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GTP Binding Protein Gtr1 Cooperating with ASF1 Regulates Asexual Development in Stemphylium eturmiunum

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Abstract: The Gtr1 protein was a member of the RagA subfamily of the Ras-like small GTPase superfamily and involved in phosphate acquisition, ribosome biogenesis and epigenetic control of gene expression in yeast. However, Gtr1 regulation sexual or asexual development in filamentous fungi is barely accepted. In the study, SeGtr1, identified from Stemphylium eturmiunum, could manipulate mycelial growth, nuclear distribution of mycelium and the morphology of conidia in SeGtr1 silenced strains compared with its overexpression transformants, while the sexual activity of SeGtr1 silenced strains were unchanged. SeASF1, a H3/H4 chaperone, participated in nucleosome assembly/disassembly, DNA replication and transcriptional regulation. Our experiments showed that deletion SeAsf1 mutants produced the hyphal fusion and abnormal conidia. Notably, we characterized that SeGtr1 was down-regulated in SeΔ asf1 mutants and SeAsf1 was also down-regulated in SiSeGtr1 strains. We further confirmed that SeGtr1 interacted with SeASF1 or SeH4 in vivo and vitro, respectively. Thus, SeGtr1 can cooperate with SeASF1 to modulate asexual development in Stemphylium eturmiunum.

Keywords: GTP binding protein Gtr1; interaction; ASF1; asexual development; Stemphylium eturmiunum

1. Introduction

The fungal hyphae are in a vegetative growth state under normal conditions. The hyphae will form various propagules and then enter the reproduction stage under a certain period. The propagation of filamentous ascomycetes mainly includes asexual reproduction (formation of asexual spores) and sexual reproduction. The main method of asexual reproduction of filamentous fungi is to produce multicellular structures called conidiophores, each bearing asexual spores called conidia [1]. Asexual spores are generated when the surroundings are favorable for growth and development. However, when nutrient availability become scarce, the sexual reproduction occurs and fruit bodies are produced to resist adverse environment [2,3]. Although it is more complicated, it has been found that the evidence of sexual reproduction is confirmed in some eukaryotic groups [4].

Previous studies have shown that fungi are a group historically considered to present a high proportion of asexual species [5]. Stemphylium, a genus of ascomycetes in Pleosporales (Dothideomycetes), is known to reproduce asexually [6]. Stemphylium is closely related to Alternaria and Ulocladium [7,8]. The conidia of Stemphylium forming on proliferating conidiophores in which produced apically swollen conidiogenous cells, which is principal distinguished it from two closely related genera [6,9]. In addition, the evolution, differentiation and asexual development of filamentous fungi are regulated by several genes, such as velvet family [10,11] and phytocromes [12]. However, another example of the GTP binding proteins with a role in biological development is the Gtr1. The Gtr1 protein is a member of the RagA subfamily of the Ras-like small GTPase superfamily [13]. Moreover,
the Gtr1, a multifunctional GTP-binding protein, is involved in phosphate acquisition [14], ribosome biogenesis [13] and epigenetic control of gene expression [15] in *Saccharomyces cerevisiae*. Notably, Gtr1 may be involved in stamen development via active GA supply in *Arabidopsis* [16]. However, Gtr1 regulation sexual or asexual development in filamentous fungi is barely accepted.

In recent years, several genes with potential functions in chromatin modification were found to be involved in asexual and sexual development [17–19]. One such factor is the histone chaperone ASF1 that was first identified in *Saccharomyces cerevisiae* [20]. ASF1, a H3-H4 chaperone, is highly conserved from yeast to mammals and involved in nucleosome assembly/disassembly [21–23], DNA replication, repair and transcriptional regulation [24]. Interestingly, in our experiments, deletion *Seasf1* mutants produced the hyphal fusion and abnormal conidia. To further investigate asexual development in *S. eturmiunum*, we hypothesized that SeGtr1 could be involved in SeASF1 regulation asexual development. Here, to address the hypothesis, we analyzed RNA transcript levels of the SeΔasf1 mutant and wild type strain at various stages, and found that SeGtr1 interacted with SeASF1 and SeH4 in *S. eturmiunum*. We confirmed that SeGtr1 could cooperate SeASF1 involved in central regulatory pathway to modulate asexual development.

2. Results

2.1. *SeASF1* Regulates Asexual Development in *S. eturmiunum*

To understand the biological functions of the SeASF1 during asexual development of *S. eturmiunum*, we obtained two *Seasf1* mutants and two complemented transformants. To determine the role of *Seasf1* in hyphal and colonial growth, these four mutants and WT strains were inoculated on CM medium and then photographed after 9 days. Colony growth rates of the two *Seasf1* mutants were distinguishable from the WT while two complemented transformants returned to normal growth (Figure 1A). Additionally, two *SeΔasf1* mutants produced the hyphal fusion and abnormal conidia compared to the complemented transformants and WT strains (Figure 1B, D). These results suggest that SeASF1 is involved in asexual development of *S. eturmiunum*.

