Monitoring global gene expression of proteases and improvement of human lysozyme production in the *nptB* gene disruptant of *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. **72:**499–505.
19. **Kitamoto, K.** 2002. Molecular biology of the Koji molds. Adv. Appl. Microbiol. **51:**129–153.
20. **Kuratsu, M., A. Taura, J. Y. Shoji, S. Kikuchi, M. Arioka, and K. Kitamoto.** 2007. Systematic analysis of SNARE localization in the filamentous fungus *Aspergillus oryzae*. Fungal Genet. Biol. **44:**1310–1323.
21. **Lemmon, S. K., and L. M. Traub.** 2000. Sorting in the endosomal system in yeast and animal cells. Curr. Opin. Cell Biol. **12:**457–466.
22. **Mabashi, Y., T. Kikuma, J. Maruyama, M. Arioka, and K. Kitamoto.** 2006. Development of a versatile expression plasmid construction system for *Aspergillus oryzae* and its application to visualization of mitochondria. Biosci. Biotechnol. Biochem. **70:**1882–1889.
23. **Machida, M., K. Asai, M. Sano, T. Tanaka, T. Kumagai, G. Terai, K. Kusumoto, T. Arima, O. Akita, Y. Kashiwagi, K. Abe, K. Gomi, H. Horiuchi, K. Kitamoto, T. Kobayashi, M. Takeuchi, D. W. Denning, J. E. Galagan, W. C. Nierman, J. Yu, D. B. Archer, J. W. Bennett, D. Bhatnagar, T. E. Cleveland, N. D. Fedorova, O. Gotoh, H. Horikawa, A. Hosoyama, M. Ichinomiya, R. Igarashi, K. Iwashita, P. R. Juvvadi, M. Kato, Y. Kato, T. Kin, A.**

Kokubun, H. Maeda, N. Maeyama, J. Maruyama, H. Nagasaki, T. Nakajima, K. Oda, K. Okada, I. Paulsen, K. Sakamoto, T. Sawano, M. Takahashi, K. Takase, Y. Terabayashi, J. R. Wortman, O. Yamada, Y. Yamagata, H. Anazawa, Y. Hata, Y. Koide, T. Komori, Y. Koyama, T. Minetoki, S. Suharnan, A. Tanaka, K. Isono, S. Kuhara, N. Ogasawara, and H. Kikuchi. 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature **438:**1157–1161.

24. **Marcusson, E. G., B. F. Horazdovsky, J. L. Cereghino, E. Gharakhanian, and S. D. Emr.** 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the *VPS10* gene. Cell **77:**579–586.
25. **Maruyama, J., and K. Kitamoto.** 2008. Multiple gene disruptions by marker recycling with highly efficient gene-targeting background (Δ*ligD*) in *Aspergillus oryzae*. Biotechnol. Lett. **30:**1811–1817.
26. **Maruyama, J., H. Nakajima, and K. Kitamoto.** 2001. Visualization of nuclei in *Aspergillus oryzae* with EGFP and analysis of the number of nuclei in each conidium by FACS. Biosci. Biotechnol. Biochem. **65:**1504–1510.
27. **Masai, K., J. Maruyama, H. Nakajima, and K. Kitamoto.** 2003. *In vivo* visualization of the distribution of a secretory protein in *Aspergillus oryzae* hyphae using the RntA-EGFP fusion protein. Biosci. Biotechnol. Biochem. **67:**455–459.
28. **Minami, Y., A. M. Weissman, L. E. Samelson, and R. D. Klausner.** 1987. Building a multichain receptor: synthesis, degradation, and assembly of the T-cell antigen receptor. Proc. Natl. Acad. Sci. U. S. A. **84:**2688–2692.
29. **Minetoki, T., Y. Nunokawa, K. Gomi, K. Kitamoto, C. Kumagai, and G. Tamura.** 1996. Deletion analysis of promoter elements of the *Aspergillus oryzae agdA* gene encoding alpha-glucosidase. Curr. Genet. **30:**432–438.
30. **Morsky, P., and E. Aine.** 1983. Determination of lysozyme in tears by immunoturbidimetric and optimized kinetic bacteriolytic methods. Clin. Chim. Acta **129:**201–209.
