國立臺灣大學生命科學院基因體與系統生物學學位學程

碩士論文

Genome and Systems Biology Degree Program

College of Life Science

National Taiwan University

Master Thesis

線蟲捕捉菌中高保守性但具可塑性之獵物感受訊息傳遞系統

Highly Conserved Yet Plastic Signaling Pathways in Prey Sensing by the Nematode-trapping Fungus *Arthrobotrys oligospora*

林宏澤

Hung-Che Lin

指導教授：薛雁冰 博士

Advisor: Yen-Ping Hsueh, Ph.D.

中華民國 108 年 7 月

July 2019

doi:10.6342/NTU201902152

國立臺灣大學碩士學位論文
口試委員會審定書

線蟲捕捉菌中高保守性但具可塑性之

獵物感受訊息傳遞系統

Highly Conserved Yet Plastic Signaling Pathways in Prey Sensing by the Nematode-trapping Fungus *Arthrobotrys oligospora*

本論文係林宏澤君（R06B48002）在國立臺灣大學基因體與系統生物學學位學程完成之碩士學位論文，於民國 108 年 7 月 23 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

蔡龍珠 （簽名）	
（指導教授）	
王忠信	毛俊方
呂俊毅	

系主任、所長 鄭石通 （簽名）

doi:10.6342/NTU201902152

致謝

Thank my advisor Ping. It has been 3 years since I was a summer student until I graduated from NTU. You gave me not only the direction of experiment but also advices of how to be a good scientist.

Thank all the member in Lab N414, IMB, Academia Sinica. Thanks Pedro, Guillermo, ChingHan, Samuel, Lucy, Ben, Vivian, Emily, Vincent, YY, Ling-Mei Hsu, and A-Mei Yang. Especially thank A-Mei Yang for the technical assistances, Pedro and Sam for reviewing the thesis.

Thank my thesis committee Dr. Jun-Yi Leu, Dr. Ting-Fang Wang, and Dr. John Wang. Thanks for making my thesis more completely, and kindly provide me precious experience for further research.

Thanks for all the people in my life.

doi:10.6342/NTU201902152

Highly Conserved Yet Plastic Signaling Pathways in Prey Sensing by the Nematode-trapping Fungus *Arthrobotrys oligospora*

Hung-Che Lin[†*], Yen-Ping Hsueh[†*]

[†]Institute of Molecular Biology, Academia Sinica
[*]Genome and Systems Biology Degree Program, National Taiwan University

Abstract

Nematode-trapping fungi develop trapping structures to capture and kill nematodes in response to nematode signals. To this day, the molecular mechanisms and evolutionary origin of nematode-sensing and trap formation in nematode-trapping fungi remain poorly understood. Mitogen-activated protein kinases (MAPKs) are highly conserved in the fungal kingdom and have been shown to be required for pathogenesis in various plant and animal pathogenic fungi. We thus hypothesize that MAPK signaling pathways might be essential for sensing the nematode-associated signals in *Arthrobotrys oligospora*. To investigate the function of the MAPK genes, we generated loss-of-function mutants for components in the evolutionary-conserved MAPK-mediated pheromone response pathway and cell wall integrity pathway through homologous recombination. Deletion of the G protein β subunit *gpb1* of the pheromone response pathway abolished trap formation upon *Caenorhabditis elegans* nematode and ascarosides, which are nematode pheromones known to trigger trap morphogenesis in nematode-trapping fungi, induction. Intriguingly, an *A. oligospora* strain lacking *ste2*, G-protein coupled receptor (GPCR) that in other fungi participates in pheromone sensing, showed identical sensitivity towards *C. elegans*. This observation suggested multiple GPCRs in *A. oligospora* are likely involved in sensing nematodes. In addition, *bck1* mutant of the other conserved cell wall integrity MAPK pathway resulted in severe defects in growth, conidiation, and trap formation.

doi:10.6342/NTU201902152

Nutrient deprivation, such as nitrogen limitation, has long been hypothesized as the selection force that drives the evolution of nematophagous fungi. In order to gain insights into the evolutionary trajectory of nematode-trapping ability in fungi, the model nematode-trapping fungus *A. oligospora* was experimentally evolved in three conditions with differences in nutrient content: rich medium, low-nutrient medium, and low-nutrient medium supplemented with *C. elegans* nematodes. Laboratory evolution under rich medium resulted in one independent evolved line that exhibited different amounts of aerial hyphae and trap formation compared to the ancestral strain. This evolved line formed more traps when exposed to *C. elegans* and ascarosides. These results suggested that prey-sensing is a plastic trait when evolving under nutrient-rich laboratory conditions. In summary, we demonstrated that the environmental nutritional status and two evolutionary-conserved MAPK signaling pathways play essential roles during nematode-sensing and trap morphogenesis in *A. oligospora*.

Key words: Nematode-trapping fungi, *Arthrobotrys oligospora*, G-protein signaling pathway, Experimental evolution, molecular evolution, Prey-predator interaction

doi:10.6342/NTU201902152

線蟲捕捉菌中高保守性但具可塑性之獵物感受訊息傳遞系統

林宏澤[†*]、薛雁冰[†*]

[†]中央研究院分子生物研究所
[*]國立台灣大學基因體與系統生物學學位學程

摘要

線蟲捕捉菌會產生由菌絲所特化之捕捉構造捕捉線蟲，線蟲與線蟲捕捉菌在野外中普遍存在，但線蟲捕捉菌與線蟲之間之交互關係卻很少人研究。本研究透過實驗室演化與基因剔除試圖探討線蟲捕捉菌與養分之間的關係與真菌是如何感知線蟲的存在。Mitogen-activated protein kinase (MAPK)訊息傳遞系統在真菌界中高度保守，且有許多研究結果證明在動植物病原真菌與致病性相關。線蟲捕捉菌會透過感知線蟲費洛蒙之訊號而產生陷阱，但其中之分子機制尚未明瞭，為了知道 MAPK 訊息傳遞系統相關基因是否參與線蟲感知與陷阱的產生，將 G 蛋白訊息傳遞系統上之 β 次單元 *gpb1* 進行基因剔除後，發現線蟲捕捉菌在加入線蟲及線蟲費洛蒙均不會產生陷阱，而高度保守之費洛蒙感知系統上游的 G 蛋白偶聯受體 *ste2* 的基因剔除株所產生的陷阱數量與野生型並沒有差別，顯示線蟲捕捉菌中有不同的 G 蛋白偶聯受體與線蟲的感知相關。另外，Cell wall integrity (CWI) MAPK 訊息傳遞系統上的 *bck1* 基因剔除株在生長、產孢與產陷阱上均有缺陷。營養缺乏如缺氮被認為是推進線蟲捕捉菌演化的動力之一，為了要對線蟲捕捉菌捕捉能力的演化有更多的了解，將線蟲捕捉菌培養在富含營養、缺乏營養、缺乏營養但給與線蟲等三種環境下，想知道線蟲捕捉菌是否會因環境中富含營養而喪失捕捉線蟲之能力。在富含營養環境下，一株獨立演化株出現不同於親代的表現型，該突變株可以產生更多的氣生菌絲與能被模式線蟲 *Caenorabditis elegans* 誘導出更多的陷阱，這說明了在營養豐富的環境下，線蟲捕捉菌的捕捉能力是具有可塑性的。綜合以上的研究結果顯示營養豐富的環境與兩個高度保守之 MAPK 訊息傳遞系統會影響線蟲捕捉菌感知線蟲與產生陷阱的能力。

doi:10.6342/NTU201902152

關鍵詞：線蟲捕捉菌，*Arthrobotrys oligospora*，G 蛋白訊息傳遞系統，實驗室演化，分子演化，獵物與獵食者間之交互作用

doi:10.6342/NTU201902152

目　錄

doi:10.6342/NTU201902152

doi:10.6342/NTU201902152

表目錄

doi:10.6342/NTU201902152

圖目錄

doi:10.6342/NTU201902152

Introduction

Nematode and nematophagous fungi are commonly existed in the world. Nematodes are the most abundant animal in the world. The fungi preying on nematode as their nutrient or nitrogen source are called nematophagous fungi. Nematophagous fungi have been mainly categorized into three groups: predatory, endoparasitic and opportunistic. Predatory fungi including nematode-trapping fungi used specialized mycelium to capture nematode. Endoparasitic fungi use spore to infect nematode. Opportunistic fungi use mycelium facultative parasite on nematodes and eggs. The most well studied nematophagous fungi is nematode-trapping fungi. Among all nematophagous fungi, nematode-trapping fungus is the best at catching efficiency (Duddington, 1957). Trapping device include adhesive network, adhesive knob, constricting ring, non-constricting ring, and adhesive column (Su *et al.*, 2017). Nematode-trapping fungi were reported to have potential against animal parasite (Waghorn *et al.*, 2003; Waller *et al.*, 2001). *Arthrobotrys oligospora*, the most common one in the wild, was the first isolated and characterized nematode-trapping fungi. The septate hyphae are usually 5 μm in diameter. Short hyphal branched out and curl back into semicircular loop. The complex three-dimensional trapping structure then formed by other branched hyphae arise. This grouped structure allowed nematode-trapping fungi to have a large surface that make the nematodes can easily be caught (Duddington, 1957). In addition, the net former *A. oligospora* can digest all of the tested carbohydrate, while the one with constrict ring can only utilize simple carbohydrate like hexoses and reduced ability in inorganic nitrogen (Gray, 1987).

