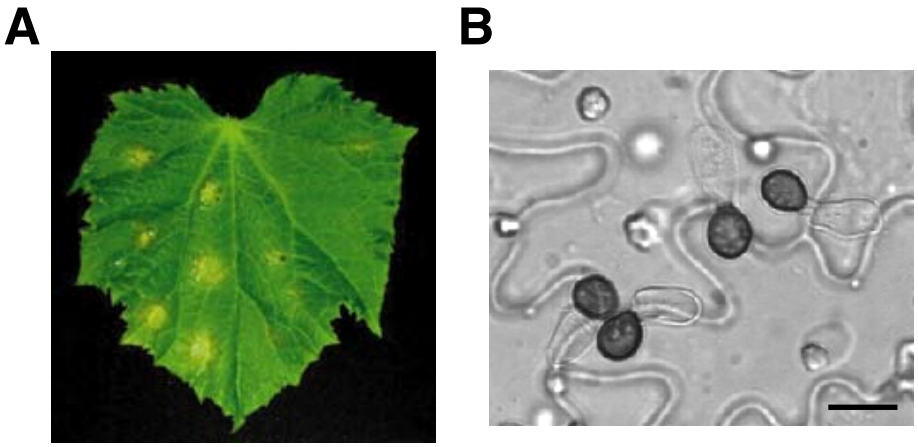
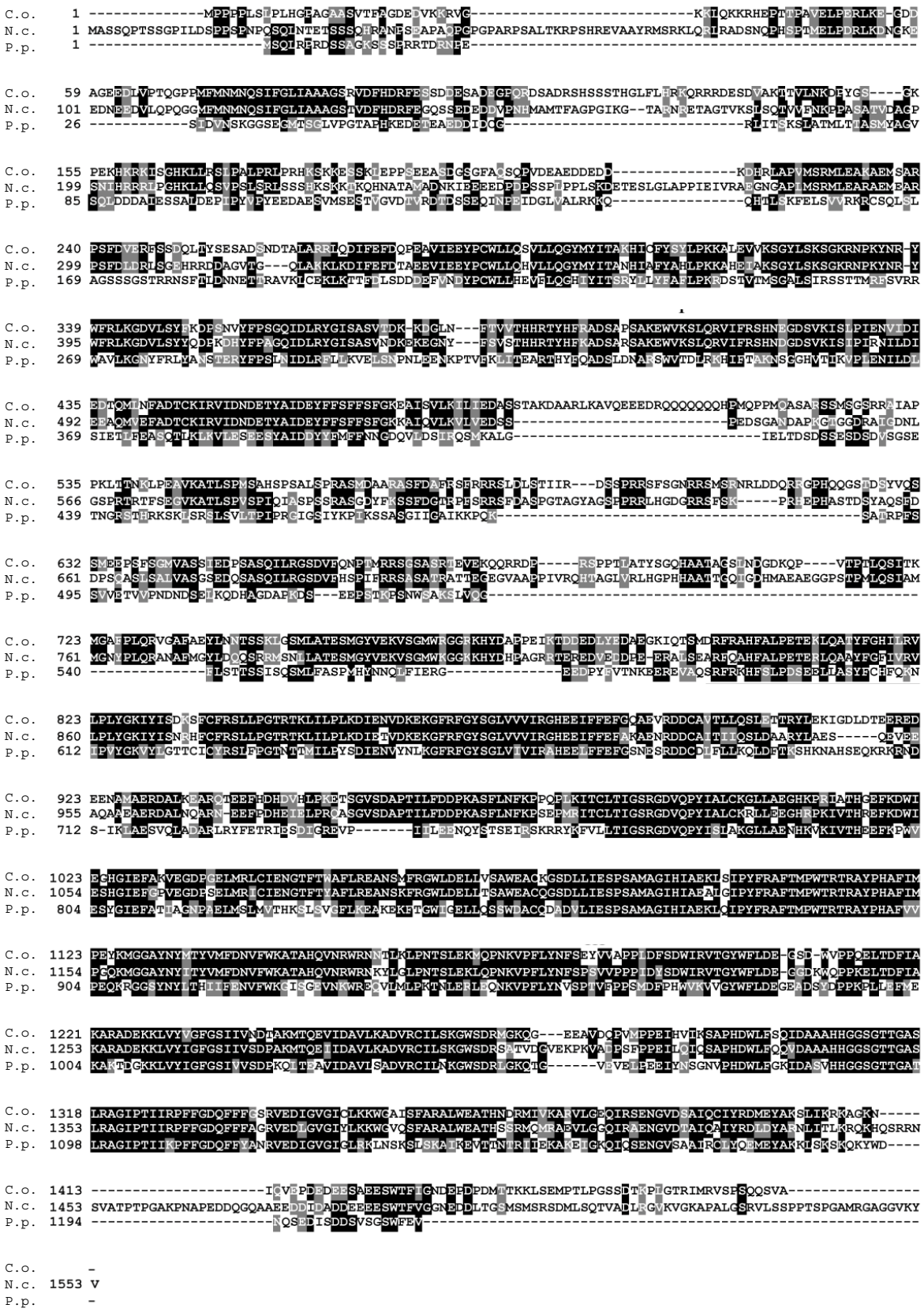
Supplemental Data. Asakura et al. (2009). Atg26-mediated pexophagy is required for host invasion by the plant pathogenic fungus *Colletotrichum orbiculare*.

