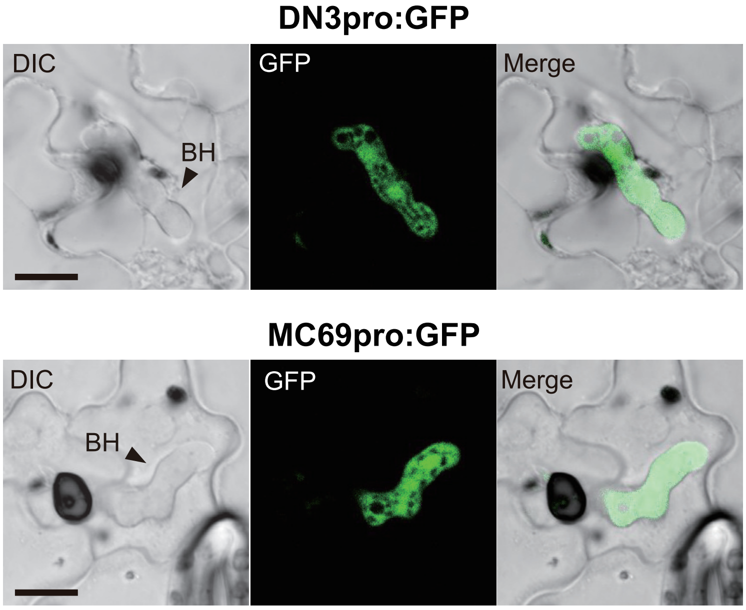
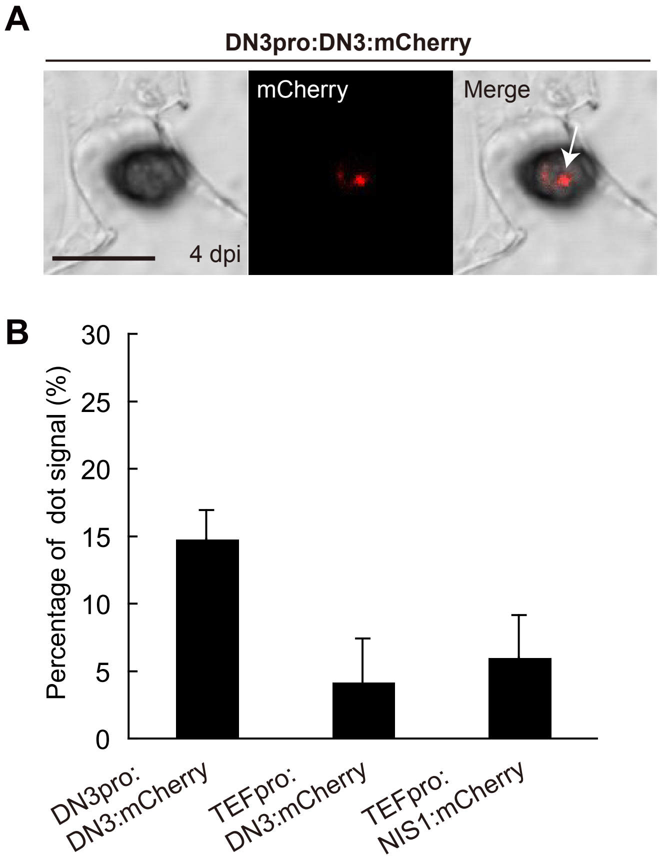


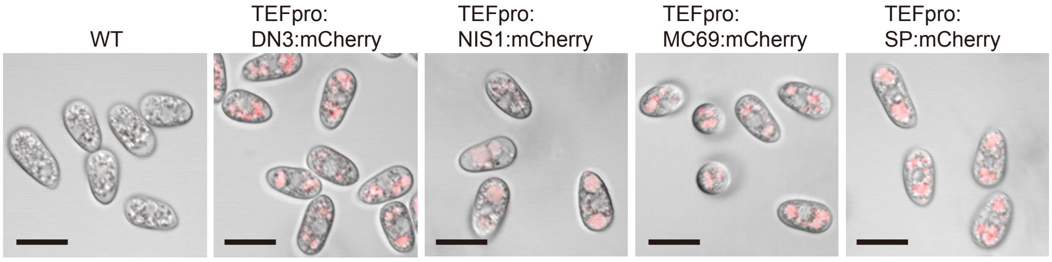
Supplemental Figure 1. Gene expression of *DN3* and *MC69* in biotrophic hyphae. *C. orbiculare* strains carrying the GFP gene with the *DN3* or *MC69* promoter were incubated on cucumber for 3 days. The GFP-based promoter analysis suggests that both *DN3* and *MC69* are highly expressed at early biotrophy. BH, biotrophic hypha. Bars = 10 µm.