Nematode can be attracted by some chemical, for example, like methyl 3-methyl-2-butenoate (MMB) mimic the sex and food cue produced by nematode-trapping fungi (Hsueh *et al.*, 2017). After receive signal from nematodes, nematode-trapping fungi in

doi:10.6342/NTU201902152

response produce specialized hyphae structure, the so-called “trap”. The trap structure contains sticky compound that will stick to nematode. After been caught, a captured nematode can struggle to release itself for 2 hrs (Duddington, 1957). Then the hyphae penetrate the body wall of nematodes. Finally, the nematodes will be paralyzed, dead and digested by the fungus. Besides the nematodes sense the fungi, how nematode-trapping fungi sense the nematodes during the prey-sensing remain unknown. Previous report (Hsueh *et al.*, 2013) showed that nematode-trapping fungi eavesdrop on nematode pheromone, ascaroside. In response to ascaroside, nematode trapping fungi will trigger trap morphogenesis. Ascarosides are the pheromone of *Caenorhabditis elegans*, and control the regulation of gender-specific attraction, repulsion, aggregation, olfactory plasticity, and entrance into a stress-resistant stage, dauer (Choe *et al.*, 2012). Ascarosides can be sensed by SRBC-64 and SRBC-66 GPCRs in *C. elegans* (Kim *et al.*, 2009). Mitogen-activated protein kinases (MAPKs) signaling pathway are highly conserved in fungi kingdom. MAPKs play an important role in many plant pathogenic fungi in response to host and environment signal (Jiang *et al.*, 2018). GTP-binding protein (G protein) on the upstream of pheromone sensing pathway of budding yeast *Saccharomyces cerevisiae* can sense the pheromone of opposite mating type. The receptor of pheromone sensing pathway is G protein coupled receptor (GPCR), it’s usually on the membrane and have a conserved seven-transmembrane domain. Pth11 is required for rice blast fungus *Magnaporthe oryzae* to have pathogenicity. Deletion of *pth11* in *M. oryzae* showed the loss of infectious structure, appressoria (DeZwaan *et al.*, 1999). *Fusarium oxysporum* using GPCR Ste2 as a receptor to sense the signal from host (Turra *et al.*, 2015). Deletion of *pth11* in *M. oryzae* and *ste2* in *F. oxysporum* lead to the loss of pathogenicity, which means G protein signaling is required for pathogenic fungi to develop infectious structure. GPCRs will activate downstream G protein. G protein is combine with 3 subunits: Gα,

doi:10.6342/NTU201902152

Gβ, and Gγ. In *M. oryzae*, it needs G-protein, including 3 Gα, 2 Gβ and 1 Gγ, to form appressoria (Kou and Naqvi, 2016). In *A. oligospora*, 3 Gα (EYR41_010456, EYR41_011543, EYR41_011720), 1 Gβ (EYR41_009480) and 1 Gγ (EYR41_001173) were found. In *Saccharomyces cerevisiae*, multiple MAPK cascades function as different signaling pathways in response to different environment signal. In mating response, GPCR Ste2/Ste3 allow sex pheromone to bind. After binding to sex pheromone, Gα will dissociate from Gβ, and Gγ subunits. The release of Gα activates the MAPKKK Ste11 and then phosphorylates downstream MAPK cascades include MAPKK Ste7, MAPK Fus3, and the transcription factor Ste12. Finally, mating-responsive genes will be turn on and start mating (Hamel *et al.*, 2012). But these highly conserved signaling pathway may not have the same pattern all around fungal kingdom. Turra *et al.* (2015) found that *F. oxysporum* used Ste2 GPCR sensing plant signal and sex pheromone at the same time. Also, they found that the downstream of Ste2 GPCR is not pheromone sensing pathway but cell wall integrity (CWI) pathway instead.

Trapping ability of nematode-trapping fungi diverge in *A. oligospora* (unpublished), showing that during natural evolution, the prey-sensing ability is plastic and there must be some reasons that changed the trapping ability. However, poorly study between their interaction. Nutrient deprivation was thought to be a selection force in making nematode-trapping fungi gain the function of catching nematodes. But experimental evidence to prove this hypothesis is lacking. Experimental evolution is a very powerful tool to monitor the process of evolution, like how novel function appear, in lab condition. People tried to use laboratory evolution to remake the evolution in real-time. Microbiologist can use microorganism to study the evolution conveniently. *A. oligospora* not only possesses a small genome of about 40 MB (Yang *et al.*, 2011), but also has short life cycle, fast growth rate, and usually can produce a lot of progeny. These advantages help researcher

doi:10.6342/NTU201902152

to have easier way to do evolution. A couple of experimental evidence showed that novel function occurs with the mutation on transcription factor (Toll-Riera *et al.*, 2016). In order to gain more insight in the nematode-trapping ability in the history of evolution, experimental evolution was used to infer the relationship between nutrient and trapping ability.

In this study, experimental evolution and gene editing were used to survey the nature of prey-predator interaction. The conserved MAPK signaling pathway play a role in nematode-sensing and trap formation. In addition, trapping ability was highly plastic when the fungi were cultured in nutrient-rich environment.

doi:10.6342/NTU201902152

Material and Method

Strain, media, and culture conditions

All *A. oligospora* strains used in this study were TWF154. Strains were grown on potato dextrose agar (PDA) and low nutrient medium (LNM) for solid cultures, and yeast nitrogen base without amino acids (YNB) and Potato Dextrose Broth (PDB) for liquid culture.

For experimental evolution, all Petri dish remained unseal and cultured in 25℃ incubator for 1 week. Experimental evolution was performed in three conditions with differences in nutrient content: rich medium (PDA), low-nutrient medium (LNM), and LNM supplemented with *C. elegans* nematode.

Trap number quantification

For *C. elegans*-induced trap formation, thirty *C. elegans* N2 strain were added on LNM plate with 2 days growth fungi and removed N2 by ddH_2O after 6 hr. After another 18 hr, photos of trap were imaged by Zeiss Stemi 305 Stereo Microscope (Zeiss) and Zeiss Axiocam ERc 5s Microscope Camera (Zeiss). Three 40X images of the mycelium (size 2.5 x 2.1 mm) per plate were captured. For ascaroside (Butcher *et al.*, 2007) -induced trap formation, one micromolar of ascaroside were added on the edge of hyphal tip, then took photo as previous describe and quantified trap number by Fungal Feature Tracker (FFT, de Ulzurrun *et al.*, 2019).

Preparation of protoplast

doi:10.6342/NTU201902152

The fungal conidia (~10^6) were inoculated in PDB liquid culture. After one-day incubation, the hyphae were blended then centrifuged at 5000 rpm for 3 minute. Discarded the supernatant and 100 mg/ml Vino Taste Pro (Novozymes) with a little amount of chitinase (Sigma-Aldrich) were added into the hyphae to digest the cell wall of fungi. After digested at 30℃ for 12 hr, the digested protoplast was filtered using a sterile Miracloth (EMD Millipore) and centrifuged at 1000×g for 10 min. The pellet was washed with sterile STC (1.2M D-Sorbitol, 10mM Tris-Hcl (pH 7.5), 50mM $CaCl_2$) buffer then centrifuged with another 1000×g for 10 min.

Transformation

10^6 of protoplast were mixed with 5 μg of knockout cassette DNA in a 50 ml centrifuge tube on ice for 40 min. Then 5 volumes of PTC (40% polyethylene glycol 3350, 10mM Tris-Hcl (pH 7.5), 50mM $CaCl_2$) buffer was added, gently mixed with inverting the tube by hand. The mixture were incubated for 20 min at room temperature. Finally, the protoplast mixture was mixed with regeneration agar (3% acid-hydrolize casein, 3% yeast extract, 205% sucrose, 10% agar) containing 100 μg/ml of hygromycin B or 200 μg/ml of nourseothricin sulfate.

Construction of *gpb1* knockout cassette

Homologous recombination was used to knockout candidate genes that were predicted to be the homolog in *A. oligospora* (Fig 1). To construct the gene knockout cassette, primer 164ste4l1, 157STE4L2, and 160STE4R1, 169ste4r2 were used to amplify the 5' and 3' UTR of Gpb1 from *A. oligospora* TWF154, while 157STE4L2 and 160STE4R1 overlapped with hygromycin resistance gene. The hygromycin resistance gene Hph was amplified by primer 158HgBL and 159HgBR from plasmid pTP0102.

doi:10.6342/NTU201902152

These first PCR products were purified by QIAquick PCR purification kit (Qiagen). The final PCR amplification used primer 164ste4l1 and 169ste4r2 to amplify the whole knockout cassette, then purified by QIAquick PCR purification kit. All the primers used were listed in Table 1.

Other primers for knockout Ste2, Ste3, and Bck1 gene were listed in Table 1.

Confirmation of gene knockout

To screen for the complete gene knockout mutants, the DNA were extracted from small amount of fungal hyphae and spore by incubating in lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5mM $MgCl_2$, 0.45% IGEPAL CA-630, 0.45% Tween 20, 0.01% (w/v) gelatin, 60 μg/ml proteinase K) for 1 hr in 65℃ and additional 10 min in 96℃ to deactivate proteinase K. Then the PCR confirmation was performed using Takara EX Taq DNA polymerase (TaKaRa). To verify the insertion of Hph gene and Gpb1, four primer pairs were used: primers 225ste4-1 and 226ste4-2 to confirm the internal region of Gpb1 gene; primers 170SO1 and 228ste4-6 to confirm the presence of Hph. Primer sequences used for other genes confirmation are listed in Table 1.

Reconstitution of Gpb1

Primers 501ste4_re_L1 and 502ste4_re_R1 were used to amplify the wild type copy of Gpb1 with 2kb upstream and 1kb downstream of the open reading frame. In-Fusion HD cloning kit (Clontech) was used to insert the wild type Gpb1 into a plasmid pRS41N containing clonNAT resistance gene. The recombinant plasmid was then transformed into *gpb1* for phenotype complementation assays.

Experimental evolution protocol

doi:10.6342/NTU201902152

For the evolution in high and low nutrition environment, 30 replicate lines (10 of each condition) were started (Fig 6). *A. oligospora* TWF154 was first cultured on PDA, then the conidia were harvested by ddH_2O to obtain conidia. Initiated the experimental evolution with inoculating 1000 conidia in the middle of a 5cm Petri dish for 7 days in 25℃. Even though the hyphae took 3 to 4 days to reach the edge of the Petri dish, 7 days were used to assure completeness growth of hyphae and enough conidia. Then conidia were collected in ddH_2O by glass spreader. One thousand conidia were transferred to start the next generation. One transfer was defined as one generation. Additionally, after every 10 transfer, the conidia were collected and stored in 25% glycerol in -80℃.

Spore morphology observation

Fungal cell wall was stained by adding 1μl of calcofluor white (Sigma-Aldrich) to 10μl of spore suspension. After being dropped onto the middle of a glass slide, spores were observed using an Axiovert 200M fluorescence microscope (Zeiss) and 400X magnification, and their morphology quantified using FFT.

Mycelium growth characterization

To quantify the mycelium growth, single spore was inoculated in the middle of twelve well plate containing LNM agar with 0.1% SCRI Renaissance 2200 (SR2200) for 2 days in dark condition. ImageXpressMicro-XL system (Molecular Devices) was used to capture the photo of fungal hyphae. Further fungal feature included number of tips, hyphal total length, and colony area were quantified by FFT.