31. **Nakajima, K., T. Asakura, J. Maruyama, Y. Morita, H. Oike, A. Shimizu-Ibuka, T. Misaka, H. Sorimachi, S. Arai, K. Kitamoto, and K. Abe.** 2006. Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by *Aspergillus oryzae*. Appl. Environ. Microbiol. **72:**3716–3723.
32. **Nemoto, T., T. Watanabe, Y. Mizogami, J. Maruyama, and K. Kitamoto.** 2009. Isolation of *Aspergillus oryzae* mutants for heterologous protein production from a double proteinase gene disruptant. Appl. Microbiol. Biotechnol. **82:**1105–1114.
33. **Ohsumi, K., Y. Matsuda, H. Nakajima, and K. Kitamoto.** 2001. Cloning and characterization of the *cpyA* gene encoding intracellular carboxypeptidase from *Aspergillus nidulans*. Biosci. Biotechnol. Biochem. **65:**1175–1180.
34. **Peñalva, M. A.** 2005. Tracing the endocytic pathway of *Aspergillus nidulans* with FM4-64. Fungal Genet. Biol. **42:**963–975.
35. **Petersen, C. M., M. S. Nielsen, A. Nykjaer, L. Jacobsen, N. Tommerup, H. H. Rasmussen, H. Roigaard, J. Gliemann, P. Madsen, and S. K. Moestrup.** 1997. Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. J. Biol. Chem. **272:**3599–3605.
36. **Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus, and C. van den Hondel.** 2002. Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol. **20:**200–206.
37. **Roggentin, P., B. Rothe, J. B. Kaper, J. Galen, L. Lawrisuk, E. R. Vimr, and R. Schauer.** 1989. Conserved sequences in bacterial and viral sialidases. Glycoconj. J. **6:**349–353.
38. **Shoji, J., M. Arioka, and K. Kitamoto.** 2006. Vacuolar membrane dynamics in the filamentous fungus *Aspergillus oryzae*. Eukaryot. Cell **5:**411–421.
39. **Suzuki, T., N. Oiso, R. Gautam, E. K. Novak, J. J. Panthier, P. G. Suprabha, T. Vida, R. T. Swank, and R. A. Spritz.** 2003. The mouse organellar biogenesis mutant buff results from a mutation in *Vps33a*, a homologue of yeast *vps33* and *Drosophila carnation*. Proc. Natl. Acad. Sci. U. S. A. **100:**1146–1150.
40. **Tabuchi, M., D. Iwaihara, Y. Ohtani, N. Ohuchi, J.-I. Sakurai, T. Morita, S. Iwahara, and K. Takegawa.** 1997. Vacuolar protein sorting in fission yeast: cloning, biosynthesis, transport, and processing of carboxypeptidase Y from *Schizosaccharomyces pombe*. J. Bacteriol. **179:**4179–4189.
41. **Tsuchiya, K., T. Nagashima, Y. Yamamoto, K. Gomi, K. Kitamoto, and C. Kumagai.** 1994. High-level secretion of calf chymosin using a glucoamylase prochymosin fusion gene in *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. **58:**895–899.
42. **Tsuchiya, K., S. Tada, K. Gomi, K. Kitamoto, C. Kumagai, Y. Jigami, and G. Tamura.** 1992. High-level expression of the synthetic human lysozyme gene in *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. **39:**738–743.
43. **Valls, L. A., C. P. Hunter, J. H. Rothman, and Y. H. Stevens.** 1987. Protein sorting in yeast; the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell **48:**887–897.
44. **Welters, P.** 1996. Vacuolar targeting: proteins of the transport machinery. Plant Physiol. Biochem. **34:**229–235.
45. **Yoon, J., S. Kimura, J. Maruyama, and K. Kitamoto.** 2009. Construction of quintuple protease gene disruptant for heterologous protein production in *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. **82:**691–701.
46. **Zhang, B., A. Chang, T. B. Kjeldsen, and P. Arvan.** 2001. Intracellular retention of newly synthesized insulin in yeast is caused by endoproteolytic processing in the Golgi complex. J. Cell Biol. **153:**1187–1198.