![Figure 1](image-url)  
*Figure 1. Seasf1 regulates asexual developmental characterization in *S. eturmiunum*. (A) Growth of two *Seasf1* mutants, two *SeΔasf1::Seasf1* transformants, and WT strains on CM medium. The cultures were photographed after 9 days of incubation. (B) Characterizations of hyphal fusion in two *SeΔasf1* mutants, two *SeΔasf1::Seasf1* transformants, and WT strains. The images were photographed after growing on PDA medium for 8 days. The fusions in the hyphae were marked with red arrows. (C, D) Conidia morphology of four mutants and WT strains were cultured on CM medium for 4 weeks. Bar = 20 \(\mu m\), 500 \(\mu m\).*
2.2. SeGtr1 Plays a Role in Asexual Development

Gtr1 (SeGtr1) was cloned from *S. eturmiunum*. SeGtr1 contains 335 amino acids with a calculated molecular mass of 37 kDa. To verify the roles of Segtr1 during the growth and development of *S. eturmiunum*, we obtained two Segtr1-silenced transformants (SiSegtr1-T63 and SiSegtr1-T65) and two overexpression transformants (OESegtr1-T3 and OESegtr1-T8) by *A. tumefaciens* mediated method (Figure 2D). Control was a negative control strain. As a result, two silenced transformants appeared the slow growth rate of colonies related to overexpression transformants or control strains (Figure 2A,C). In addition, the nuclei were anomalously distributed in mycelia of SiSegtr1 strains (Figure 2B). To further observe the roles of Segtr1 during the asexual and sexual development of *S. eturmiunum*, all transformants and WT strains were in the dark condition at 25 °C for 5 weeks on CM medium by inserting double slides. For two silenced strains, the conidiophores turned into bead-like and the conidia grew subglobose, which were significantly different from overexpression transformants and WT strains (Figure 3A). Furthermore, the expression levels of genes involved in the central regulatory pathway, including the *brl*, *aba*, and *wet*, were significantly down-regulated in the two SeΔasf1 mutants and two Segtr1-silenced transformants (Supplementary Figure S1). However, two silenced strains still produced perithecia that were unanimous with overexpression transformants and WT strains (Figure 3B). These results indicate that SeGtr1 can affect the expression of related genes (*brl*, *aba* and *wet*) in the central regulatory pathway and modulate asexual development.

2.3. The Expression Patterns of Seasf1, SeH4, and Segtr1 in Knockout Mutants, Silenced Lines or Overexpression Strains

Due to SeGtr1 can also control the asexual development in *S. eturmiunum*, we verify whether can occur the relation among SeASF1, SeH4 and SeGtr1. Two SeH4-silenced strains were SiSeH4-T8 and SiSeH4-T20. The transcript levels of Segtr1 and SeH4 were detected in two SeΔasf1 mutants and two complemented transformants. The expressions of Segtr1 and Seasf1 were measured in two SiSegtr1 lines and two OESegtr1 strains. As a result, Segtr1 and SeH4 showed down-regulation and up-regulation in two SeΔasf1 mutants, while Segtr1 was up-regulation in SeΔasf1::Seasf1 strains, respectively (Figure 4A). At the same time, Seasf1 displayed up-regulation in two SiSeH4 lines, but Segtr1 did not change (Figure 4B). Furthermore, Seasf1 and SeH4 showed down-regulation and up-regulation in two SiSegtr1 lines, respectively. However, Seasf1 showed up-regulation in two OESegtr1 strains (Figure 4C). Summary, SeGtr1 could interact with SeASF1 or SeH4.