Genomic DNA extraction

doi:10.6342/NTU201902152

For Illumina sequencing of evolved line e14(2), fungal hyphae and conidia were first harvested by ddH_2O, introduced into YNB liquid culture in 25℃ incubator under 200 rpm for 2~3 days. The fungal hyphae were centrifuged under 5000 rpm and washed with ddH_2O once. All water was removed and lyophilized overnight. Dried fungal hyphae were broken down by glass beads. Thirteen milliliter extraction buffer (20% CTAB, 20% PVP, 100mM Tris-HCl, 25mM EDTA, 2M NaCl, 0.5g/L spermidine (Sigma-Aldrich), 2% 2-Mercaptoethanol (β-ME, Sigma-Aldrich), 40μg/ml proteinase K) were added into the mix and incubated under 60℃ for 45 min until dissolved. One volume of chloroform was added to the tube, then centrifuged at 5000 rpm for 15 min at 4℃. The upper layer was transferred to a new tube, followed by another one volume of isopropanol added and gently inverted. After 20 min incubated in room temperature, DNA were centrifuged at 5000 rpm for 20 min at 4℃. The DNA pellet was washed with 70% EtOH twice, and allowed to air-dry at room temperature. Finally, the DNA was dissolved in STE (100mM NaCl, 10mM Tris-HCl, 1mM EDTA) buffer. For higher quality of DNA for sequencing, 420 μg/ml RNaseA were added into DNA at 42℃ for 30 min. One volume of phenol chloroform was added and mixed gently. Tubes were centrifuged at 15000 rpm for 15 min at 4℃. The supernatant was transferred to a new tube, 0.1 volume of 3M sodium acetate and 2 volume of 100% cold EtOH were added and gently inverted. The DNA were pelleted at 15000 rpm for 10 min at 4℃, washed with 70% EtOH twice. Finally, the pellet was air-dried then dissolved in 100 μl of ddH_2O.

Remapping of evolved line

Mapped the reference genome TWF154 (Accession: PRJNA523398) and Illumina sequencing data with bwa-0.7.15 (Li and Durbin, 2009) and converted to BAM files and mark duplicates with picard. Applied picard and GATK (McKenna *et al.*, 2010) for indel

doi:10.6342/NTU201902152

calling, local realignment and SNP calling. Finally, the output CSV file showed the position of mutations.

Reconstitution and overexpression of mutated EYR41_009099 in TWF154

To examine if the mutation on EYR41_009099 effect on prey-sensing and trap formation, EYR41_009099 on e14(2) with 2kb upstream and 1kb downstream of the open reading frame was amplified by primer 1147_9099F1 and 1148_9099R1. After purified by QIAquick PCR purification kit, PCR product was fused into plasmid pRS41N by In-Fusion HD cloning kit.

Overexpression was also employed to examine the function of EYR41_009099. A gpdA promoter, amplified by primer 1149_oe_GPDAF1 and 1150_oe_GPDAR1 from the *Aspergillus* vector pAN7-1, was fused with EYR41_009099 on e14(2) with 1kb downstream, then cloned into pRS41N by In-Fusion HD cloning kit. Primer sequences used are listed in Table 1.

doi:10.6342/NTU201902152

Results

Identification of homolog genes in *A. oligospora* TWF154

Since MAPKs pathway are highly conserved among fungi kingdom, a reverse genetic approach was used to investigate the molecular mechanism of trapping process. The homologs of *A. oligospora* were identified by Blast2GO 5 Pro performing a Local BLAST using the protein sequences of homologs from *Saccharomyces cerevisiae* and other filamentous fungi. EYR41_009480, which has 58.9% similarity to *S. cerevisiae* Ste4, was identified as the *A. oligospora* Gpb1. GPCRs Ste2 and Ste3 in *S. cerevisiae* has 46.4% and 48.7% to EYR41_011505 and EYR41_007468. EYR41_003178, shares 66.2%, 70.4%, 68.5%, 69.2%, and 65.6% from *Botrytis cinerea* (BC1T_11345), *Tuber melanosporum* (GSTUMT00006322001), *Saccharomyces cerevisiae* (YJL095W), *Cochliobolus heterostrophus* (estExt_Genewise1Plus.C_260058), and *Alternaria brassicicola* (AB02044.1) was identified as *A. oligospora* Bck1.

Deletion of *A. oligospora gpb1* gene abolished trap formation

To determine the function of G protein in *A. oligospora*, two independent G protein β subunit (*gpb1*) deletion mutants were acquired with homologous recombination. A rapid genomic PCR experiment was performed to confirmed the deletion of the Gpb1 gene.

The two independent *gpb1* deletion strains showed no obvious growth difference on rich medium PDA. Interestingly, the *gpb1* mutants showed strong defect in trap morphogenesis in response to both nematodes and ascarosides. Trap morphogenesis was rescued in GPB1 reconstitute line (Fig 2).

doi:10.6342/NTU201902152

Deletion of *A. oligospora* GPCR *ste2* and *ste3* didn't affect trap morphogenesis

In *A. oligospora*, only one GPCR STE2 and STE3 were found. Two independent *ste2* deletion mutants and one *ste3* deletion mutant showed no difference in sensing nematodes compare to wild type (Fig 3).

Deletion of *A. oligospora* CWI MAPKKK *bck1* results in severe growth defect

Eighteen out of 175 transformants were founded to have deletion of *bck1*, confirmed by PCR screening of primer 426bck1-1 and 427bck1-2. The *bck1* gene knockout mutants resulted in severe defects in growth, conidiation, and trap formation (Fig 4).

One evolved line in PDA can form more aerial hyphae

In order to gain more insights into the coevolution of nematodes and nematode-trapping fungi, 30 independent lines were evolved in PDA, LNM, and LNM supplement with N2 every 3 transfers (Fig 6). After 14 transfers (e14), one evolving line, e14(2), evolved in rich medium PDA, started to form different hyphal growth pattern compared to ancestral strain TWF154 (WT): e14(2) generated a lot more aerial hyphae after one week on PDA plate (Fig 7A). Quantitatively measuring the spore number in LNM for 7 days showed that although the spore number of e14(2) is more than ancestral strain without statistical significance (Fig 7B, *p*-value = 0.1029). In the mycelia growth test, e14(2) showed an increase in total hyphae length and numbers of tips, but decreased area in LNM (Fig 7C, D, E).

Trap morphogenesis was tested by *C. elegans* and ascarosides. Trap quantification showed e14(2) can generate almost twice the number of traps as wild type when exposed to *C. elegans* N2. For ascaroside-induced trap formation, e14(2) formed more than 4-fold of trap compared to WT (Fig 8).

doi:10.6342/NTU201902152

Until 40 transfer, 20 independent evolving lines (10 in LNM and 10 in LNM supplied with *C. elegans*) didn't show significant difference of trap number induced by *C. elegans* (Fig 9).

Identifying mutations associated with enhanced trap morphogenesis

Whole genome was sequenced to identify the causal mutations of enhanced trap morphogenesis. By remapping to the reference genome of ancestral strain, almost 2000 mutations were found. In summary, there are 62 (3.24%), 199(10.41%), 129 (6.75%), 247 (12.92%), 285 (14.91%), 205 (10.72%), 144 (7.53%), 637 (33.32%), 3 (0.16%), and 1 (0.05%) mutations from exon, intron, upstream and downstream, downstream, UTR3, upstream, UTR5, intergenic, splicing, and UTR5, UTR3. One evolved strain e10(8) evolved from rich medium with reduced aerial hyphae production but no difference with trap morphogenesis was also whole-genome sequenced. Since the mutation evolved in trap formation was only in e14(2), identified and deleted the same mutation in both lines by Linux "diff" code. Filtered out 87% mutations coexist in 2 evolved lines. Finally, 3 (1.18%), 31 (12.20%), 20 (7.87%), 45 (17.72%), 29 (11.42%), 35 (13.78%), 21 (8.27%), and 70 (27.56%) mutations were found from exon, intron, upstream and downstream, downstream, UTR3, upstream, UTR5, intergenic in e14(2). No specific mutations were found in splicing and UTR5, UTR3 (Fig 10). Three mutations from e14(2) were found on exon. NCBI BLAST were used to find out the conserved domain from those 3 genes.

In e14(2), mutation on EYR41_009099 cause nonsynonymous single nucleotide variation (SNV). EYR41_009099 contains a conserved DNA binding domain which is hypothesized to be a transcription factor (Table 2). Others 2 genes EYR41_003931 and EYR41_008926 disrupted by frameshift insertion found no conserve domain on NCBI Conserved Domain Database (CDD).

doi:10.6342/NTU201902152

Mutated EYR41_009099 involved in hyphae production

To find out which mutation cause the enhanced trap morphogenesis in e14(2), EYR41_009099 from e14(2) was used to knock in ancestral strain. Two out of twelve and one out of eleven transformants are from reconstitution and overexpression lines, all of those transformants can generate a lot of aerial hyphae on both rich medium (Fig11A-E) and low nutrient medium.

In trap quantification assay, only overexpression line (31-2-2-1) rescued both the aerial hyphae production and *C. elegans*-induced trap formation phenotype (Fig 11F, G).

doi:10.6342/NTU201902152

Discussion

Nematode and nematophagous fungi are ubiquitous in nature environment. Nematodes usually habitat in soil, consuming fungi and bacteria as their nutrient source (Yeates *et al.*, 1993). Nematophogous fungi are characterized in the ways they prey on nematodes, including nematode-trapping/predators, opportunistic, endoparasites, and toxin-producing fungi; these fungi are distributed in Deuteromycetes, Discomycetes, Basidiomycetes, Chytridiomycetes, and Zygomycetes (Khan, 2015), suggesting that nematophagous fungi are independently evolved and have coevolved with nematodes for a long time. However, how and why nematophagous fungi evolved the ability to prey on nematode remains elusive.

Mitogen-activated protein kinase (MAPK) pathways play a role during trap morphogenesis

Nematode-trapping fungi can sense nematodes and produce specialized trap structures to capture nematodes as their nitrogen source. Previous finding showed that one of the signals that trigger trap morphogenesis is ascarosides (Hsueh *et al.*, 2013). In *C. elegans* nematode, the SRBC subfamily was used as the receptor for sensing ascarosides (Kim *et al.*, 2009). However, the molecular mechanism of how nematode-trapping fungi sense the signal of nematode remains unknown. Since nematodes are one of the environment signals, the highly conserved MAPK signaling pathways were the way fungi sense outside signal. Conserved MAPKs pathways have been characterized as the key signaling pathways in sensing host and environment signals in plant and animal signal (Jiang *et al.*, 2018). Pth11 in rice blast fungus *Magnaporthe grisea* is the GPCR-like receptor and it is required for pathogenesis. In nematode-trapping fungi, G protein

doi:10.6342/NTU201902152

subunit and MAPK cascade components were found to upregulate in response to nematodes by RNA-seq (Pandit *et al.*, 2017). Their results suggested G-protein plus MAPKs play a role in sensing the presence of prey in *A. conoides*, but no experimental evidence supports this hypothesis. In this study, the deletion of G protein β subunit (*gpb1*) showed severe defect in trap formation after induced by nematodes or ascarosides, demonstrating that Gpb1 plays an essential role in the signaling pathways that govern nematode-sensing and trap morphogenesis.