Supplemental Figure 1



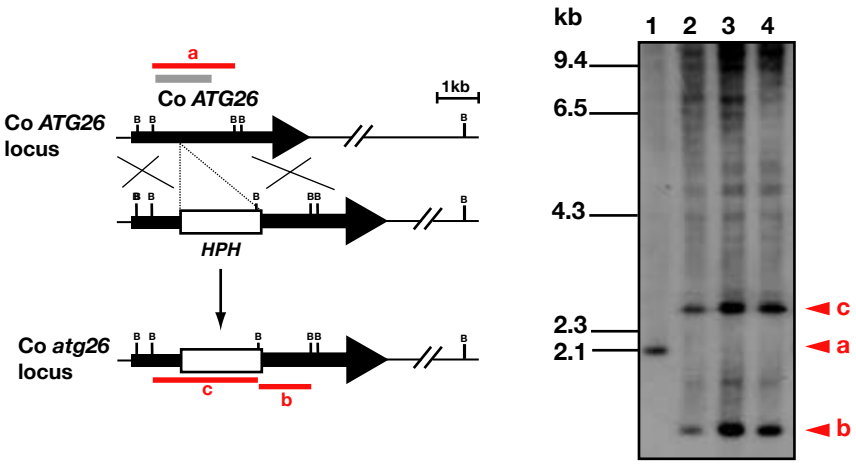
Supplemental Figure 1. The REMI mutant NP71 of *C. orbiculare*. (A) Pathogenicity assay of the REMI mutant NP71 on cucumber. Conidial suspensions were inoculated onto cucumber leaves. On the left halves of the leaves, the wild-type strain 104-T (WT) was inoculated as a positive control. On the right halves, NP71 was inoculated. Inoculated leaves were incubated for seven days. (B) Appresorium formation of NP71. Conidia of NP71 were inoculated on cucumber cotyledons and incubated for 24 h. Bar = 10 μm.

Supplemental Figure 2



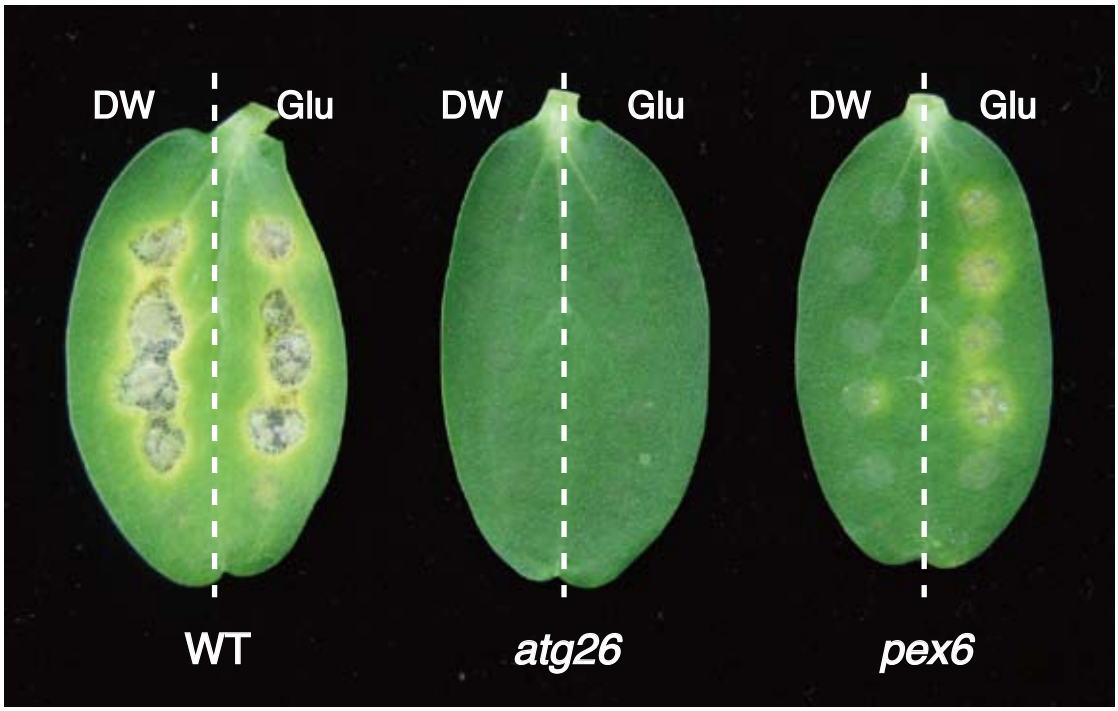
Supplemental Figure 2. Alignment of the deduced amino acid sequence of *C. orbiculare* (C. o.) Atg26 with a putative Atg26 homolog of the filamentous fungus *Neurospora crassa* (N. c.) and *P. pastoris* Atg26 (P. p.). The sequence alignment was generated using the CLUSTAL W program (Thompson et al., 1994). Identical amino acids are indicated as white letters on a black background. Similar residues are shown on gray backgrounds, and the gaps introduced for alignment are indicated by hyphens.