2.4. SeASF1 Interaction with SeH4, and SeGtr1 Interaction with SeASF1 and SeH4

To test whether can occur the interaction between SeASF1 and SeGtr1, SeH4 and SeGtr1. Y2H revealed that SeASF1 interacted with SeH4, and SeGtr1 interacted with SeASF1 and SeH4 (Figure 5A). On the basis of GST pull-down, SeASF1 was specifically interacted with SeH4 (Figure 5B), while SeGtr1 could interact with SeASF1 and SeH4, respectively (Figure 5C). To further assure those results of the Y2H and pull-down experiments, SeASF1-GFP and SeH4-Flag, SeGtr1-Flag and SeASF1-GFP, and SeGtr1-Flag and SeH4-GFP were expressed in *F. graminearum* protoplasts, respectively, and then the immune complexes were estimated using Co-IP assays (Figure 5D,E). Thus, SeASF1 interacted with SeH4, and SeGtr1 interacted with SeASF1 and SeH4. Altogether, SeGtr1 could cooperate with SeASF1 and SeH4 to modulate asexual development of *S. eturmiunum*. 
Figure 2. The colonial phenotypes and nuclear distribution of Segtr1 silenced transformants. (A) Colonial growth of two Segtr1 silenced transformants (SiSegtr1-T63 and SiSegtr1-T66) and two Segtr1 overexpression transformants (OESegtr1-T3 and OESegtr1-T8) was observed on PDA medium. WT was S. eturmiunum strain and Control was a negative control strain. The cultures were photographed after 1 day, 3 days, 5 days, 7 days and 9 days. (B) The mycelium of these four transformants was grown on PDA medium for 6 days and examined by DIC and fluorescence microscopy. The nuclei of the mycelia were discovered under the fluorescence microscopy after staining by DAPI. Bar = 20 μm. (C) Colony diameters were measured in each independent biological replication.
were grown on PDA medium for 6 days and examined by DIC and fluorescence microscopy. The nuclei of the mycelia were discovered under the fluorescence microscopy after staining by DAPI. Bar = 20 µm. (C) Colony diameters were measured in each independent biological experiment at 1–9 days of growth on PDA medium. Rates of colonial growth were calculated for each treatment. (D) qRT-PCR was used to measure the expression levels of \( \text{segtr1} \) in silenced transformants, overexpression transformants, Control and WT. The degree of WT was assigned to value 1.0. \( \text{Actin} \) gene of \( S. eturmiunum \) was used as endogenous control. The bars indicated statistically significant differences (ANOVA; ** \( p < 0.01 \)). Each experiment was repeated at least three times.

**Figure 3.** \( \text{SeGtr1} \) plays a role in asexual development, but the sexual activity was unchanged. (A) For the microscopic investigation of conidiophores and conidia development, two silenced transformants (\( \text{SiSegtr1-T63} \) and \( \text{SiSegtr1-T66} \)), two overexpression transformants (\( \text{OESegtr1-T3} \) and \( \text{OESegtr1-T8} \)), Control and WT strains were grown on CM medium for 35 days, respectively. Control was a negative control strain. Red arrowheads indicated abnormal conidiophores. (B) To further observe the role of \( \text{Segtr1} \) during the sexual development of \( S. eturmiunum \), all transformants and WT strains were cultured on PDA medium for inducing perithecia production. At 35 days, all transformant strains produced abundant perithecia. Bar = 20 µm and 500 µm.

**Figure 4.** The expression patterns of \( \text{Seasf1}, \text{SeH4}, \) and \( \text{Segtr1} \) in knockout mutants, silenced lines or overexpression strains. (A) The expression levels of \( \text{Segtr1} \) and \( \text{SeH4} \) in two \( \text{Seasf1} \) mutants and two
complemented transformants were measured by qRT-PCR. (B) The expression levels of \textit{Segtr1} and \textit{Seasf1} in two \textit{SiSeH4} lines were measured by qRT-PCR. (C) The expression levels of \textit{Seasf1} and \textit{SeH4} in two \textit{SiSegtr1} lines and two \textit{OE Segtr1} lines were measured by qRT-PCR. The degree of WT was assigned to value 1.0. Two \textit{Seasf1} deleted mutants were \textit{Seasf1-0} and \textit{Seasf1-5}, two complemented transformants were \textit{Seasf1::Segtr1-1} and \textit{Seasf1::Segtr1-2}. Two \textit{SeH4} silenced lines were \textit{SiSeH4-T8} and \textit{SiSeH4-T20}. Two \textit{Segtr1} silenced lines were \textit{SiSegtr1-T63} and \textit{SiSegtr1-T66}, two \textit{OE Segtr1} lines were \textit{OE Segtr1-T3} and \textit{OE Segtr1-T8}. The \textit{Actin} in \textit{S. etarumium} was used as endogenous control. The bars indicated statistically significant differences (ANOVA; ** \( p < 0.01 \)).