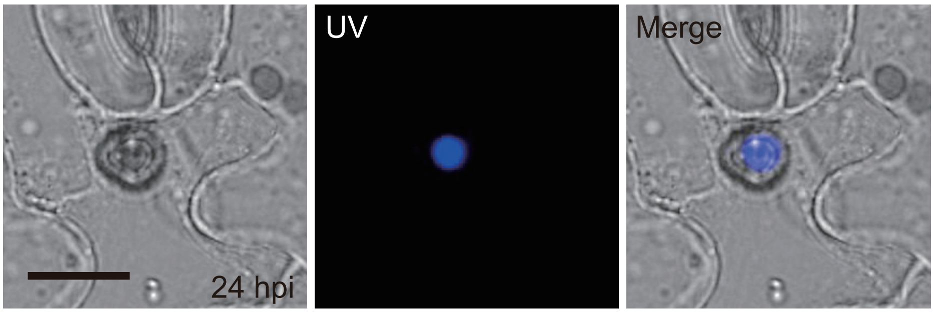
Supplemental Figure 2. The DN3:mCherry signal was detected frequently as a dot signal at the bases of *C. orbiculare* appressoria before invasion.

(A) Focal accumulation of DN3:mCherry in the basal region of appressoria. *C. orbiculare* strain with DN3pro:DN3:mCherry was inoculated on cucumber cotyledons and the inoculated plants were observed at 4 dpi. The DN3:mCherry signal was detected frequently as a dot signal at the bases of appressoria that did not develop biotrophic invasive hyphae (represented by an arrow). Bar = 10 μm.

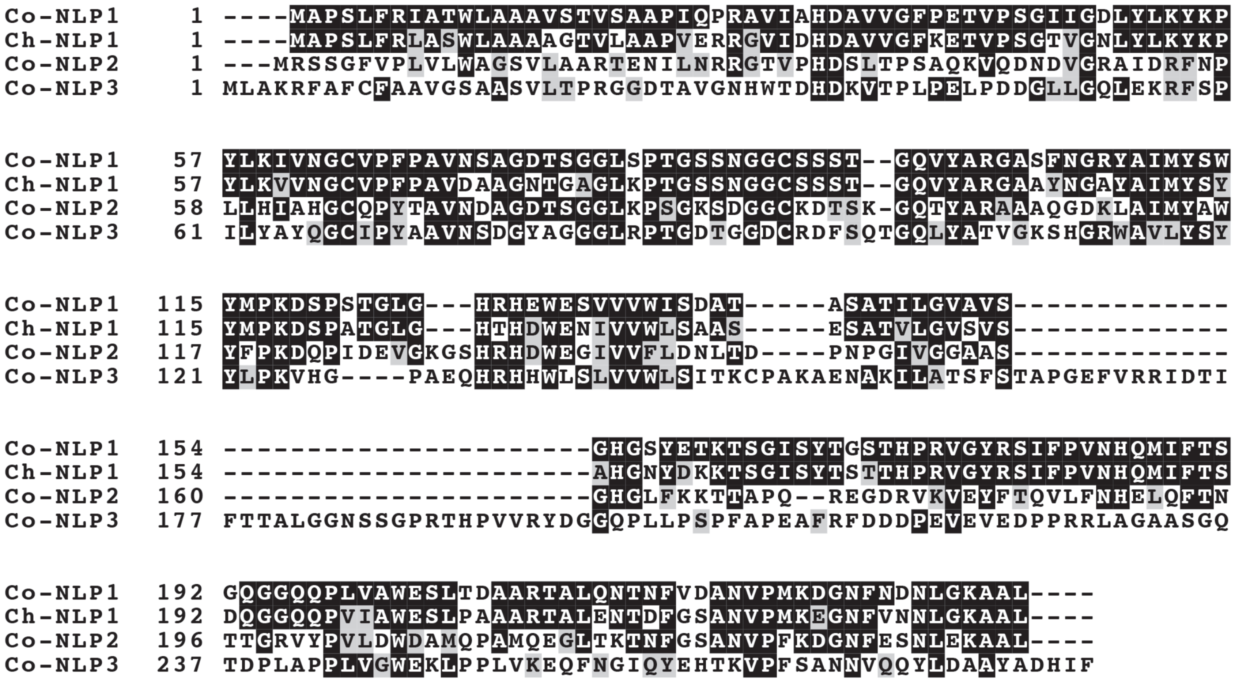
(B) Quantification of effector dot signals at the appressoria bases of *C. orbiculare* with DN3pro:DN3:mCherry, TEFpro:DN3:mCherry, or TEFpro:NIS1:mCherry. Each strain was incubated on cucumber for 4 days. At least 50 appressoria without biotrophic hyphae were investigated in each plant sample. The mean and SD were calculated from three independent plant samples.



Supplemental Figure 3. The mCherry fluorescence in pre-incubated conidia of the wild type and each reporter strain. mCherry fluorescence is equally detected in conidia of all tested reporter strains. Bars = 10 μm.



Supplemental Figure 4. Typical deposition of papillary callose beneath the melanized appressorium.
C. orbiculare strain carrying the TEFpro:DN3:mCherry gene cassette were incubated for 24 h on cucumber. The infected cucumber samples were treated with aniline blue fluorochrome for 20 min before UV excitation. Bar = 10 µm.