In *Fusarium oxysporum*, which cause severe vascular wilt disease in crop, seven-transmembrane (7TM) protein Ste2 GPCR was identified as a receptor to sense the signal from host and pheromone of mating factor (Turra *et al.*, 2015). Although in this case, the downstream components of Ste2 were not conserved pheromone sensing pathway. Instead, cell wall integrity (CWI) pathway switches to be the downstream of pheromone sensing receptor GPCR Ste2. Prey-sensing in nematode-trapping fungi can also be the same as *F. oxysporum*. In fact, deletion of *ste2* homolog in *A. oligospora* didn't show any defect on trap morphogenesis while the deletion of G protein β subunit showed severe defect in trap formation. This result suggests that the upstream receptor of Gpb1 is not the conserved GPCR Ste2, or that the redundancy of receptors for sensing nematodes masked the effect of single GPCR deletion.

Zhen *et al.* (2018) have found that MAPK Slt2, which is the MAPK of CWI pathway, play a role in trap formation. But *slt2* showed severe growth defect, the defect of growth maybe the reason why *slt2* didn't form trap after triggered by *C. elegans*. Here, deletion of *bck1*, CWI MAPKKK homolog in *A. oligospora* also showed strong trap morphogenesis defect, at the same time, also showed strong growth defect. Again to prove that there is a concern about whether the trap-forming defect actually causing by the disruption of CWI or the overall fitness defect. Together, we experimentally prove that G

doi:10.6342/NTU201902152

protein β subunit Gpb1 in nematode-trapping fungi is required for prey-sensing and trap morphogenesis, however the upstream receptor and downstream transcription factor needs more information like RNA sequencing of *gpb1* to make the molecular mechanism more complete. In addition, CWI MAPK play an unknown function in hyphal growth and trap formation to decrease the overall fitness (Fig 4).

Experimental evolution reveals the plasticity of prey-sensing

Nematode-trapping fungi can utilize carbohydrates and inorganic nitrogen. They can undergo saprophytic stage in soil. Nematode-trapping fungi, especially the trap former *A. oligospora*, can consume various carbohydrates, including cellulose, starch, and glycogen, and nitrogen source. They can well-grown in the absence of nematodes, although in some case, *Dactylella bembicodes*, which produce constricting ring to physically catch nematodes, is not good at utilizing nutrients. Some species even need to maintain in the lab with the supplement of nematodes (Gray, 1987). In lab condition, the *C. elegans*-induced trap morphogenesis in nematode-trapping fungi absents when the environment is full of nutrient (Hsueh *et al.*, 2013). This indicates that nematode-trapping fungi don't require to digest worms as their nutrient or nitrogen source to survive. In this study, nematode-trapping fungi were experimentally evolved in response to high or low nutrient level to know the effect of nutrient to the plasticity of trapping ability. Two out of ten independent evolved lines in rich medium showed a significant change in aerial hyphae growth: one with increased, and one with decreased aerial hyphae biomass (not shown). The former evolved line from only 14 transfer in rich medium also performed strong trap formation in response to both *C. elegans* N2 strain and ascarosides, showing higher sensitivity to their prey. The hypothesis of evolved in nutrient-rich environment is not matched to what have evolved probably cause by the protocol of evolution experiment.

doi:10.6342/NTU201902152

In this study, conidia were transferred to the next generation, so the fungi evolved to produce more conidia will have higher possibility to pass to next transfer. Increasing spore production were reported to increase mating success in mushroom-forming fungus *Schizophyllum commune*. An additional production of spore also linked directly to increase fitness, though no trade-offs were found (Nieuwenhuis and Aanen, 2018). But in nematode-trapping fungi, trade-off was found when growing in low nutrient environment. Although e14(2) have higher ability to sense nematodes and form more trap, slightly lower colonizing ability were found in LNM (Fig 7E).

Functional innovation occurs when there are mutations in transcription regulator and metabolic genes in *Pseudomonas aeruginosa* (Toll-Riera *et al.*, 2016). By the whole genome sequencing data, one mutated gene, EYR41_009099, has identified as a transcription factor. Knocking in the mutated gene to ancestral strain found that both the reconstitute strains and overexpression strain have phenotype on aerial hyphae production; however, only the overexpression line recovered the enhanced trap morphogenesis in response to *C. elegans*. Further study needed to understand the molecular mechanism of how this mutated gene enhance the trap morphogenesis. Since EYR41_009099 has a conserved DNA binding domain, ChIP-seq technology can be used to specifically bind to the downstream target gene. Which gene activated by this transcription factor can be identified. Gene knockout and rescue experiment can also employ to know if the mutation is causing by loss of function or gain of function.

In conclusion, by gene engineering and evolution experiment, G protein β subunit in the conserved G protein signaling pathway is required for prey-sensing and the following trap morphogenesis; trapping ability is highly plastic in high nutrient environment. The

doi:10.6342/NTU201902152

results here allow us to gain more insights into the interaction of prey-predator and how these organisms have coevolved.

doi:10.6342/NTU201902152

Reference

Butcher, R.A., Fujita, M., Schroeder, F.C., and Clardy, J. (2007). Small-molecule pheromones that control dauer development in *Caenorhabditis elegans*. Nat Chem Biol *3*, 420.

Choe, A., von Reuss, S.H., Kogan, D., Gasser, R.B., Platzer, E.G., Schroeder, F.C., and Sternberg, P.W. (2012). Ascaroside signaling is widely conserved among nematodes. Curr Biol *22*, 772-780.

de Ulzurrun, G.V.-D., Huang, T.-Y., Chang, C.-W., Lin, H.-C., and Hsueh, Y.-P. (2019). Fungal Feature Tracker (FFT): A tool for quantitatively characterizing the morphology and growth of filamentous fungi. bioRxiv, 659672.

DeZwaan, T.M., Carroll, A.M., Valent, B., and Sweigard, J.A. (1999). *Magnaporthe grisea* pth11p is a novel plasma membrane protein that mediates appressorium differentiation in response to inductive substrate cues. The Plant Cell *11*, 2013-2030.

Duddington, C. (1957). The predacious fungi and their place in microbial ecology, Vol 218 (Cambridge University Press, Cambridge).

Gray, N. (1987). Nematophagous fungi with particular reference to their ecology. Biological Reviews *62*, 245-304.

Hamel, L.P., Nicole, M.C., Duplessis, S., and Ellis, B.E. (2012). Mitogen-activated protein kinase signaling in plant-interacting fungi: distinct messages from conserved messengers. Plant Cell *24*, 1327-1351.

doi:10.6342/NTU201902152

Hsueh, Y.-P., Mahanti, P., Schroeder, F.C., and Sternberg, P.W. (2013). Nematode-trapping fungi eavesdrop on nematode pheromones. Current biology : CB *23*, 83-86.

Hsueh, Y.P., Gronquist, M.R., Schwarz, E.M., Nath, R.D., Lee, C.H., Gharib, S., Schroeder, F.C., and Sternberg, P.W. (2017). Nematophagous fungus *Arthrobotrys oligospora* mimics olfactory cues of sex and food to lure its nematode prey. Elife *6*.

Jiang, C., Zhang, X., Liu, H., and Xu, J.R. (2018). Mitogen-activated protein kinase signaling in plant pathogenic fungi. PLoS Pathog *14*, e1006875.

Khan, M.R. (2015). Nematophagous Fungi: Ecology, Diversity and Geographical Distribution. In.

Kim, K., Sato, K., Shibuya, M., Zeiger, D.M., Butcher, R.A., Ragains, J.R., Clardy, J., Touhara, K., and Sengupta, P. (2009). Two chemoreceptors mediate developmental effects of dauer pheromone in *C. elegans*. Science (New York, NY) *326*, 994-998.

Kou, Y., and Naqvi, N.I. (2016). Surface sensing and signaling networks in plant pathogenic fungi. Paper presented at: Semin Cell Dev Biol (Elsevier).

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics *25*, 1754-1760.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., and Daly, M. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res *20*, 1297-1303.

Nieuwenhuis, B.P.S., and Aanen, D.K. (2018). Nuclear arms races: Experimental

doi:10.6342/NTU201902152

evolution for mating success in the mushroom-forming fungus *Schizophyllum commune*. PLoS One *13*, e0209671.

Pandit, R., Patel, R., Patel, N., Bhatt, V., Joshi, C., Singh, P.K., and Kunjadia, A. (2017). RNA-Seq reveals the molecular mechanism of trapping and killing of root-knot nematodes by nematode-trapping fungi. World J Microbiol Biotechnol *33*, 65.

Su, H., Zhao, Y., Zhou, J., Feng, H., Jiang, D., Zhang, K.Q., and Yang, J. (2017). Trapping devices of nematode-trapping fungi: formation, evolution, and genomic perspectives. Biological Reviews *92*, 357-368.

Toll-Riera, M., San Millan, A., Wagner, A., and MacLean, R.C. (2016). The Genomic Basis of Evolutionary Innovation in *Pseudomonas aeruginosa*. PLoS Genet *12*, e1006005.

Turra, D., El Ghalid, M., Rossi, F., and Di Pietro, A. (2015). Fungal pathogen uses sex pheromone receptor for chemotropic sensing of host plant signals. Nature *527*, 521-524.

Waghorn, T.S., Leathwick, D.M., Chen, L.Y., and Skipp, R.A. (2003). Efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against three species of gastro-intestinal nematodes in laboratory faecal cultures from sheep and goats. Vet Parasitol *118*, 227-234.

Waller, P.J., Knox, M.R., and Faedo, M. (2001). The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: feeding and block studies with *Duddingtonia flagrans*. Vet Parasitol *102*, 321-330.

Yang, J., Wang, L., Ji, X., Feng, Y., Li, X., Zou, C., Xu, J., Ren, Y., Mi, Q., Wu, J., *et al.* (2011). Genomic and Proteomic Analyses of the Fungus *Arthrobotrys oligospora* Provide Insights into Nematode-Trap Formation. PLoS Path *7*, e1002179.

doi:10.6342/NTU201902152

Yeates, G.W., Bongers, T., De Goede, R., Freckman, D., and Georgieva, S. (1993). Feeding habits in soil nematode families and genera—an outline for soil ecologists. J Nematol *25*, 315.

Zhen, Z., Xing, X., Xie, M., Yang, L., Yang, X., Zheng, Y., Chen, Y., Ma, N., Li, Q., and Zhang, K.-Q. (2018). MAP kinase Slt2 orthologs play similar roles in conidiation, trap formation, and pathogenicity in two nematode-trapping fungi. Fungal Genet Biol *116*, 42-50.

doi:10.6342/NTU201902152

Table 1. Oligonucleotides Used in This Study.