**Figure 5.** \textit{SeASF1} interaction with \textit{SeH4}, and \textit{SeGtr1} interaction with \textit{SeASF1} or \textit{SeH4}. (A) \textit{SeASF1} interacted with \textit{SeH4}, and \textit{SeGtr1} interaction with \textit{SeASF1} or \textit{SeH4} using Y2H. \textit{SeASF1} or \textit{SeH4} was cloned into plasmid pGBKT7 (BD). \textit{SeH4} or \textit{SeGtr1} was cloned into plasmid pGADT7 (AD). Yeast transformants were first grown on SD/-Trp/-Leu, and selected on SD/-Trp/-Leu/-His/-Ade/X-\( \alpha \)-gal. A positive interaction results in the activation of the \textit{lacZ} reporter, which turned the blue in the presence of X-\( \alpha \)-galactosidase. The images were photographed at 3 days after incubation. (B) \textit{SeASF1} was cloned into plasmid pGEX-6P-1. \textit{SeH4} was cloned into plasmid pET28a. \textit{SeASF1-GST} was expressed in \textit{E. coli} and incubated with \textit{SeH4-His}, purified (pull-down) by glutathione sepharose beads. Recombinant GST was control. \textit{SeH4-His} was pulled down by \textit{SeASF1-GST}. (C) \textit{SeGtr1} was cloned into plasmid pGEX-6P-1. Flag-\textit{SeASF1} or \textit{SeH4} was cloned into plasmid pET28a. \textit{SeH4-His} and Flag-\textit{SeASF1-His} were both retained by \textit{SeGtr1-GST}. (D) \textit{SeASF1} was cloned into plasmid pDL2, \textit{SeH4} was cloned into plasmid pFL7. Total proteins were extracted from \textit{F. graminearum} protoplasts expressing \textit{SeASF1-GFP} and \textit{SeH4-Flag}. Recombinant GFP or Flag was control. The immune complexes were immunoprecipitated with \( \alpha \)-Flag antibody (\( \alpha \)-Flag IP). Co-precipitation of \textit{SeH4-Flag} was detected by immunoblotting. (E) \textit{SeH4} was cloned into plasmid pDL2. \textit{SeGtr1} was cloned into plasmid pFL7. Total proteins were extracted from \textit{F. graminearum} protoplasts expressing \textit{SeASF1-GFP}, \textit{SeH4-GFP}, and \textit{SeGtr1-Flag}. Coprecipitation of \textit{SeGtr1-Flag} was detected by immunoblotting. Membranes were stained with Ponceau S to confirm equal loading. Protein sizes are indicated in kDa. Each experiment was repeated at least three times.
and incubated with SeH4-His, purified (pull-down) by glutathione sepharose beads. Recombinant GST was control. SeH4-His was pulled down by SeASF1-GST. (C) Segtr1 was cloned into plasmid pGEX-6P-1. Flag-SeASF1 or SeH4 was cloned into plasmid pET28a. SeH4-His and Flag-SeASF1-His were both retained by SeGtr1-GST. (D) SeASF1 was cloned into plasmid pDL2, SeH4 was cloned into plasmid pFL7. Total proteins were extracted from F. graminearum protoplasts expressing SeASF1-GFP and SeH4-Flag. Recombinant GFP or Flag was control. The immune complexes were immunoprecipitated with α-Flag antibody (α-Flag IP). Coprecipitation of SeH4-Flag was detected by immunoblotting. (E) SeH4 was cloned into plasmid pDL2. Segtr1 was cloned into plasmid pFL7. Total proteins were extracted from F. graminearum protoplasts expressing SeASF1-GFP, SeH4-GFP, and Segtr1-Flag. Coprecipitation of Segtr1-Flag was detected by immunoblotting. Membranes were stained with Ponceau S to confirm equal loading. Protein sizes are indicated in kDa. Each experiment was repeated at least three times.

3. Discussion

Stemphylium was a dematiaceous hyphomycete that was established with S. botryosum as type species [25]. Until now, there were more than 150 Stemphylium species had been described [26–29]. S. eturmiunum, a typical species of Stemphylium genus, was an important homothallic filamentous fungus, and it could produce both conidia and perithecia. In a previous study, ASF1 could manipulate the sexual reproduction in Sordaria macrospora effectively [30]. However, in this study, deletion of Seasf1 carried out asexual development characters, such as hyphal fusion and abnormal conidia. A possible explanation for the SeASF1 in asexual development was functional redundancy because some genes were down-regulated or up-regulated expression when Seasf1 was deleted. Therefore, we hypothesized that other proteins might be involved in SeASF1 regulation asexual development.

In summary, through the comparative analysis of transcriptome data (Supplementary Table S1), we characterized that the expression of Segtr1 (No. TR23646-c0_g1) and Seasf1 (No. KX033515) was down-regulated in SeΔasf1 and SiSegtr1 strains, respectively. Meanwhile, we found that SeASF1 or Segtr1 participated in the central regulatory pathway and regulated the expression of related genes, such as brl, aba and wet. In addition, we verified that Segtr1 could effectively stimulate asexual activity of S. eturmiunum in Segtr1 silenced strains compared with its overexpression strains. A model of the process is shown in Figure 6. SeASF1 coupled to SeH4 is translocated into the nucleus. In previous studies, ASF1 was reported to regulate DNA replication and damage repair in nucleosome. In this study, we found that Segtr1 interacted with SeASF1 or SeH4 in vivo and vitro, and Segtr1 cooperated with SeASF1, which is involved in the central regulatory pathway, to regulate asexual development in S. eturmiunum.