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Co-NLP1     1 ----MAPSLFRIATWLAAAVSTVSAAPIQPRAVIAHDAVVGFPETVPSGIIGDLYLKYKP
Ch-NLP1     1 ----MAPSLFRLASWLAAAAGTVLAAPVERRGVIDHDAVVGFKETVPSGTVGNLYLKYKP
Co-NLP2     1 ---MRSSGFVPLVLWAGSVLAARTENILNRRGTVPHDSLTPSAQKVQDNDVGRAIDRFNP
Co-NLP3     1 MLAKRFAFCFAAVGSAASVLTPRGGDTAVGNHWTDHDKVTPLPELPDDGLLGQLEKRFSP

Co-NLP1    57 YLKIVNGCVPFPAVNSAGDTSGGLSPTGSSNGGCSSST--GQVYARGASFNGRYAIMYSW
Ch-NLP1    57 YLKVVNGCVPFPAVDAAGNTGAGLKPTGSSNGGCSSST--GQVYARGAAYNGAYAIMYSY
Co-NLP2    58 LLHIAHGCQPYTAVNDAGDTSGGLKPSGKSDGGCKDTSK-GQTYARAAAQGDKLAIMYAW
Co-NLP3    61 ILYAYQGCIPYAAVNSDGYAGGGLRPTGDTGGDCRDFSQTGQLYATVGKSHGRWAVLYSY

Co-NLP1   115 YMPKDSPSTGLG---HRHEWESVVVWISDAT-----ASATILGVAVS-------------
Ch-NLP1   115 YMPKDSPATGLG---HTHDWENIVVWLSAAS-----ESATVLGVSVS-------------
Co-NLP2   117 YFPKDQPIDEVGKGSHRHDWEGIVVFLDNLTD----PNPGIVGGAAS-------------
Co-NLP3   121 YLPKVHG----PAEQHRHHWLSLVVWLSITKCPAKAENAKILATSFSTAPGEFVRRIDTI

Co-NLP1   154 ----------------------GHGSYETKTSGISYTGSTHPRVGYRSIFPVNHQMIFTS
Ch-NLP1   154 ----------------------AHGNYDKKTSGISYTSTTHPRVGYRSIFPVNHQMIFTS
Co-NLP2   160 ----------------------GHGLFKKTTAPQ--REGDRVKVEYFTQVLFNHELQFTN
Co-NLP3   177 FTTALGGNSSGPRTHPVVRYDGGQPLLPSPFAPEAFRFDDDPEVEVEDPPRRLAGAASGQ