Supplemental Figure 3



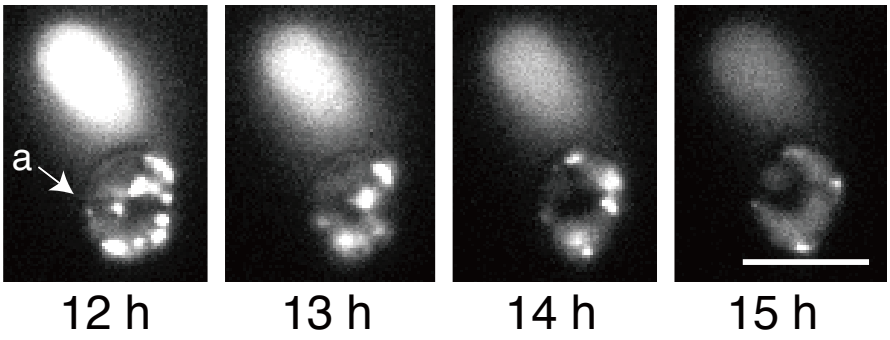
Supplemental Figure 3. Gene disruption of Co *ATG26*. (A) Gene disruption vector pKOATG26. Bars marked a-c indicate fragments detected in the gel blot analysis shown in B. (B) DNA gel blot analysis of the *atg26* strains. Genomic DNAs were isolated from the wild-type strain 104-T (lane 1) or the *atg26* strains DAT26-1, DAT26-2, and DAT26-3 (lanes 2-4). Isolated DNAs were digested with *Bgl*II. The DNA blot was hybridized to the 1.4kb *Bgl*II fragment indicated by a gray bar in A. The *atg26* strains do not contain the 2.1-kb fragment detected in the wild type, but they contain common 1.4-kb and 2.7-kb fragments, which is consistent with the lengths expected from a gene disruption event.

Supplemental Figure 4



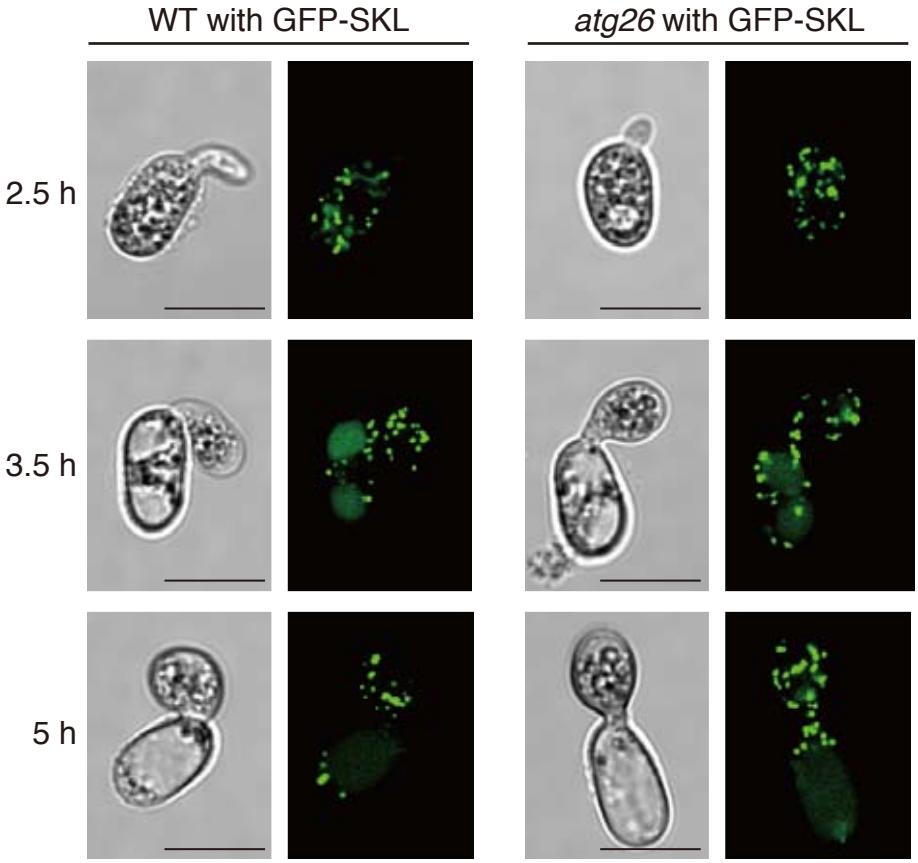
Supplemental Figure 4. Glucose did not restore pathogenicity of the *atg26* mutant. Conidia of tested strains were suspended in water (DW) or 1mM glucose (Glu) to approximately $5x10^5$ conidia /ml, and inoculated on cucumber cotyledons. Photo was taken at 7 dpi. Glucose partially restored pathogenicity of the *pex6* mutant but not the *atg26* mutant.