![Figure 6](image)

**Figure 6.** A model for ASF1 binding H4 (ASF1-H4) to interact with GTR1 and then to mediate asexual reproduction in *Stemphylium eturmiunum*. ASF1 interacts with H4 and then they are translocated into
nuclei through the nuclear pore. The dimer of ASF1-H4 regulates DNA replication and damage repair in previous studies. Herein, ASF1-H4 combines with GTR1 to constitute a trimeric complex which in turn is involved in the central regulatory pathway to modulate asexual reproduction.

4. Materials and Methods

4.1. Strains and Culture Conditions

Stemphylium eturmiunum strain (EGS 29-099) (WT) and all transformants strains were cultured in the dark condition at 25 °C on complete medium (CM), or potato dextrose agar (PDA) medium for mycelial growth assays. Escherichia coli DH5α or Agrobacterium tumefaciens AGL-1 was incubated in LB (Luria-Bertani) medium at 37 °C or 28 °C, respectively [31].

4.2. Plasmid Construction

Deletion strains for Seasf1 was generated by homologous recombination. The Seasf1 flanking regions, 1500 bp upstream and 1500 bp downstream, were amplified using primer pairs (Table 1). The resulting PCR products were ligated to the Hygromycin cassette and then transformed into WT. Transformants were screened by PCR with primers (Table 1). In addition, Seasf1 was cloned into eGPF-pHDT vector for complementation analysis (Table 2). The recombinant plasmid eGFP-pHDT-Seasf1 was transformed into the ScΔasf1 mutants by A. tumefaciens mediated transformation (ATMT) method [32]. Transformants were screened by PCR and western blot.

Table 1. Primers used in this study.