Co-NLP1   192 GQGGQQPLVAWESLTDAARTALQNTNFVDANVPMKDGNFNDNLGKAAL----
Ch-NLP1   192 DQGGQQPVIAWESLPAAARTALENTDFGSANVPMKEGNFVNNLGKAAL----
Co-NLP2   196 TTGRVYPVLDWDAMQPAMQEGLTKTNFGSANVPFKDGNFESNLEKAAL----
Co-NLP3   237 TDPLAPPLVGWEKLPPLVKEQFNGIQYEHTKVPFSANNVQQYLDAAYADHIF
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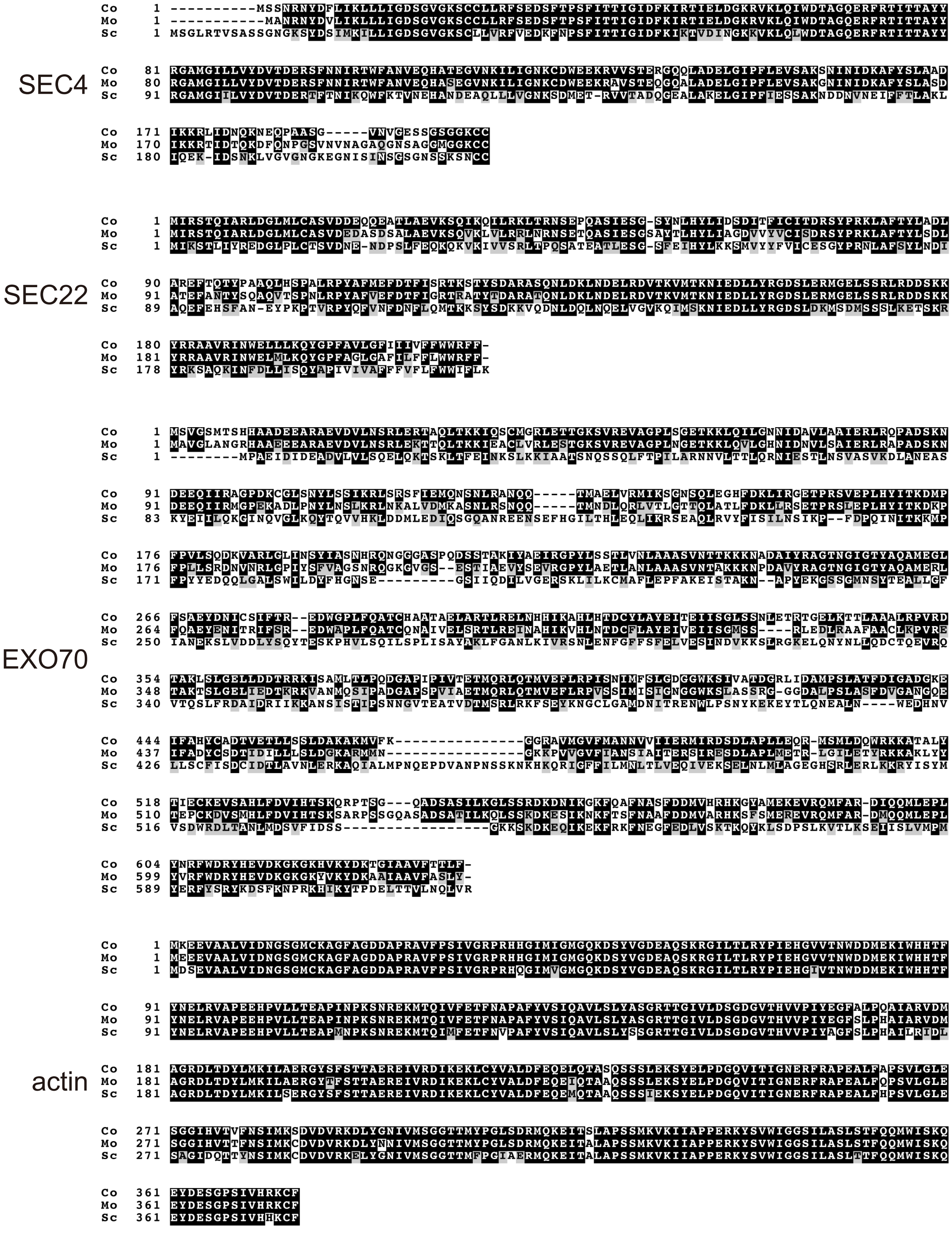
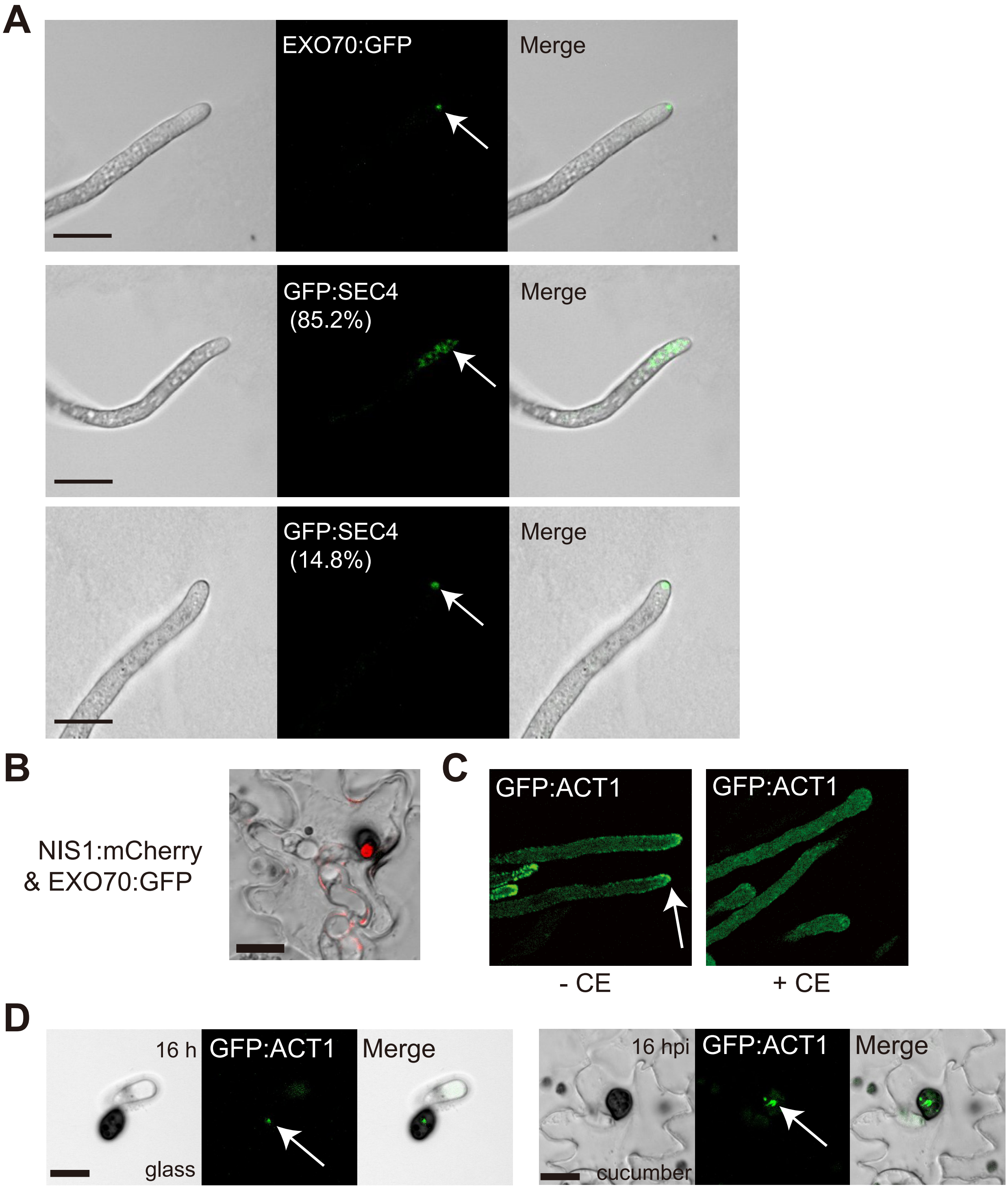
Supplemental Figure 5. Amino acid sequence alignment of three NLP homologs of *C. orbiculare* and Ch-NLP1 of *C. higginsianum.*