Supplemental Figure 5

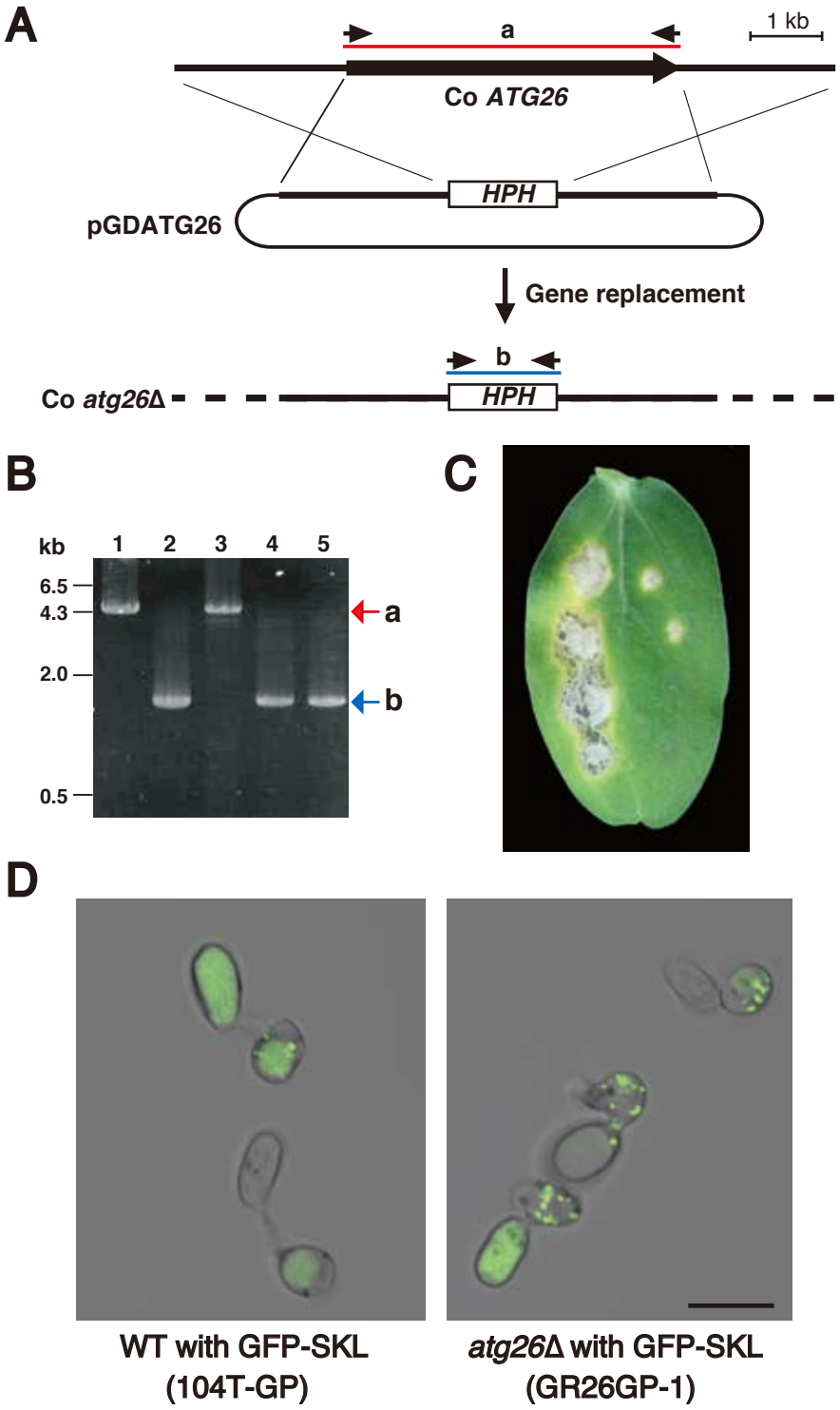


Supplemental Figure 5. Time lapse analysis of peroxisome degradation in appressorium. Conidia of the wild-type strain expressing GFP-SKL were incubated on glass. a, appressorium. Bar = 10 μm.