| Primer          | Sequence(5’-3’)                          | Application                        |
|-----------------|------------------------------------------|------------------------------------|
| Seasf1-F        | ATGTCTGTGTTCGCTTC                        | Amplify Seasf1 sequence            |
| Seasf1-R        | CTAGTGAACCATGACATCTGC                    |                                     |
| Seasf1-5f       | CGCGTCGAGCAATCCAGGGGCCGATAAAG            | Amplify Seasf1 upstream sequence   |
| Seasf1-5r       | GGAAGATCTGCTGAGGGGTAGATAGAG             | Amplify Seasf1 downstream sequence |
| Seasf1-3f       | CGCGGATCCCGCCGCTCTGTTAGTCTTT            | For identification of Seasf1 deletion strains |
| Seasf1-3r       | CTAGTCTAGACAGAGAGATGCTTGCTTGTGC         | For identification of Hph in deletion transformants |
| Seasf1-f        | TCTGTCTGTTGCCGCTTC                     |                                     |
| Seasf1-r        | TGAACCATGACATGCGCC                      |                                     |
| Seasf1-3D       | CCTCGTCGTCTCTCGATACCT                      |                                     |
| Hph-3D          | GAGATTCTTCGCCCTCCGAG                     |                                     |
| Hph-5D          | AATTTCGATGACAGCTTGGG                     |                                     |
| Seasf1-5D       | CAAGACTCCGCTTCCTCTCATCGT                |                                     |
| Hph-F           | CGACAGCGTCTCCGACCTGA                    | For identification of Hph           |
| Hph-R           | CCGCCAAGCTGACATCGAA                      | For identification of Seasf1        |
| Seasf1-PHDT-F   | CGCGTCGAGATGCTGTTGCCGCTTCTC             | Seasf1 complementation expression  |
| Seasf1-PHDT-R   | GCTCTGACTAGGAACCATGACATCTGC             |                                     |
| PHDT-F          | GATCATGCTTCTGCTG                        | Vector construction of sequencing primer |
| PHDT-R          | CACCAACGATCTTATATCCAG                   |                                     |
| Segtr1-pCIT-F(BamHI/ClaI) | CGCGGATCCATGAGATGCTGACCTGTTGATGAGGC | Primer for Segtr1 silence          |
| Segtr1-pCIT-R(PstI/EcoRV) | AACTGCAGGATATGCACACGCCACCGCAGCCATGTTACATCC | For identification of Seasf1        |
| Seasf1-QRT-F    | ACAACGAGTACACAGATGAGG                    | QRT-PCR for Seasf1                  |
| Seasf1-QRT-R    | TCGTCAAGTCACATGATA                     |                                     |
| SeH4-QRT-F      | AGCCATGACAGCGAAAGAAGA                    | QRT-PCR for SeH4                    |
| SeH4-QRT-R      | CGAGAGAGATGACGTCGCTTCTCAC               |                                     |
| Primer                        | Sequence (5′-3′)                                                                 | Application                                      |
|------------------------------|---------------------------------------------------------------------------------|--------------------------------------------------|
| Segtr1-QRT-F                 | AAGCTAGCGAGGGTTTCAAG                                                            | QRT-PCR for Segtr1                               |
| Segtr1-QRT-R                 | GCCGTTCGATATCGGCGGGTATG                                                         | QRT-PCR for Segtr1                               |
| Seactin-F                    | GTGCAATGGGAAGGAGGAGGAGT                                                         | QRT-PCR for Seactin                              |
| Seactin-R                    | GTCTTCCTTGGCAGGCCCAGA                                                          | QRT-PCR for Seactin                              |
| Segtr1-QRT-F                 | AAGCTAGCGAGGGTTTCAAG                                                            | QRT-PCR for Segtr1                               |
| Segtr1-QRT-R                 | GCCGTTCGATATCGGCGGGTATG                                                         | QRT-PCR for Segtr1                               |
| Seactin-F                    | GTGCAATGGGAAGGAGGAGGAGT                                                            | QRT-PCR for Seactin                              |
| Seactin-R                    | GTCTTCCTTGGCAGGCCCAGA                                                          | QRT-PCR for Seactin                              |
| Seasf1-BD-F (NdeI)           | GGAATTCATTGATATCGGCGGGTATG                                                       | Seasf1 recombined into pGBK7                    |
| Seasf1-BD-R (BamHI)          | CGCGGATCTCTTGGTATGAGGAGGAGGAGT                                                 | Seasf1 recombined into pGBK7                    |
| SeH4-BD-F (SmaI)             | TCCCCCGGGGATACGGCTTCCTGTTGTTGCGGTATG                                         | SeH4 recombined into pGBK7                      |
| SeH4-BD-R (BamHI)            | CGCGGATCTCTTGGTATGAGGAGGAGGAGT                                                 | SeH4 recombined into pGBK7                      |
| SeH4-AD-F (SmaI)             | TCCCCCGGGGATACGGCTTCCTGTTGTTGCGGTATG                                         | SeH4 recombined into pGBK7                      |
| SeH4-AD-R (BamHI)            | CGCGGATCTCTTGGTATGAGGAGGAGGAGT                                                 | SeH4 recombined into pGBK7                      |
| Seasf1-pET28a-Flag-F (NdeI)  | GGAATTCATTGATATCGGCGGGTATG                                                       | Seasf1 recombined into pET28a                   |
| Seasf1-pET28a-Flag-R (HindIII)| CCCAAGCTTCTGATGAGGAGGAGGAGT                                                   | Seasf1 recombined into pET28a                   |
| Seasf1-pGEX-F (BamHI)        | CGCGGATCTCTTGGTATGAGGAGGAGGAGT                                                 | Seasf1 recombined into pEX                      |
| Seasf1-pGEX-R (NotI)         | ATGACAAAAATTTCGCGGGTATGAGGAGGAGT                                               | Seasf1 recombined into pEX                      |
| Segtr1-pGEX-F (NdeI)         | GGAATTCATTGATATCGGCGGGTATG                                                       | Segtr1 recombined into pGEX                     |
| Segtr1-pGEX-R (NotI)         | ATGACAAAAATTTCGCGGGTATGAGGAGGAGT                                               | Segtr1 recombined into pGEX                     |
| Seasf1-pDL2-GFP-F            | CAGATCTTGGCGTCTGAGGAGGAGGAGGAGT                                               | SeaH4 recombined into pDL2                      |
| Seasf1-pDL2-GFP-R            | CACCCGGGATGTCGCGGGGGAAGGAGGAGGAGT                                              | SeaH4 recombined into pDL2                      |
| Seasf1-pDL2-GFP-F            | CAGATCTTGGCGTCTGAGGAGGAGGAGGAGT                                               | SeaH4 recombined into pDL2                      |
| Seasf1-pDL2-GFP-R            | CACCCGGGATGTCGCGGGGGAAGGAGGAGGAGT                                              | SeaH4 recombined into pDL2                      |
| Seasf1-pFL7-Flag-F           | CAGATCTTGGCGTCTGAGGAGGAGGAGGAGT                                               | SeaH4 recombined into pFL7                      |
| Seasf1-pFL7-Flag-R           | CAGATCTTGGCGTCTGAGGAGGAGGAGGAGT                                               | SeaH4 recombined into pFL7                      |
| Segtr1-pFL7-Flag-F           | CAGATCTTGGCGTCTGAGGAGGAGGAGGAGGAGT                                              | Segtr1 recombined into pFL7                     |
| Segtr1-pFL7-Flag-R           | CAGATCTTGGCGTCTGAGGAGGAGGAGGAGGAGT                                              | Segtr1 recombined into pFL7                     |
| Segtr1-PHDT-F (SmaI)         | CCTACGTA TGAGGAGGAGGAGGAGGAGGAGT                                               | Primer for Segtr1 overexpression                |
| Segtr1-PHDT-R (XbaI)         | GGCTAGACATTCTACGGCGGGGAGGAGGAGGAGT                                              | Primer for Segtr1 overexpression                |