Primers	Sequence (5'→ 3')	Gene	Purpose
158HgBL	TTTTTGGGCTTGGCTGGAGC	Hph	Amplify hygromycin resistance gene
159HgBR	CTATTCCTTTGCCCTCGGACG		
164ste4l1	TTTTGGCATGGCAGTTTCGT	Gpb1	Amplify 5' homolog flanking region
157STE4L2	GCTCCAGCCAAGCCCAAAAATGTCTGTTCACAGTTGATATATATTTGGGA		
160STE4R1	CGTCCGAGGGCAAAGGAATAGTCACACATCGCCCTATTCACTAAAAGT		Amplify 3' homolog flanking region
169ste4r2	GAATGGCTTGCCCTTATCGA		
225ste4-1	ACAGCCATACAGGAGCACTGACC		PCR confirmation for gene knockout
226ste4-2	CGTTCTGCTACGGAACAGGATCT		
170SO1	TCCCAAATATATATCAACTGTGAACAGACA		PCR confirmation for cassette insertion
228ste4-6	TCGATATCGATGCTTCGGTAGAA		
501ste4_re_L1	CGGTATCGATAAGCTTTGAAATTTGCCTCTGTTGAGC		reconstitution of WT gene
502ste4_re_R1	TAGAACTAGTGGATCCTTCCTTGCTGTTGGCCGTAG		
195STE2L1	CTTCCTCCCCTTCGAACTTCAG	Ste2	Amplify 5' homolog flanking region
196STE2L2	GCTCCAGCCAAGCCCAAAAATTCCGCCATCGGGATAAAAG		
197STE2R1	CGTCCGAGGGCAAAGGAATAGTTTTTATCGCACCTGCTTCATTC		Amplify 3' homolog flanking region
198STE2R2	GCTTTGGCGAATATGGCTTTT		
199STE2SO1	TGGCCCAGATTGACTCATTCC		PCR confirmation for gene knockout
200STE2SO2	GCCATGTCCTGCTTCAAGGTCTT		
201STE21	CTTTTATCCCGATGGCGGAA		PCR confirmation for cassette insertion
202STE22	CCCCCCTCTTGAATTTTCGGGTA		

doi:10.6342/NTU201902152

203STE3L1	GGGCCACGAGACTAGATTTA	Ste3	Amplify 5' homolog flanking region
204STE3L2	GCTCCAGCCAAGCCCAAAAAAATACACAGCCGTGAAGACG		
205STE3R1	CGTCCGAGGGCAAAGGAATAGCCTAGAGTAAAACGGGTCGGAAG		Amplify 3' homolog flanking region
206STE3R2	ATGCAGGTAGTGGCCAGACTGG		
207STE3SO1	CGTCTTCACGGCTGTGTATT		PCR confirmation for gene knockout
208STE3SO2	CTTCCGACCCGTTTTACTCTAGG		
209STE31	ATGCCCCGACTCCTTGTTTTC		PCR confirmation for cassette insertion
210STE32	TTTTTCTTGGGAATCTCTGGGG		
402Bck1L1	CCCACCGAATTACCGATTCC	Bck1	Amplify 5' homolog flanking region
403Bck1L2	GCTCCAGCCAAGCCCAAAAAATATGCGATCCTACGCCCTC		
404Bck1R1	CGTCCGAGGGCAAAGGAATAGAACAACAAGCTCCAGAGACAAG		Amplify 3' homolog flanking region
405Bck1R2	TCTTGCTTCAAGGCTGTTCG		
426bck1-1	ATCTAGCGAAGCAGTTGCAG		PCR confirmation for gene knockout
427bck1-2	ACGACAGTGACGACTTCTTC		
1147_9099F1	TCGACGGTATCGATAGATCCAAACTGGACGGCCTG	EYR41_009099	Reconstitution of EYR41_009099
1148_9099R1	TAGAACTAGTGGATCCGATCTCTTCAGGTGCCTGG		
1149_ oe_GPDAF1	TCGACGGTATCGATACGAGCTCCCAAATCTGTCCA		Amplification of gpdA promoter from pAN7-1
1150_ oe_GPDAR1	ATTAACTGCCGCGATATGGTGATGTCTGCTCAAGC		
1151_ oe_9099InF1	AGCAGACATCACCATATCGCGGCAGTTAATAGAGC		Overexpression of EYR41_009099

doi:10.6342/NTU201902152

Table 2. Functional prediction of mutated genes on exon from e14(2).

Mutated gene	Type of mutation	Position of mutation	Gene function prediction
EYR41_009099	nonsynonymous SNV	exon3:c.A1666T:p.I556F	Maltose O-acetyltransferase, GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain
EYR41_003931	frameshift insertion	exon2:c.548dupA:p.X183delinsX	Protein of unknown function
EYR41_008926	frameshift insertion	exon1:c.175dupG:p.E59fs	Protein of unknown function

doi:10.6342/NTU201902152

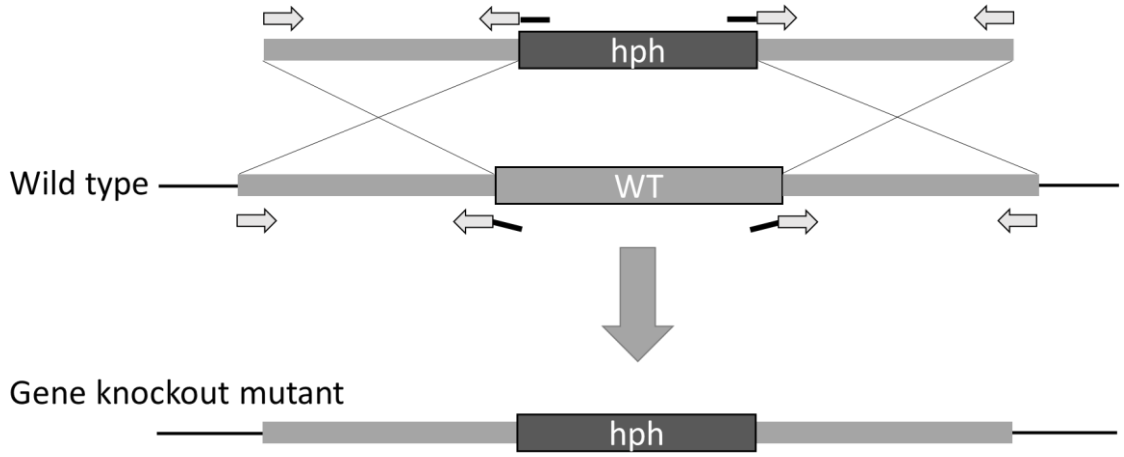

Fig 1. Map of target gene knockout. 5' and 3' flanking region of target gene were overlapped with hygromycin resistance gene, fused by overlap PCR then. The gene knockout cassette was transformed into WT, knockout target gene during DNA replication.

doi:10.6342/NTU201902152

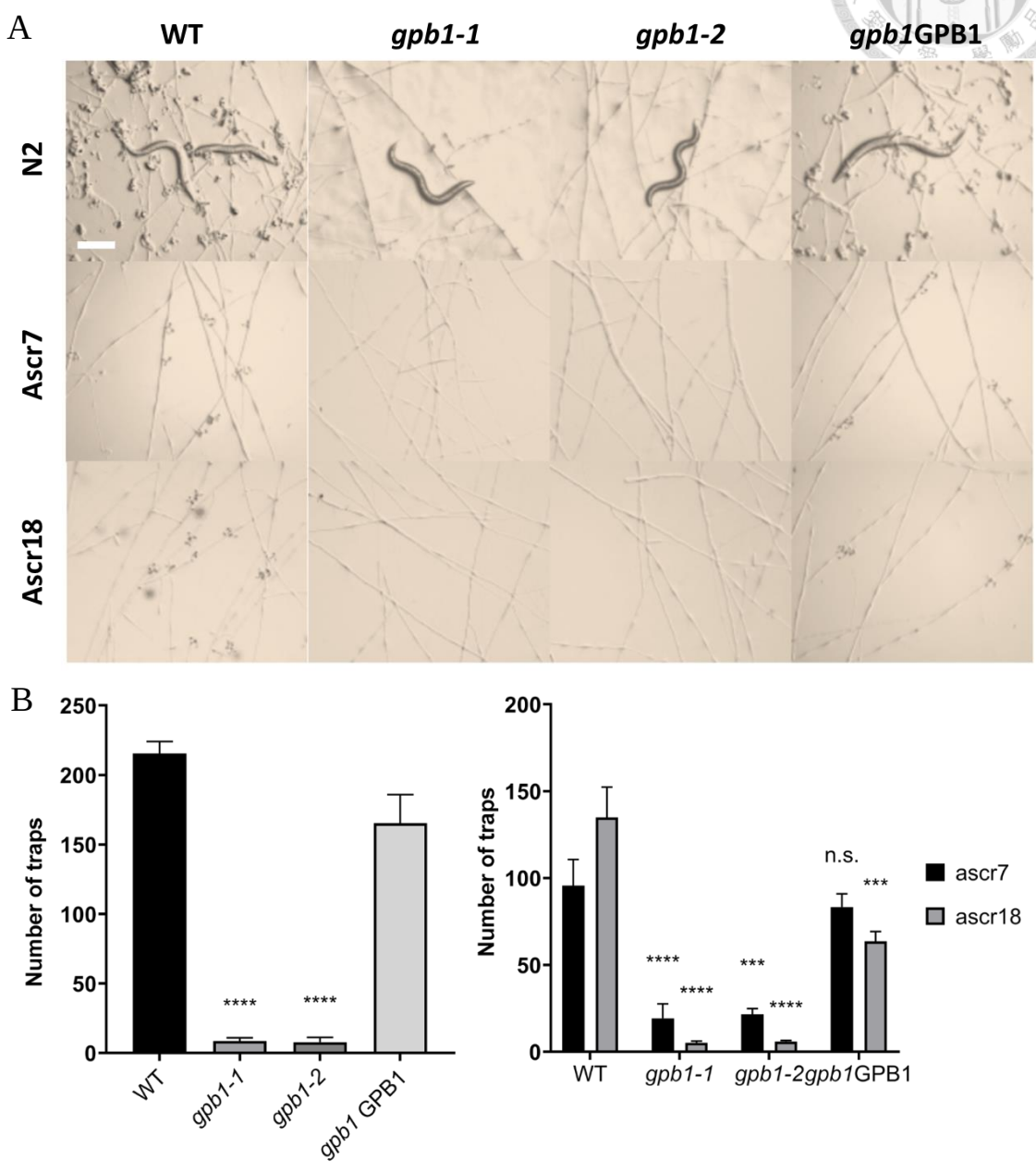

Fig 2. G protein signaling is required for prey sensing. (A) Images of the traps induced by *C. elegans* lab strain N2 or ascaroside (ascr7 and ascr18) in wild type (TWF154), *gpb1* deletion mutants, and GPB1 reconstitute strain (*gpb1*GPB1). Bar = 100 μm. (B) Quantification of traps number.