Supplemental Figure 6

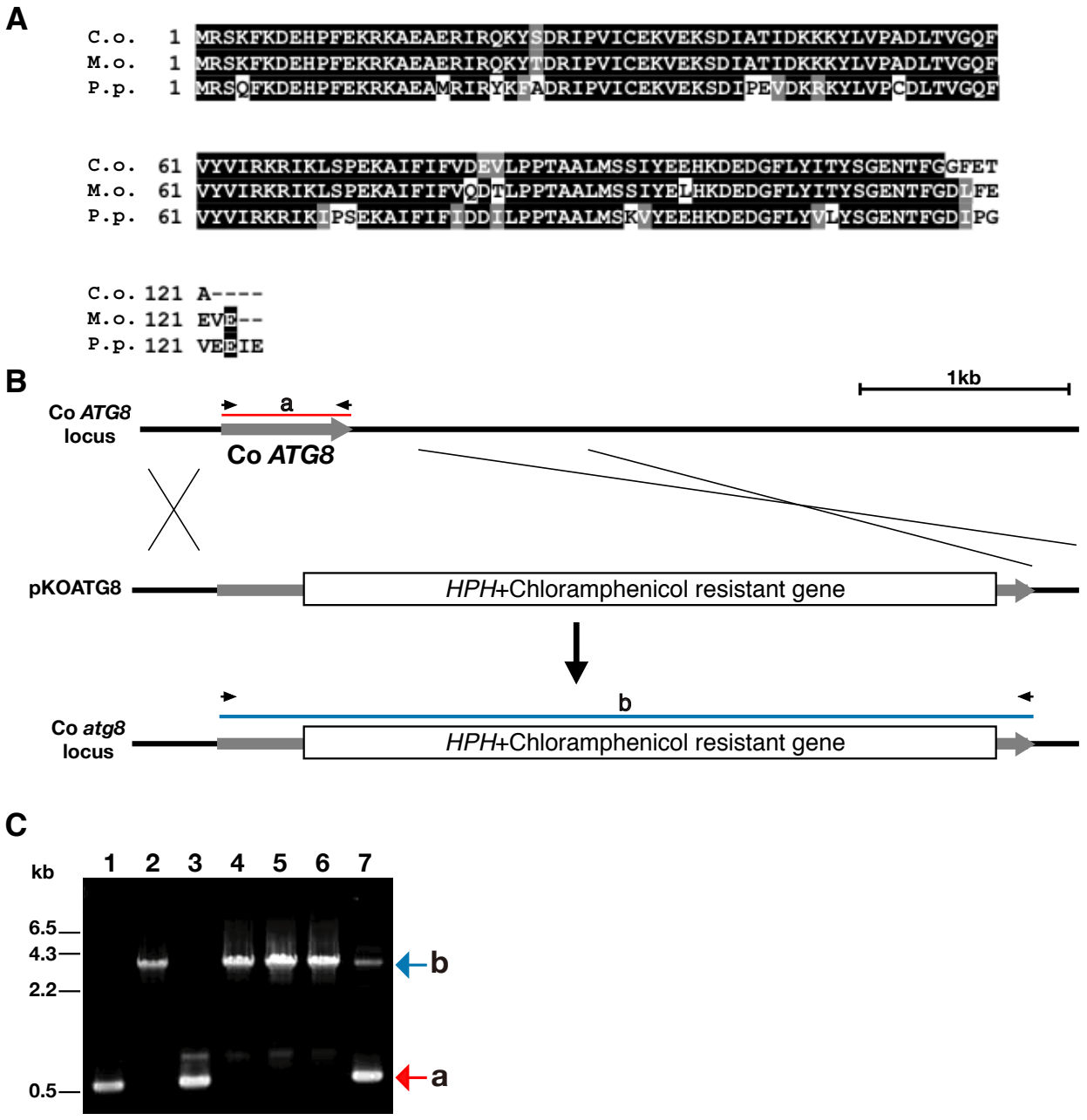


Supplemental Figure 6. Peroxisome degradation of the *atg26* mutant during conidial germination and appressorium differentiation. Conidia of the wild-type strain expressing GFP-SKL and the *atg26* mutant expressing GFP-SKL were incubated on glass. At 2.5 h when both strains germinated, peroxisome degradation was not clearly detected. At 3.5 h when germ tubes were differentiating appressoria, peroxisomes were commonly degraded in vacuoles of conidia in both strains. Bars = 10 μm.