RNA interference [33] was used for Segtr1 silencing. The 525 bp cDNA fragments of Segtr1 was amplified from S. eturnium with primers (Table 1) and inserted into vector pCIT that flanked to the intron to form silencing construct, respectively [33] (Table 2). The constructed plasmid pCH-Segtr1 was transformed into S. eturnium strain by A. tumefaciens mediated transformation (ATMT) method [32].
### Table 2. Plasmids used in this study.

| Name               | Origin of Target Genes | Construction Path                  | Purpose                                      |
|--------------------|------------------------|------------------------------------|----------------------------------------------|
| Seasf1-pXEH        | *S. eturmiunum*        | Seasf1 recombined into pXEH        | Knockout asf1                                |
| Seasf1-pHDT        | *S. eturmiunum*        | Seasf1 recombined into pHDT        | asf1 complementation expression             |
| Segtr1-pCIT        | *S. eturmiunum*        | Segtr1 recombined into pCIT        | Segtr1 silence                              |
| Seasf1-pGBKT7      | *S. eturmiunum*        | Seasf1 recombined into pGBKT7      | Yeast two-hybrid assays                      |
| SeH4-pGBKT7        | *S. eturmiunum*        | SeH4 recombined into pGBKT7        | Yeast two-hybrid assays                      |
| Segtr1-pGADT7      | *S. eturmiunum*        | Segtr1 recombined into pGADT7      | Yeast two-hybrid assays                      |
| SeH4-pGADT7        | *S. eturmiunum*        | SeH4 recombined into pGADT7        | Yeast two-hybrid assays                      |
| SeH3-pGADT7        | *S. eturmiunum*        | SeH3 recombined into pGADT7        | Yeast two-hybrid assays                      |
| Seasf1-pET28a      | *S. eturmiunum*        | Seasf1 recombined into pET28a      | Pull-down assays                             |
| Segtr1-pGEX-6P-1   | *S. eturmiunum*        | Segtr1 recombined into pGEX-6P-1   | Pull-down assays                             |
| Seasf1-pDL2        | *S. eturmiunum*        | Seasf1 recombined into pDL2        | CO-IP assays                                 |
| SeH4-pDL2          | *S. eturmiunum*        | SeH4 recombined into pDL2          | CO-IP assays                                 |
| Segtr1-pFL7        | *S. eturmiunum*        | Segtr1 recombined into pFL7        | CO-IP assays                                 |
| SeH4-pFL7          | *S. eturmiunum*        | SeH4 recombined into pFL7          | CO-IP assays                                 |
| Segtr1-pHDT        | *S. eturmiunum*        | Segtr1 recombined into pHDT        | Segtr1 over expression                      |

For overexpression analysis, *Segtr1* was cloned from *S. eturmiunum* with primers (Table 1), and then cloned into eGFP-pHDT vector. Subsequently, recombinant plasmid eGFP-pHDT-*Segtr1* was transformed into the Si*Segtr1* lines by ATMT method. Overexpression transformants were screened by qRT-PCR and western blot.

For co-immunoprecipitation (Co-IP) analysis, SeASF1, SeH4, and SeGtr1 were amplified from *S. eturmiunum* with primers (Table 1), and cloned into the pDL2 or pFL7 in yeast (KK125) by recombination approach [34] (Table 2). Recombinant plasmids were then co-transformed into the protoplasts of *Fusarium graminearum* wild-type strain (PH-1). Transformants were also screened by western blot.

### 4.3. RNA Extraction and qRT-PCR

Total RNA was extracted from mycelia of *S. eturmiunum* growing in PDB (Potato Dextrose Broth) cultures using the Fungal RNA Kit (OMEGA Biotechnology, USA). cDNA was generated using the HiScript II QRT SuperMix for qPCR (Vazyme, Nanjing, China). The qRT-PCR was carried out using the 2 × ChamQ SYBR Color qPCR Master Mix (Vazyme, China) and performed on an ABI QuantStudio™ 6 Quantitative Real-Time PCR System (Applied Biosystems). The specific primers of qRT-PCR listed in the Table 1. Changes in the relative expression level of each gene were calculated by the $2^{-\Delta\Delta CT}$ method [35]. Gene expression levels were normalized using the housekeeping gene actin. This experiment was repeated at least three times.