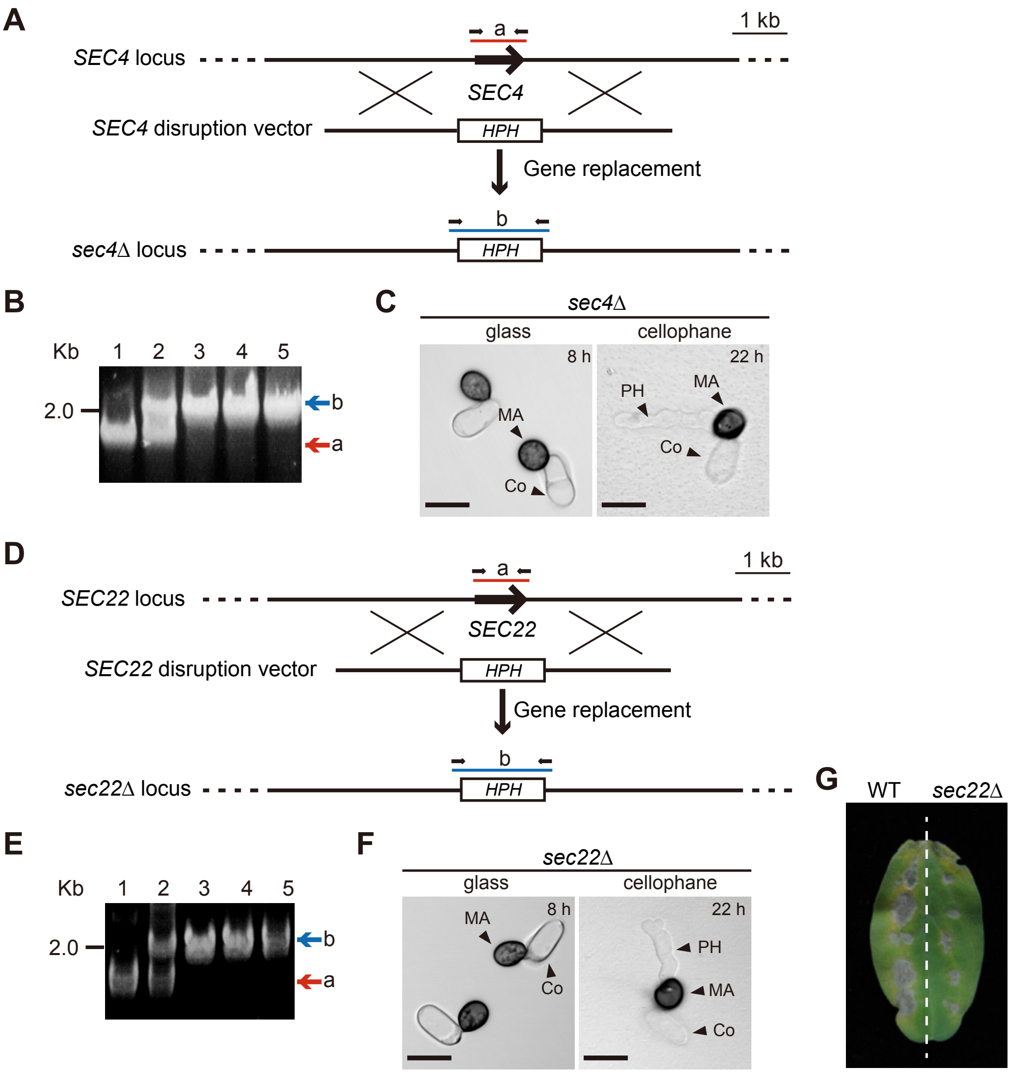
Sequence data of each NLP homologs can be found in the GenBank/EMBL data libraries under accession numbers ENH78932.1 (Cob_11657) for Co-NLP1, ENH81601.1 (Cob_09815) for Co-NLP2, ENH81388.1 (Cob_00931) for Co-NLP3, and CCF46728.1 (CH063_15391) for Ch-NLP1. Amino acid sequences were aligned using the ClustalW program (Thompson et al., 1994). Amino acids identical to *C. orbiculare* Co-NLP1 are indicated as white letters on black ground; similar residues are indicated on gray background; and gaps introduced for alignments are indicated by hyphens.



Supplemental Figure 6. Amino acid sequence alignments of SEC4, SEC22, EXO70, and actin orthologs of *C. orbiculare* with corresponding orthologs of other organisms. The SEC4, SEC22, EXO70, and actin orthologs of *C. orbiculare* (Co) were aligned with the corresponding orthologs of *M. oryzae* (Mo) and *S. cerevisiae* (Sc). Amino acid sequences were aligned using the ClustalW program (Thompson et al., 1994). Amino acids identical to each *C. orbiculare* ortholog are indicated as white letters on black ground; similar residues are indicated on gray background; and gaps introduced for alignments are indicated by hyphens. Each ortholog sequence data can be found in the GenBank/EMBL data libraries under accession numbers EHA52200 (Mo-*SEC4*), DAA12434 (Sc-*SEC4*), EHA47424 (Mo-*SEC22*), DAA09582 (Sc-*SEC22*), EHA54952 (Mo-*EXO70*), DAA08714(Sc-*EXO70*), EHA47504 (Mo-actin), DAA12401 (Sc-actin).