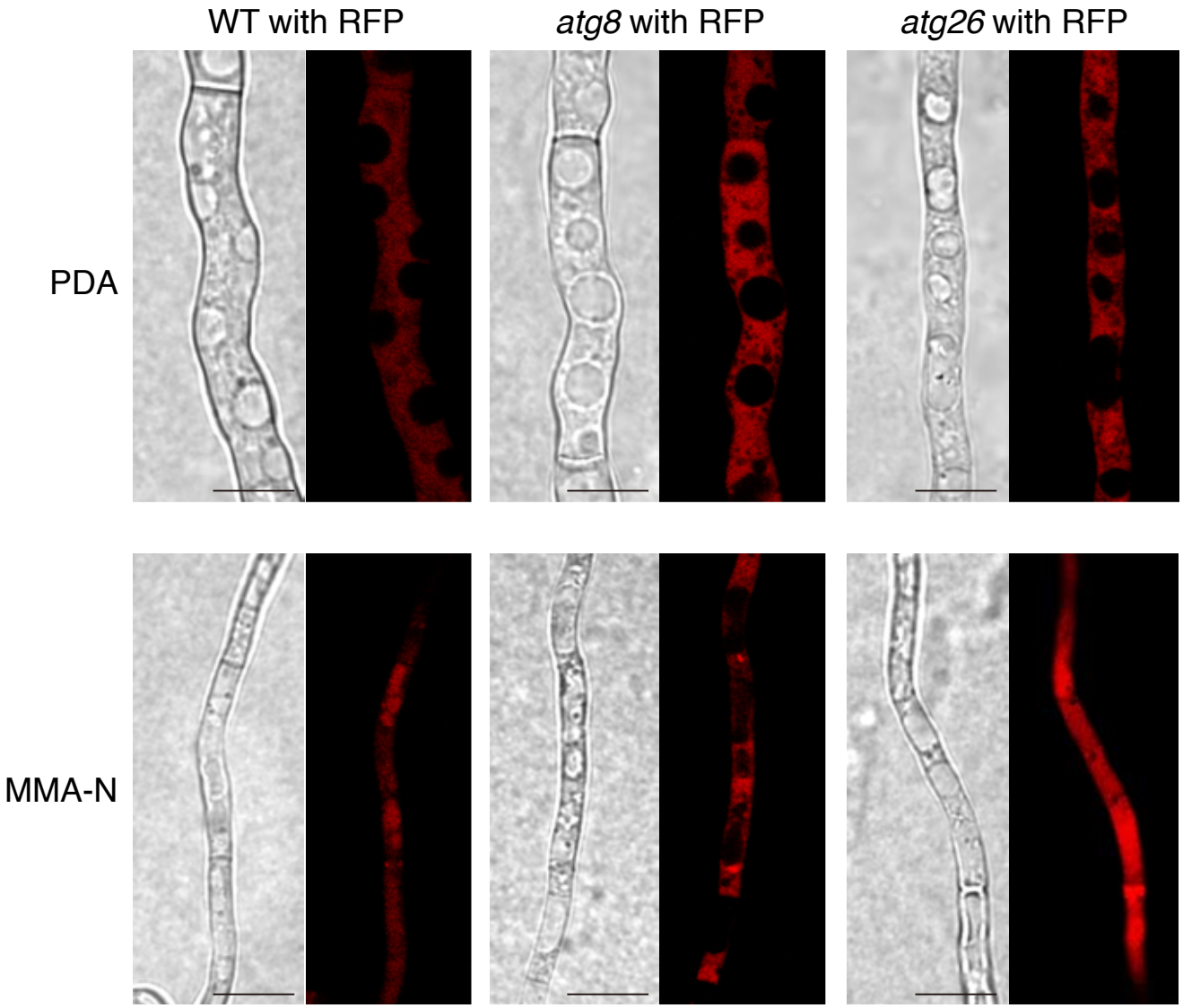
Supplemental Figure 7. Gene replacement of Co *ATG26*. **A,** Co *ATG26* locus and the gene replacement vector pGDATG26. pGDATG26 contains a hygromycin phosphotransferase gene (*HPH*) cassette flanked by border sequences of Co *ATG26*. **B,** Genomic PCR analysis of the Co *ATG26* gene replacement mutants. Genomic DNAs were isolated from the wild-type strain 104-T and the *atg26*Δ mutant strains GR26GP-1, GR26GP-2. The 4.6-kb fragment was amplified from the genomic clone pCOSATG26 containing *ATG26* (lane 1) and 104-T (lane 3), whereas the 1.5-kb fragment was amplified from pGDATG26 (lane 2), GR26GP-1 (lane 4) and GR26GP-2 (lane 5). Primers used for PCR were indicated by arrows shown in A. Fragments amplified by PCR were indicated by bars (a and b) in A. **C**, Pathogenicity test of the *atg26*Δ mutant. On the left half of the cucumber cotyledon, the wild-type strain expressing GFP-SKL (104-TGP) was inoculated as a positive control. On the right half, the *atg26*Δ mutant expressing GFP-SKL (GR26GP-1) was inoculated. Inoculated leaves were incubated for 10 days. **D**, Peroxisome degradation in the *atg26*Δ mutant. Tested strains were incubated on glass for 24 h in the presence of a melanin biosynthesis inhibitor carpropamid. Bar = 10 μm.