### 4.4. Yeast Two-Hybrid

To test whether SeASF1 and SeH4 interact with SeGtr1, Y2H assay was performed according to the Yeast Protocols Handbook (Clontech) using the Y2H Gold yeast reporter strain (Clontech). The *Seasf1* or *SeH4* was amplified and cloned into pGBKT7 (BD). The
Segtr1 or SeH4 was amplified and cloned into pGADT7 (AD) (Table 2). The primers used are listed in Table 1. Pairwise interaction was tested using AD and BD to transform the yeast strain Y2H gold. The yeast transformants were grown on SD/-Trp/-Leu mediums (TaKaRa Bio) for 3–5 days and then cultured on selection mediums (SD/-Trp/-Leu/-His/-Ade/X-α-gal) to detect the protein-protein interaction. Each experiment was repeated at least three times.

4.5. GST Pull-Down

The Seasf1 was cloned into the pET28a vector after adding a 1 × FLAG tag to the 5′-terminal of Seasf1 to make the Flag-SeASF1 fusion protein. The Segtr1 was cloned into the pGEX-6P-1 vector to make the GST-SeGtr1 fusion protein (Table 2). Flag-SeASF1-His, GST-SeGtr1, pET28a or pGEX-6P-1 was expressed in BL21 strain of E. coli, and were then affinity purified with a Ni-affinity column (GE) or GST-affinity column (glutathione sepharose™ 4B beads GE Healthcare, Little Chalfont, Buckinghamshire, UK). For glutathione S-transferase (GST) pull-down in vitro, GST-SeGtr1 and Flag-SeASF1-28a were expressed in E. coli strain BL21 (DE3). Total proteins of GST-SeGtr1 and Flag-SeASF1-His were then incubated with 4000 µL of glutathione sepharose™ 4B beads at 4 °C for 2 h. The supernatant was removed, and the beads was washed by GST-lysis buffer three times. Finally, the beads were eluted by GST-elution buffer. Pull-down of GST-SeGtr1 with Flag-SeASF1-His was detected using an anti-Flag (Invitrogen, Waltham, MA, USA). Each experiment was repeated at least three times.

4.6. Co-IP

Fusarium graminearum protoplasts were transfected with the indicated combination plasmids and empty construct. Proteins of F. graminearum were extracted in an extraction buffer (50 mM HEPES, 130 mM NaCl, 10% glycerin, pH 7.4) with 25 mM Glycerol phosphate, 1 mM Sodium orthovanada and protease inhibitor (100 mM PMSF). For FLAG IP, protein extracts were incubated with 30 µL of Anti-Flag® M2 Affinity Gel beads (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 4 h. The beads were washed five times with by Co-IP washing buffer (50 mM HEPES, 130 mM NaCl, 10% glycerin, pH 7.4). The bound proteins retained on the beads were separated by 12% SDS–PAGE gels and detected using immunoblotting with anti-FLAG (Sigma-Aldrich) or anti-GFP antibody (Invitrogen). Each experiment was repeated at least three times.

4.7. Western Blot

Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Immobilon®-P PVDF membrane for 1.5 h at 230 mA. The PVDF membranes were blocked with TBST (0.02 M Tris-base, 0.14 M NaCl, 0.1% Tween-20, pH 7.4) with 5% non-fat milk for 1 h at room temperature. Co-immunoprecipitated proteins were analyzed by incubating the membranes with 1:5000 diluted GFP or FLAG antibodies (Sigma-Aldrich) at room temperature for 1–1.5 h. The membranes were washed three times with TBST and then were incubated for 1 h with Goat anti-Mouse-HRP secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA, no. 31430) at 1:7500 dilution. The specific proteins were visualized by the ECL Chemiluminescence Detection Kit (Vazyme) and imaged using a Tanon-5200 System. Each experiment was repeated at least three times.

4.8. Microscopy

To observe the morphology of conidia and conidiophores, all the transformants and WT strains were grown in the dark condition at 25 °C for 4 weeks on PDA medium by inserting double slides. Microscopic examination of nuclear distribution in mycelia, the transformants and WT strains were stained using 4,6-diamidino-2-phenylindole (DAPI). To image the sexual structures including perithecia and asci, all these test strains were cultured on CM medium at 25 °C for 6 weeks in dark condition. Perithecia were sectioned by using a double-edged blade in a dissecting microscope (Olympus, SZX10). The asci,
Conidia and conidiophores were all captured with 20× or 40× objectives of Olympus microscope (Olympus BX53, Tokyo, Japan) using differential interference contrast (DIC) and fluorescence illumination. Microscopic characters of asexual structures were further determined by measurements of 50 mature conidia and 50 conidiophores. The experiment was repeated at least three times.

4.9. Statistical Analysis

All determinations were carried out in triplicate and the results are expressed as mean ± standard deviation (SD). The data were subjected to one-way analysis of variance (ANOVA). Statistically significant differences were determined by two-tailed Student’s t test: *p < 0.05, **p < 0.01.

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