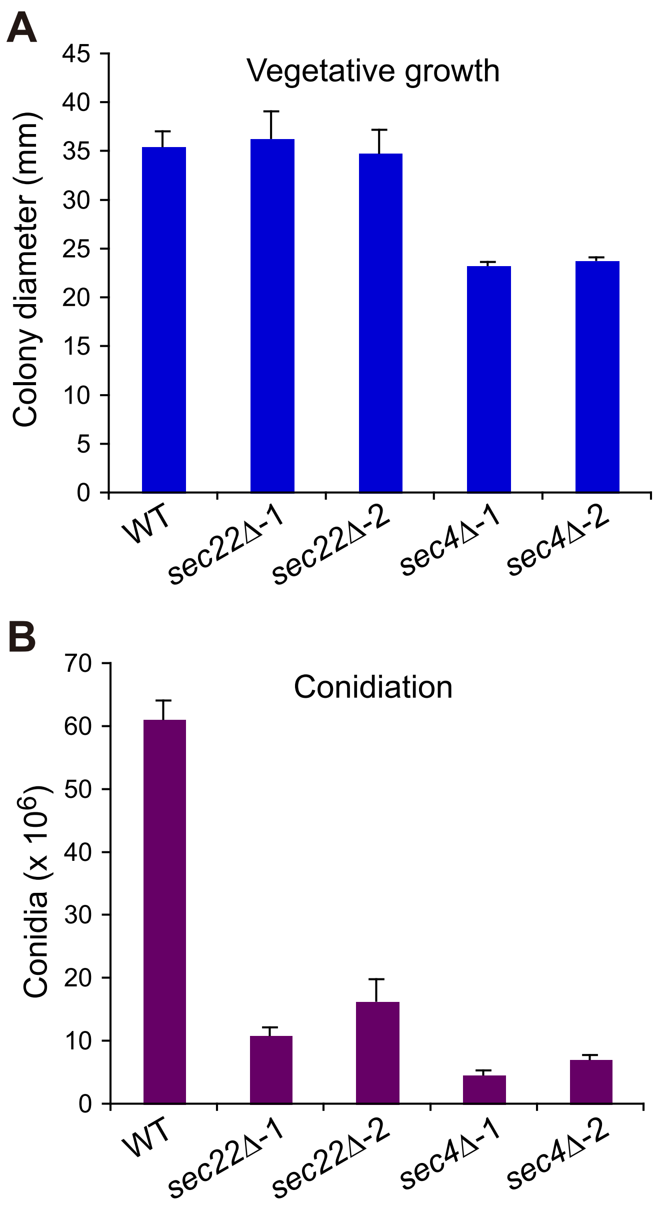


Supplemental Figure 7. Localization of exocytosis-related components and actin in vegetative hyphae and appressoria.
(A) Apical localization patterns of EXO70 and SEC4 in vegetative hyphae (white arrows). The vegetative hyphae of each strain carrying SCD1pro:EXO70:GFP or SCD1pro:GFP:SEC4 gene casette were observed. Bars = 10 μm.
(B) No fluorescence of EXO70:GFP in the cavity region of effector ring. The strain carrying both SCD1pro:EXO70:GFP and TEFpro:NIS1:mCherry was inoculated onto cucumber cotyledons, and the inoculated plants were analyzed. Bar = 10 μm.
(C) Apical localization pattern of ACT1 (actin) in vegetative hyphae (white arrow). The vegetative hyphae of the strain with SCD1pro:GFP:ACT1 were observed with or without cytochalasin E (CE).
(D) Dot signals of GFP:ACT1 at the bases of appressoria formed on glass and cucumber (white arrows). The *C. orbiculare* strain with SCD1pro:GFP:ACT1 was incubated for 16 h. Bars = 10 μm.



Supplemental Figure 8. Gene disruptions of *SEC4* and *SEC22* in *C. orbiculare*.