doi:10.6342/NTU201902152

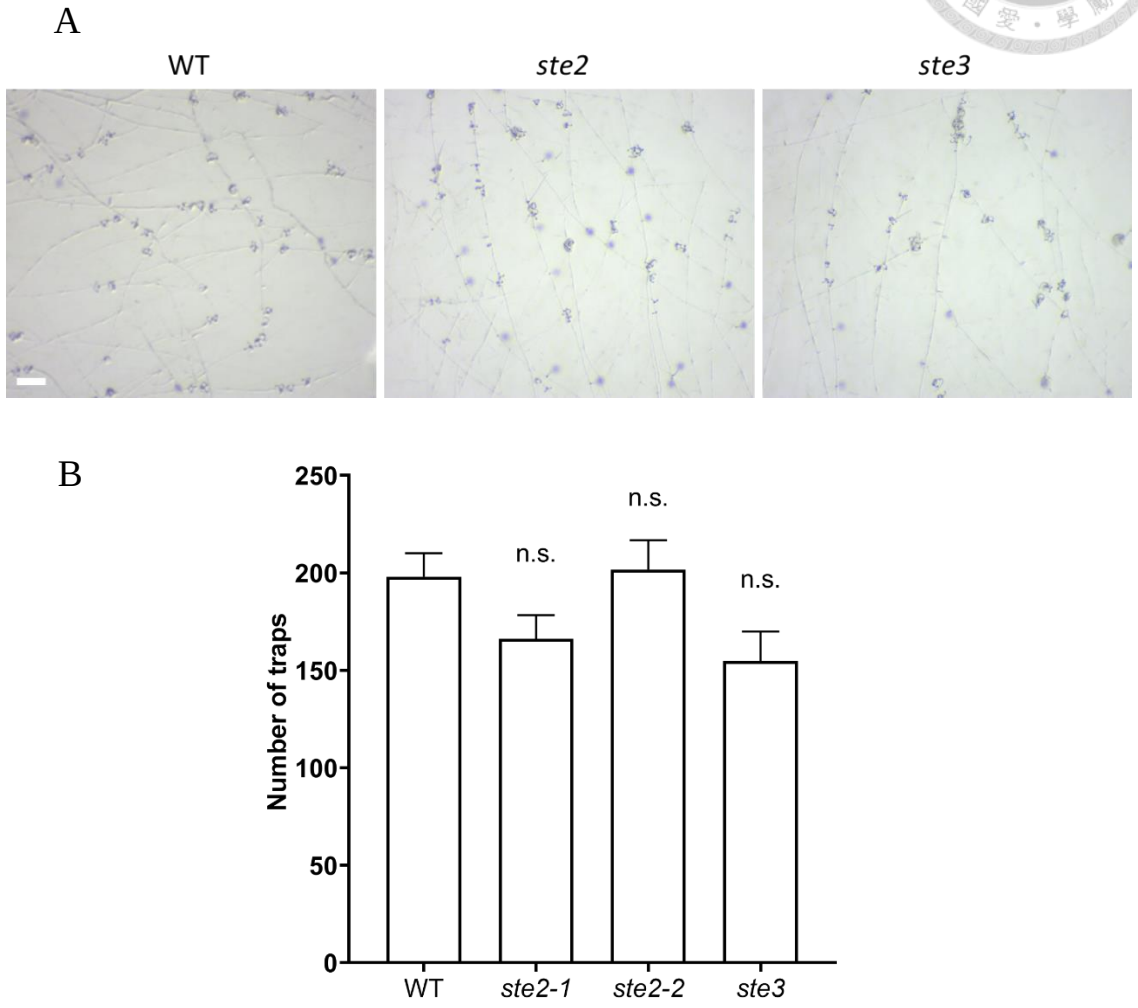

Fig 3. GPCR of conserved pheromone sensing pathway is not related to prey-sensing. (A) Images of the traps induced by *C. elegans* lab strain N2 in wild type, *ste2* and *ste3* deletion mutants. Bar = 200 μm. (B) Quantification of traps number.

doi:10.6342/NTU201902152

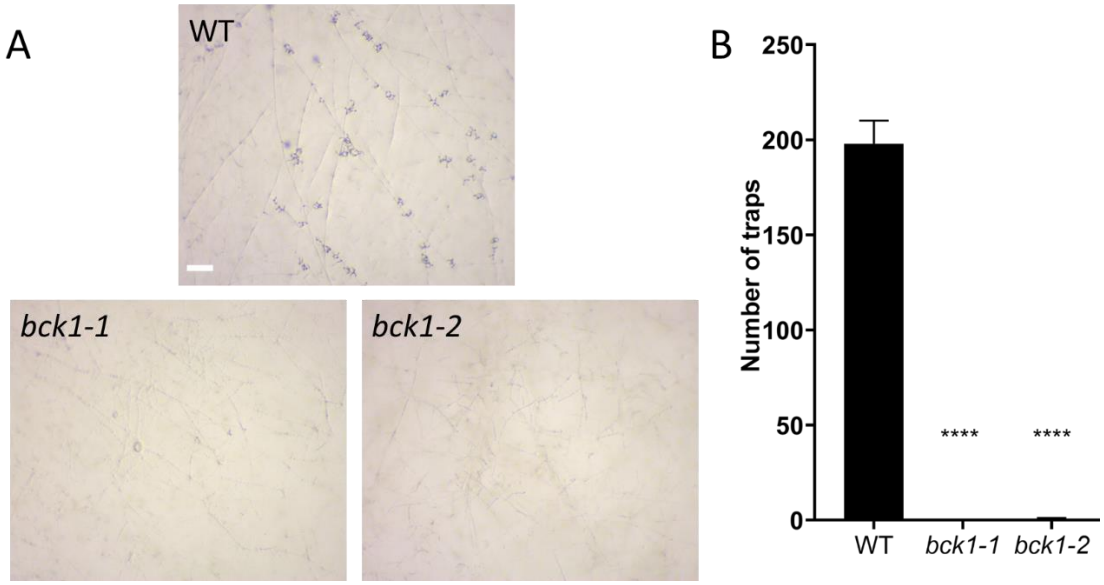

Fig 4. CWI MAPKKK *bck1* showed severe trap morphogenesis defect. (A) Images of *C. elegans*-induced trap formation. Bar = 200 μm. (B) Quantification of traps number.

doi:10.6342/NTU201902152

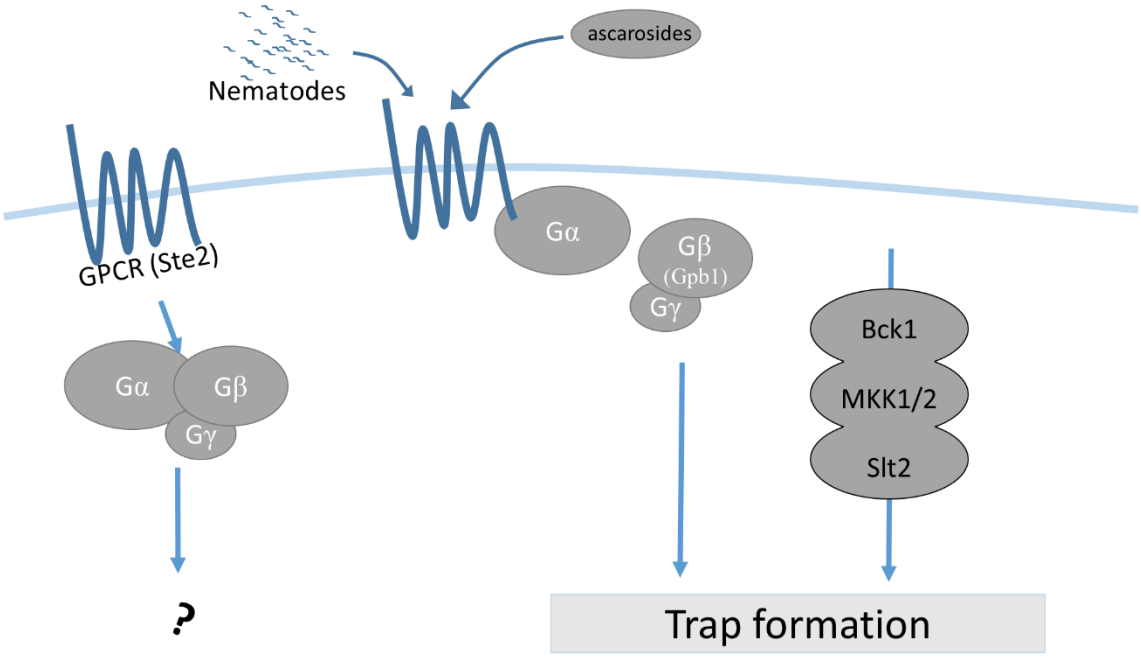

Fig 5. Schematic signaling pathway in *A. oligospora* in nematode- or ascarosides-sensing. *C. elegans* and ascarosides sensing require G protein β subunit Gpb1 and Slt2 MAPK pathway, while the receptor is not the conserved GPCR of pheromone sensing pathway, Ste2. MAPKKK Bck1 from CWI pathway also play an essential role in trap morphogenesis.

doi:10.6342/NTU201902152

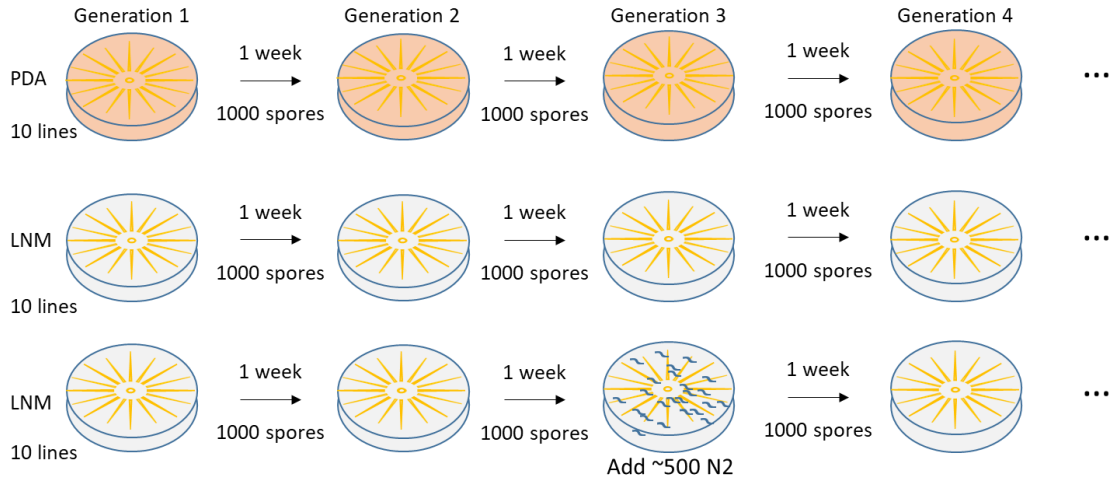

Fig 6. Setup for experimental evolution of *A. oligospora*. Three condition were setup: rich medium (PDA), low nutrient medium (LNM), and LNM supplied with *C. elegans* every 3 transfer. Conidia (~1000) were inoculated in the middle of agar plate, then the conidia were collected and transfer 1000 conidia to the new plate after one week.