Supplemental Figure 8



Supplemental Figure 8. Identification and knockout analysis of Co *ATG8* in *C. orbiculare*. (A) Alignment of the deduced amino acid sequence of the *C. orbiculare* Atg8 (C.o.) with a putative Atg8 homolog of *M. oryzae* (M. o.) and Atg8 of *P. pastoris* (P. p.). Co *ATG8* was isolated by PCR using degenerate primers designed from the conserved sequences in putative Atg8 orthologs in filamentous fungi. The sequence alignment was generated using CLUSTAL W. (B) Gene disruption of Co *ATG8*. pKOATG8 was generated by inserting the transposon into Co *ATG8* in the genomic clone pCOSATG8. The transposon contains both the hygromycin- and chloramphenicol-resistance genes. (C) Genomic PCR analysis of the Co *atg8* strains. Genomic DNAs were isolated from the wild-type strain 104-T and the Co *atg8* strains DAT8-1, DAT8-2, and DAT8-3, as well as the complemented strain GAT8-1 carrying the GFP-*CoATG8*. The entire Co *ATG8* gene was amplified with the two primers indicated by arrows, from pCOATG8 (lane 1), pKOATG8 (lane 2), genomic DNAs from 104-T (lane 3), DAT8-1 (lane 4), DAT8-2 (lane 5), DAT8-3 (lane 6), and GAT8-1 (lane 7). The 0.6 kb product (a) was amplified from the intact Co *ATG8* gene. In contrast, the 0.6 kb product was not amplified when the transposon was inserted into Co *ATG8*; however, in that case, the 3.9 kb product (b) was amplified.

Supplemental Figure 9



Supplemental Figure 9. Nitrogen starvation assay. Tested genotypes (WT, *atg8*, *atg26*) expressing cytosolic RFP were grown on potato dextrose agar plate (PDA) for 1 week, and mycelial plugs of each strain were transferred to a minimal medium agar plate lacking a nitrogen source (MMA-N) and incubated for three days. Under nitrogen starvation condition (MMA-N), cytosolic RFP was degraded inside vacuoles of the wild-type strain and the *atg26* strain, but the RFP degradation was impaired in the *atg8* strain. Bars = 10 μm.

Supplemental Table 1. Characteristics of the Co *atg26* and Co *atg8* mutants

Strain	Genotype	PDA (mm)*	Conidia ($x10^6$)*
104-T	Wild type	36.3 ± 0.6	32.9 ± 3.1
DAT26-1	Co *atg26*	32.1 ± 0.5	5.3 ± 1.0
AT26Com	Co *atg26* + Co *ATG26*	36.1 ± 0.6	32.0 ± 2.0
GR26GP-1	Co *atg26*Δ	32.8 ± 0.3	6.3 ± 1.5
DAT8-1	Co *atg8*	31.3 ± 1.2	1.6 ± 2.2

*Tested strains were grown on each medium for 7days. For growth assay, colony diameter was investigated on PDA medium. Total number of conidia were investigated on culuture grown on PDA. Means and standard deviations were calculated from three independent experiments.

Supplemental Table 2. Strains and plasmids used in this study.

Strain	Genotype (explanation, plasmid used for transformation)	Reference
104-T	Wild type	Ishida and Akai, 1969
104-TGP	Wild type / pBAGFPPTS1(GFP-SKL)	Kimura et al., 2001
MRPTS1	Wild type / pBATPMRPTS1 (RFP-SKL)	Asakura et al., 2006
104-TR	Wild type / pBATTEFPMR (RFP)	This study
DPE1	Co *pex6* (pGDPEX6)	Kimura et al., 2001
PEGP1	Co *pex6* /pBAGFPPTS1(GFP-SKL)	Kimura et al., 2001
NP71	Insertional mutant of Co *ATG26* (pCB1636, REMI mutant)	This study
DAT26-1	Co *atg26* (pKOATG26)	This study
DAT26-2	Co *atg26* (pKOATG26)	This study
DAT26-3	Co *atg26* (pKOATG26)	This study
AT26Com	Co *atg26* / pBATG26FL	This study
AT26GP	Co *atg26* / pBAGFPPTS1(GFP-SKL)	This study
AT26RP	Co *atg26* / pBATPMRPTS1(RFP-SKL)	This study
AT26GP1FL	Co *atg26* / pBAGFPPTS1(GFP-SKL) / pIIATG26FL	This study
AT26GP1ΔCAT	Co *atg26* / pBAGFPPTS1(GFP-SKL) / pIIATG26ΔCAT	This study
AT26GP1ΔPBD	Co *atg26* / pBAGFPPTS1(GFP-SKL) / pIIATG26ΔPBD	This study
AT26GP1CAT	Co *atg26* / pBAGFPPTS1(GFP-SKL) / pIIATG26CAT	This study
AT26R	Co *atg26* / pBATTEFPMR (RFP)	This study
AT26GAFL1	Co *atg26* / pHGFPATG26FL/pII99	This study
AT26GADP1	Co *atg26* / pHGFPATG26ΔPBD/pII99	This study
DAT8-1	Co *atg8* (pKOATG8)	This study
DAT8-2	Co *atg8* (pKOATG8)	This study
DAT8-3	Co *atg8* (pKOATG8)	This study
GAT8-1	Co *atg8* / pCB16GFPATG8 (GFP-*CoATG8*)	This study
AT8R	Co *atg8* / pBATTEFPMR (RFP)	This study
AT8RPGAT8	Co *atg8* / pBATPMRPTS1 / pCB16GFPATG8	This study
GR26GP-1	Co *atg26Δ* (pGDATG26) / pBAGFPPTS1(GFP-SKL)	This study
GR26GP-2	Co *atg26Δ* (pGDATG26) / pBAGFPPTS1(GFP-SKL)	This study