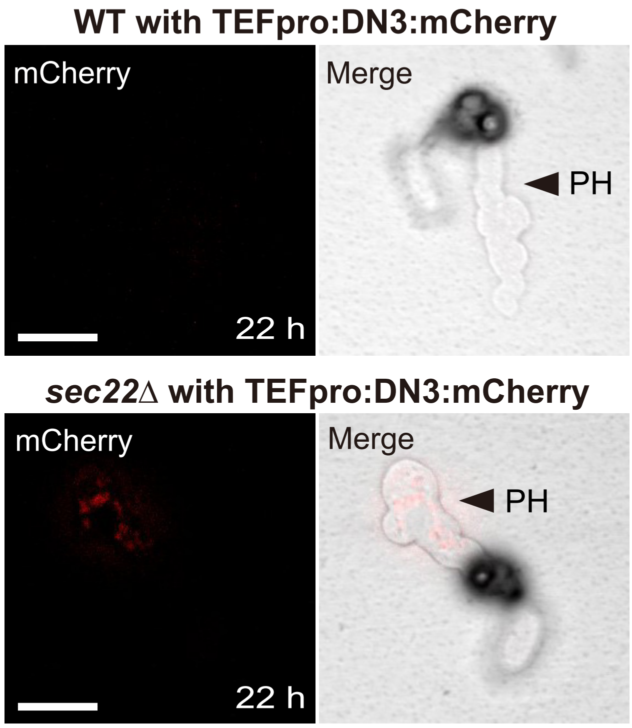
(A) *SEC4* locus of *C. orbiculare* and the *SEC4* gene disruption vector containing a hygromycin phosphotransferase gene (*HPH*) cassette flanked by the border sequences of *SEC4*. The primers used for the genomic PCR are indicated by arrows. The fragments amplified by PCR are indicated by bars (a and b). **(B)** The genomic PCR analysis was performed using DNA isolated from wild-type strain 104-T or strains transformed with the *SEC4* gene disruption vector. A 1.2-kb fragments (a) was amplified from both the wild-type strain (lane 1) and an ectopic transformant (lane 2), whereas a 1.8-kb fragment (b) was amplified from the ectopic transformant (lane 2), the two *sec4*Δ strains (lanes 3 and 4) and the *SEC4* gene disruption vector (lane 5). **(C)** Morphological behaviors of *sec4*Δ strains incubated on glass and cellophane. The *sec4*Δ strains exhibited normal appressorium formation on glass and cellophane, and the appressoria of the mutant developed pseudo-biotrophic hyphae on cellophane. Co, conidium; MA, melanized appressorium; PH, pseudo-biotrophic hypha. Bars = 10 μm. **(D)** *SEC22* locus of *C. orbiculare* and *SEC22* gene disruption vector containing the *HPH* cassette flanked by the border sequences of *SEC22*. **(E)** The genomic PCR analysis was performed using DNA isolated from wild-type strain 104-T or strains transformed with the *SEC22* gene disruption vector. A 1.2-kb fragments (a) was amplified from both the wild-type strain (lane 1) and an ectopic transformant (lane 2), whereas a 1.8-kb fragment (b) was amplified from the ectopic transformant (lane 2), the two *sec22*Δ strains (lanes 3 and 4) and the *SEC22* gene disruption vector (lane 5). **(F)** Morphological behaviors of *sec22*Δ strains incubated on glass and cellophane. *sec22*Δ strains exhibited normal appressorium formation on glass and cellophane, and the appressoria of the mutant developed pseudo-biotrophic hyphae on cellophane. Co, conidium; MA, melanized appressorium; PH, pseudo-biotrophic hypha. Bars = 10 μm. **(G)** The *sec22*Δ strain developed lesions on cucumber cotyledons treated with heat shock. For the heat shock treatment, the detached cucumber cotyledons were immersed in distilled water at 50˚C for 50s, and then they were subjected to the inoculation assay. WT, the wild-type strain.



Supplemental Figure 9. Vegetative growth and conidiation of *sec4*Δ and *sec22*Δ mutants.

(A) Colony diameter of each strain grown on PDA for 7 days was measured. The means and standard deviations were calculated from three independent experiments.

(B) Conidia of each strain were harvested from colonies grown on PDA medium for 7 days. Numbers of conidia were counted. The means and standard deviations were calculated from three independent experiments.



Supplemental Figure 10. The retention of the effector:mCherry signal inside pseudo-biotrophic hyphae developed in cellophane.

WT and *sec22*Δ strains with TEFpro:DN3:mCherry were incubated on cellophane for 22 h. PH, pseudo-biotrophic hypha. Bars = 10 µm.