doi:10.6342/NTU201902152

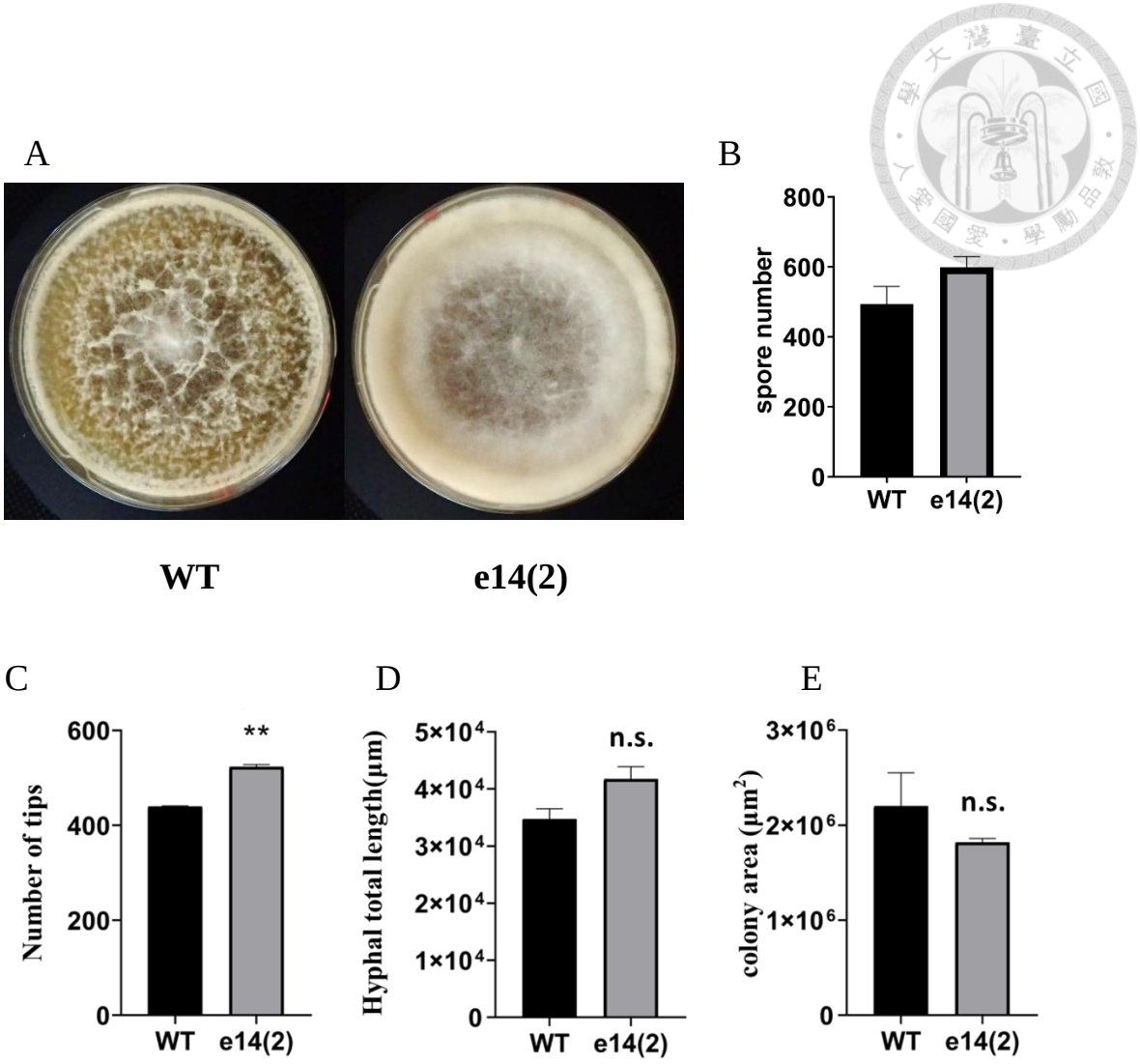

Fig 7. Morphology of evolved line. (A) Morphology of the evolved line e14(2) on PDA. Bar = 1 cm. (B) Spore numbers from one week LNM plate. (C, D, E) Comparison of evolved line and WT on LNM of (C) number of tips, (D) hyphal total length, and (E) colony area.

doi:10.6342/NTU201902152

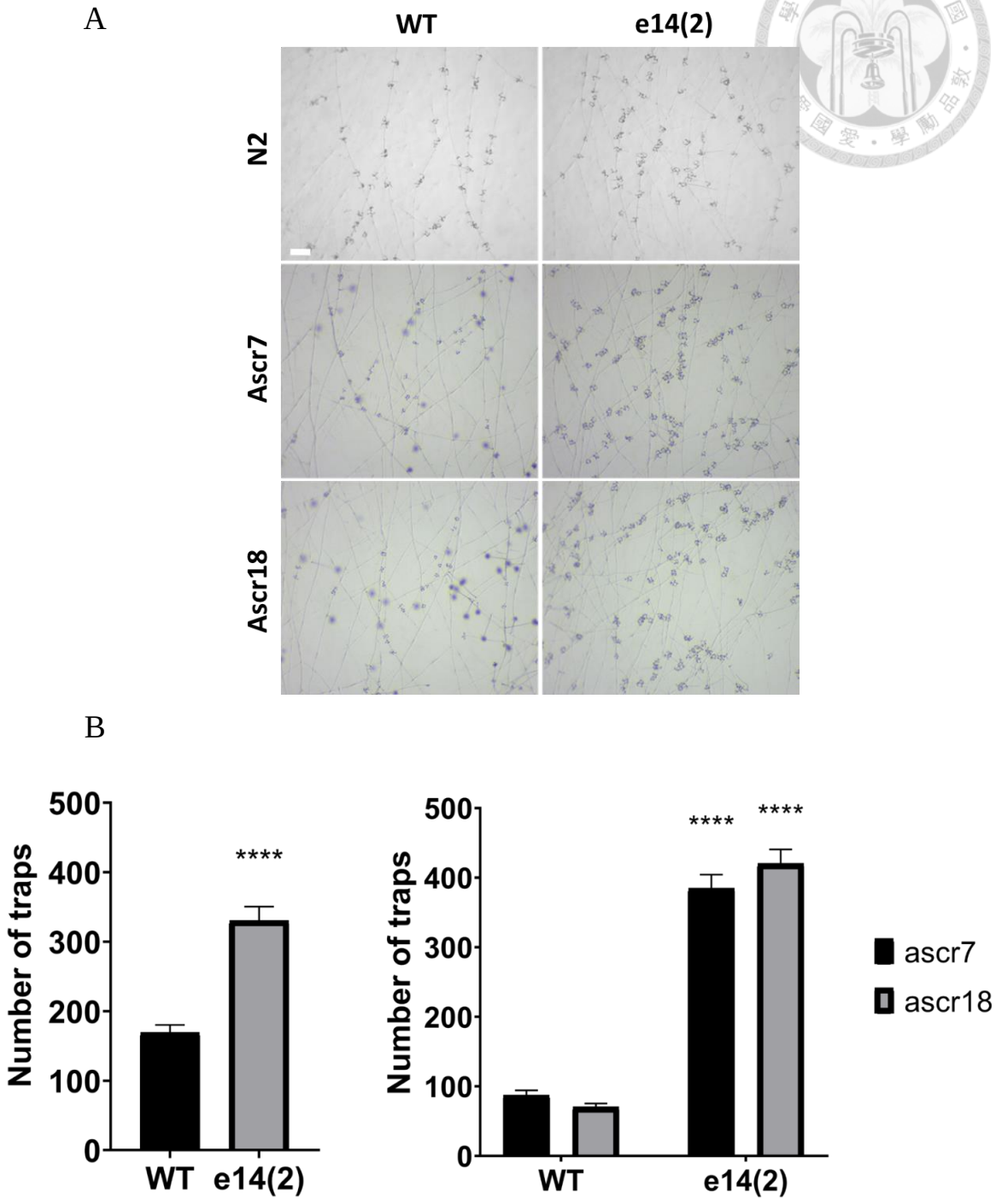

Fig 8. Prey-sensing ability improved in one evolved line, e14(2). (A) Images of the traps induced by *C. elegans* lab strain N2 or ascaroside (ascr7 and ascr18) in wild type (WT, TWF154) and e14(2). Bar = 200 μm. (B) Quantification of traps number showed e14(2) can generate more trap significantly (*p*-value < 0.0001).