Supplemental Table 3. Primers used in this study

Name	Sequence
26GS1	5'-GCCCTCTAGATCGTCGCCATGCCACCGCCGCCGCTA-3'
26GAS1	5'-GGACTAGTCTAAGCGACCGACTGCTGAGAAGG-3'
26GRD1	5'-CCACTAGTCTAAGGCTTGAAATTGAGAAATGAGGC-3'
26GS2	5'-GCCCTCTAGAGGCTTTAGGTTTGGCTACTCCGGC-3'
26GAS2	5'-GCCCTCTAGAGGCAAGAGCGGTATCGTTGGAGTC-3'
26CAS1	5'-GCCCTCTAGATCGTCGCCATGCCAGAGGACGAGGAGAACGCCATGGCT-3'
AT8FGB	5'-CGGGATCCTCGGTGGTATGCGATCCAAGTTCAAGGACGAGCAC-3'
AT8FASE	5'-CGGCGGAATTCTTACGCCGTCTCAAACCCGCCGAAAGT- 3'
ATG8koS1	5'-CATGCGATCCAAGTTCAAGG- 3'
ATG8koAS1	5'-CTTACGCCGTCTCAAACC- 3
ATG26FSB	5'-CGGGATCCTCGGTGGTATGCCACCGCCGCCGCTATCGCTGCCG-3'
ATG26FASE	5'-CGGCGGAATTCCTAAGCGACCGACTGCTGAGAAGGAGA-3'
ATG26asA	5'-CCAATCTTTTCCAGGTAGCGGGTGGTCTC-3'
ATG26sB	5'-GGAGAAGGGCTTTAGGTTTGGCTAC-3'
TEFNS1	5'- CAGGTTGCGGCCGCGGGTAGCAAACGGTGGTCAAAGGA-3’
TEFXAS1	5'-CGGGTCTAGAGTGATGTATGGAAGATGGAGTGAA-3'
MRFPSTOPB	5'-GGCGGATCCTTAGGCGCCGGTGGAGTGGCGGCC-3'
ATG26d1X	5'-GCTCTAGACGAAGTGAACCTACGGCCTC-3’
ATG26d2B	5'-CGCGGATCCGGCGACGATGGATATCTGGG-3’
ATG26d3K	5'-CGGGGTACCGATGCTGCCGACACACAGAC-3’
ATG26d4	5'-GGTCAAACATGAGAATTCGCGGCCGCATAATAC-3’
ATG26dS1	5'-CTTGCCATCGCCGAACCCCAGATATCC-3’
ATG26dAS1	5'-CGATTCTTCGTTACGTGAGGCCGACC-3’

Supplemental Methods

Nitrogen starvation assay

Minimal medium agar (MMA) contains (per liter): 6 g $NaNO_3$, 0.52 g KCl, 0. 52 g $MgSO_4 \cdot 7H2O$, 1.52 g KH_2PO_4, 10 mg thiamine, 1 ml trace elements (Hutner et al., 1950), 10 g glucose, and 15 g agar. For the nitrogen starvation assay, mycelial plugs from a 1 week old culture of test strains expressing cytosolic RFP were taken from PDA cultures using a cork borer (internal diameter 4 mm). Plugs were placed on MMA lacking $NaNO_3$ (designated MMA-N) and incubated for 3 days. RFP fluorescence in mycelia barely growing on MMA-N was investigated by confocal microscopy.

Isolation of gene disruptants

To isolate gene disruptants, total DNAs of candidate transformants were isolated from the mycelia using the DNeasy plant mini kit (QIAGEN, Hilden, Germany). Isolated DNA was subjected to DNA blot analysis or PCR analysis to investigate gene disruption. To identify the *coatg26* insertional mutant, we performed DNA blot analysis. DNA blot analysis was performed as described previously (Takano et al., 1997). To identify the *coatg8* mutant and the *coatg26Δ* mutant, we performed genomic PCR analysis. To identify the *coatg8* mutant, the entire *ATG8* ORF was amplified with the primers ATG8koS1 and ATG8koAS1. PCR conditions were follows: preincubation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 4 min. To identify the *coatg26Δ* mutant, the primers ATG26dS1 and ATG26dAS1 were used. PCR conditions were follows: preincubation at 94 °C for 2 min, followed by 29 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 4.5 min.

Inoculation of *C. orbiculare* on cucumber cotyledons with glucose

Conidia were collected from 7-day-old PDA cultures and suspended in water or 1mM glucose to a concentration of 5 x 10^5 conidia per ml. Conidial suspensions (20 ul) were drop-inoculated onto detached cucumber cotyledons (*Cucumis sativus* L. 'Suyo'), and placed for 1 week.

Supplemental References

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