Supplemental Table 1. Primers used in this study

Name	Sequence	destination
CoDN3pro-NotI-f	5´-ATAAGAATGCGGCCGCTAGCCGCTTCAGCGTCAGTC-3´	DN3pro:DN3:mCherry, DN3pro:GFP, DN3pro:SP:mCherry
CoDN3-XhoI-r(c)	5´-CCGCTCGAGAGGTCCCTTTTTCCCGGGAG-3´	DN3pro:DN3:mCherry, TEFpro:DN3:mCherry
CoDN3pro-XbaI-r(c)	5´-GCTCTAGAGGTGAATGGGAGGCGTCTGTC-3´	DN3pro:GFP, DN3pro:SP:mCherry
CoNIS1pro-NotI-f	5´-ATAAGAATGCGGCCGCATAGAATGAGTTTCGTTATTG-3´	NIS1pro:NIS1:mCherry
CoNIS1-XhoI-r(c)	5´-CCGCTCGAGGATTTTCCTCGTGTACCCG-3´	NIS1pro:NIS1:mCherry
CoMC69pro-NotI-f	5´-ATAAGAATGCGGCCGCGTCTTTCGTCTTTTCGGTCT-3´	MC69pro:MC69:mCherry
CoMC69-XhoI-r(c)	5´-CCGCTCGAGTGACTTTCTCAGAGGACTACAG-3´	MC69pro:MC69:mCherry, TEFpro:MC69:mCherry
CoNLP1pro-NotI-f	5´-ATAAGAATGCGGCCGCGTCTGTTTTCGAGACAGCAAAGAGCGG-3´	NLP1pro:GFP
CoNLP1pro-XbaI-r(c)	5´-GCTCTAGATGTCTGTGTGAAGGACCTTTTGGGTTCTGCC-3´	NLP1pro:GFP
mCherry-BamHI-BglII-XhoI-f	5´-GAGAGGAGAAGGATCCAGATCTCTCGAGACCATGGTGAGCAAGGGCGAGGAG-3´	Effector:mCherry constructs (for *Xho*I joint)
mCherry-EcoR I-r(c)	5´-CCGGAATTCTTACTTGTACAGCTCGTCCATGCC-3´	Effector:mCherry constructs, SP:mCherry constructs
SPcodn3A-mCherry-XbaI-f	5'-GCTCTAGAATGTACGCCTCAAGCTTCGTCGTCATGCTGCTCGCTATCCCCTTTGCGGCTGCAGTGAGCAAGGGCGAGGAG-3'	SP:mCherry constructs
CoDN3-XbaI-f	5´-GCTCTAGACAGACACAATGTACGCCTCAAGCTTCGTCG-3´	TEFpro:DN3:mCherry
CoNIS1-XbaI-f	5´-GCTCTAGAATGCAGTTCCTCACCTCCCTC-3´	TEFpro:NIS1:mCherry
CoNIS1-BamH I-r(c)	5´-CGGGATCCGATTTTCCTCGTGTACCCGC-3´	TEFpro:NIS1:mCherry
CoMC69-XbaI-f	5´-GCTCTAGAATGAAGTTTACACTCGCTCTCC-3´	TEFpro:MC69:mCherry
mCherry-BamHI-f	5´-GCGGATCCATGGTGAGCAAGGGCGAGGAGGATAAC-3´	Effector:mCherry constructs (for *Bam*HI joint)
NIS1ORFXIs1	5´-GCCCTCTAGACAGACACAATGCAGTTCCTCACCTCCCTCG-3´	TEFpro:NIS1:GFP
NIS1GFPrev	5´-GCCCTCTAGACCTCCACCACCACCGATTTTCCTCGTGTACCCGC-3´	TEFpro:NIS1:GFP
eGFP-EcoRV-f	5´-CCGATATCATGGTGAGCAAGGGCGAGGAG-3´	pBAT-eGFP, pHYPT-eGFP
eGFP-HindIII-r(c)	5´-CCCAAGCTTTTACTTGTACAGCTCGTCCATGC-3´	pBAT-eGFP, pHYPT-eGFP
CoExo70-BamHI-f	5´-CGGGATCCATGTCGGTCGGCTCAATGACCAGTC-3´	SCD1pro:EXO70:GFP
CoExo70-PstI-r(c)	5´-CATTGGTTCTGCAGACCACCACCACCAAATAGGGTCGTAAACACGGCGG-3´	SCD1pro:EXO70:GFP
CoSEC4-BamHI-f	5´-CGGGATCCCGGGTGGTATGTCGAGCAACCGCAACTACGACTTTC-3´	SCD1pro:GFP:SEC4
CoSEC4-PstI-r(c)	5´-CATTGGTTCTGCAGTTAACAGCACTTTCCGCCCGATCCGC-3´	SCD1pro:GFP:SEC4
SEC22SB1	5´-CGGGATCCCGGGTGGTATGATTCGCTCAACGCAAATAGCAAGG-3´	SCD1pro:GFP:SEC22
SEC22ASB1	5´-CGGGATCCTCAAAAGAAGCGCCACCAGAAAAACAC-3´	SCD1pro:GFP:SEC22
A7ACT1B	5´-CGGGATCCCGGCTGCTGCTGCTGCTGCTGCAATGAAGGGTATGTGACGCCAT-3´	SCD1pro:GFP:ACT1
A7ACT1E	5´-CGGCGGAATTCTTAGAAGCACTTGCGGTGAAC-3´	SCD1pro:GFP:ACT1
Sec4-3UTR-SA1	5´-GAAGGGCCCAGCAAAGGGTCCTTTGGTTGATAC-3´	*SEC4* gene disruption
Sec4-3UTR-ASK1	5´-GGGGTACCCATCACGAAGAGACTAAGAAGTGG-3´	*SEC4* gene disruption
Sec4-5UTR-SB1	5´-CGGGATCCAAGAGCTTGTCTTTGGCTTCGTTG-3´	*SEC4* gene disruption
Sec4-5UTR-ASE1	5´-CGGAATTCTTTCAGAGAGGAAATGGGGTATTC-3´	*SEC4* gene disruption
CoSEC4OUTS	5´-CGACGACGACATCGCGATAC-3´	Confirmation of *SEC4* gene disruption
CoSEC4OUTAS	5´-CGTGGTCTGCGAAAGTGCAAATC-3´	Confirmation of *SEC4* gene disruption
Sec22-5UTRSN1	5´-ATAAGAATGCGGCCGCCTCTGCGTAAGAGCCATGGTCGAC-3´	*SEC22* gene disruption
Sec22-5UTRASE1	5´-CGGAATTCGAGTGTTTCTGGTGTTTGGTGGCG-3´	*SEC22* gene disruption
Sec22-3UTRSA1	5´-GAAGGGCCCGTGGAATATCATACGGCATCGC-3´	*SEC22* gene disruption
Sec22-3UTRASK1	5´-GGGGTACCACAGTGGAGTTCCATACGTCTCTC-3´	*SEC22* gene disruption
Sec22-OUTS	5´-TACCTGCATACCTGGTCACG-3´	Confirmation of *SEC22* gene disruption
Sec22-OUTAS	5´-TACTCCACAGGCAGATGTCC-3´	Confirmation of *SEC22* gene disruption
XCNU1	5’-GCTCTAGAATGCAGTTCCTCACCTCCCTCG-3’	pCB-Ppwl2-NIS1-mCherry-NLS
BCNL1	5’-CGGATCCGATTTTCCTCGTGTACCCGCCC-3’	pCB-Ppwl2-NIS1-mCherry-NLS