doi:10.6342/NTU201902152

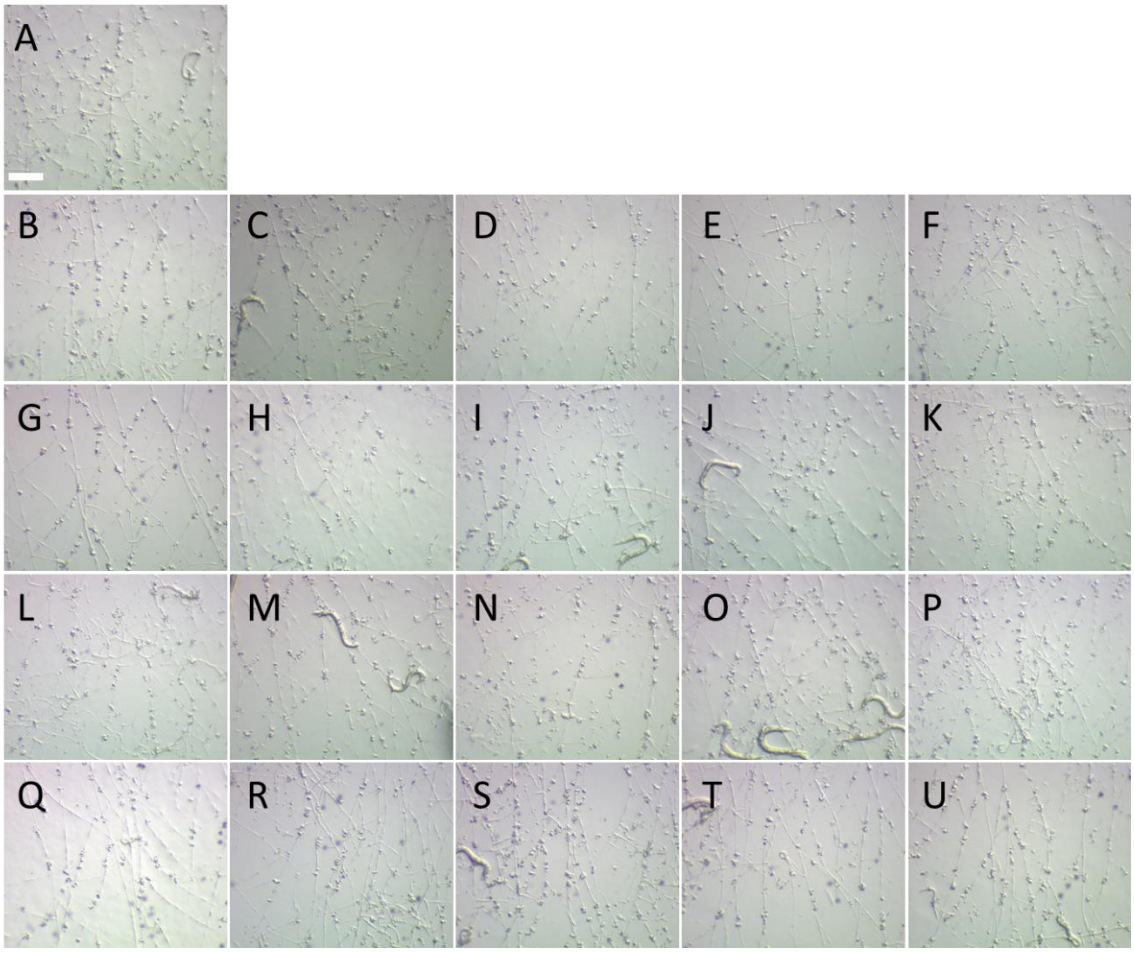

Fig 9. *C. elegans*-induced trap morphogenesis of 20 independent evolving lines in LNM and LNM supplied with nematodes. (A) Ancestral strain (TWF154); (B-K) 10 evolving lines in LNM; (L-U) 10 evolving lines in LNM supplied with nematodes. Bar = 1 mm.

doi:10.6342/NTU201902152

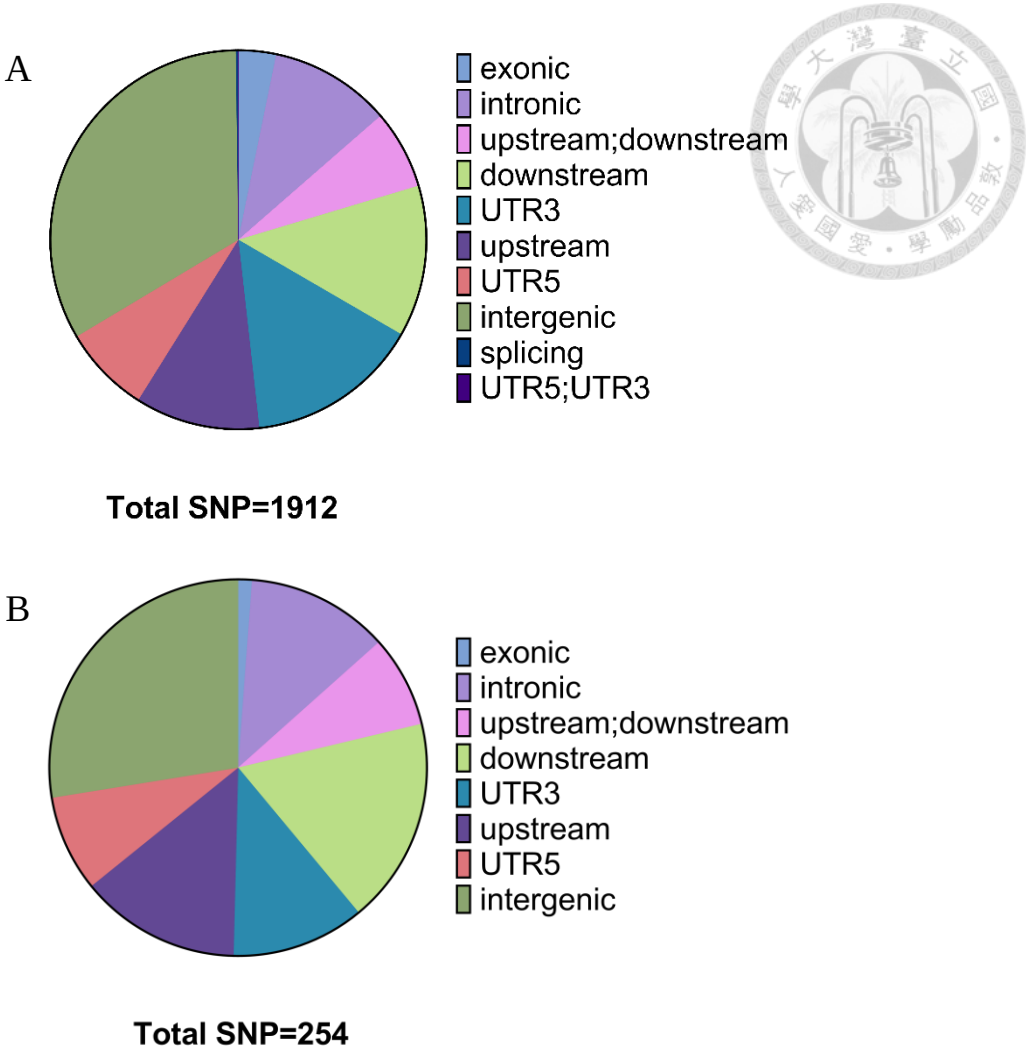

Fig 10. Remapping and filtering of genetic variants of e14(2). (A) Sixty-two, 199, 129, 247, 285, 205, 144, 637, 3, and 1 mutations were found on exon, intron, upstream and downstream, downstream, UTR3, upstream, UTR5, intergenic, splicing, and UTR5 and UTR3 with a total 1912 mutations; (B) Filtered out mutations coexist with another evolved line. Three, 31, 20, 45, 29, 35, 21, 70 mutations were found on exon, intron, upstream and downstream, downstream, UTR3, upstream, UTR5, and intergenic.

doi:10.6342/NTU201902152

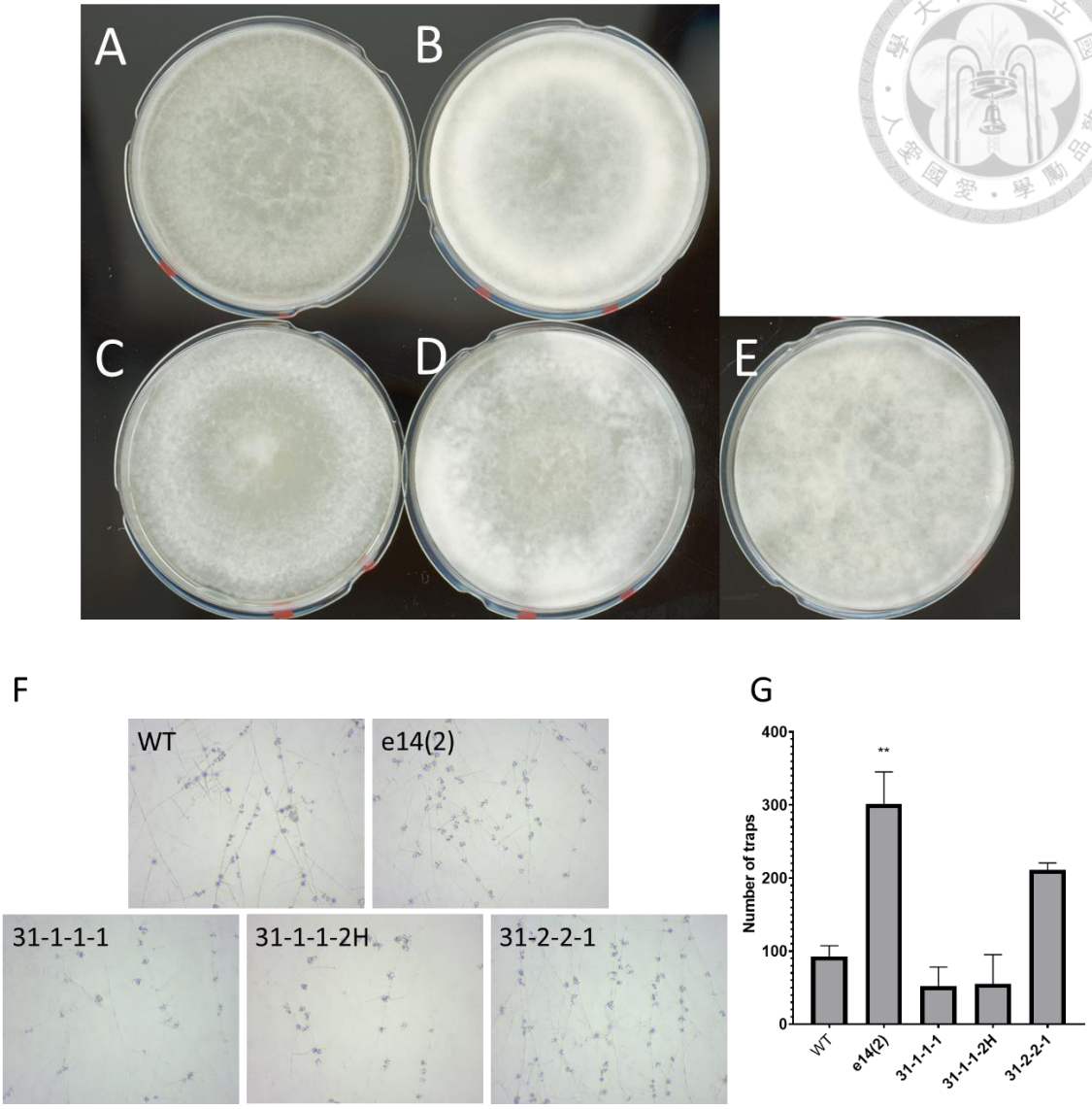

Fig 11. Mutation on EYR41_009099 in e14(2) govern aerial hyphae over-production phenotype. (A-E) Hyphal growth phenotype on PDA of (A) WT, (B) e14(2), (C, D) mutated EYR41_009099 knock-in strain 33-1-1-1 and 33-1-1-2H, and (E) overexpression of EYR41_009099 strain 31-2-2-1; (F) trap number quantification induced by *C. elegans*; (G) Quantification of traps number.

doi:10.6342/NTU201902152