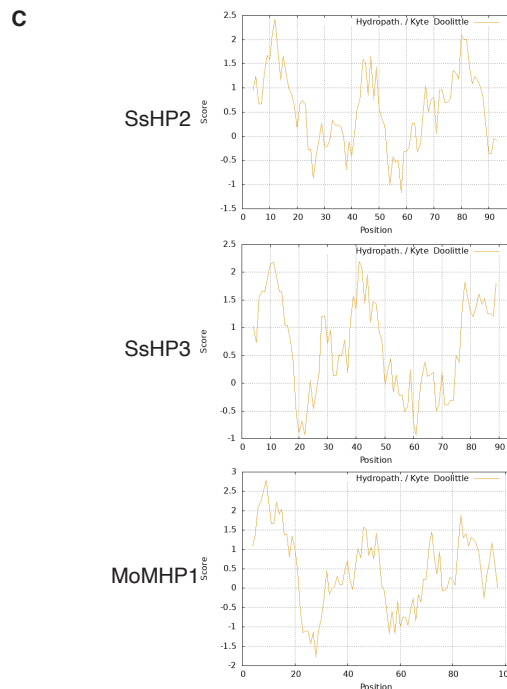
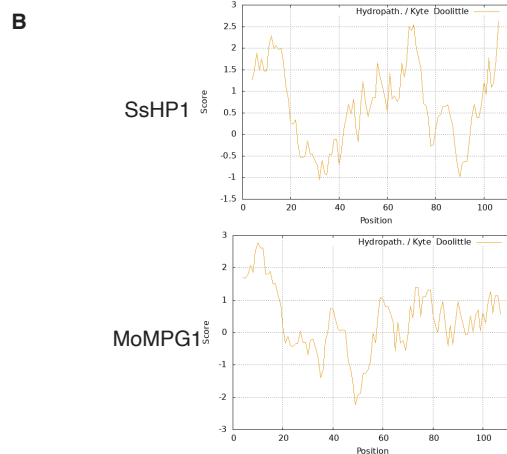
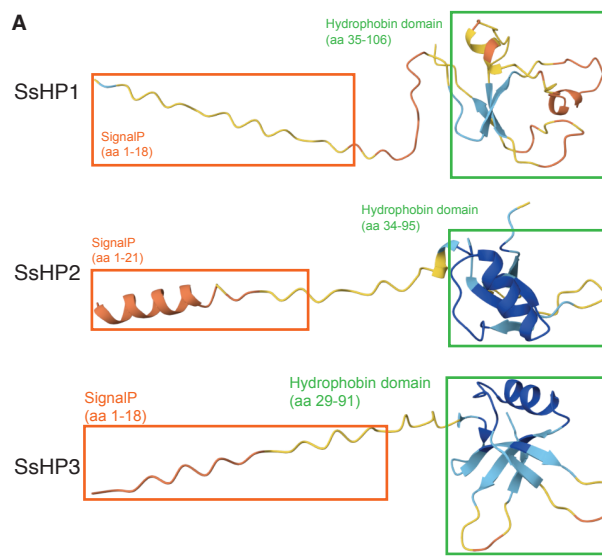


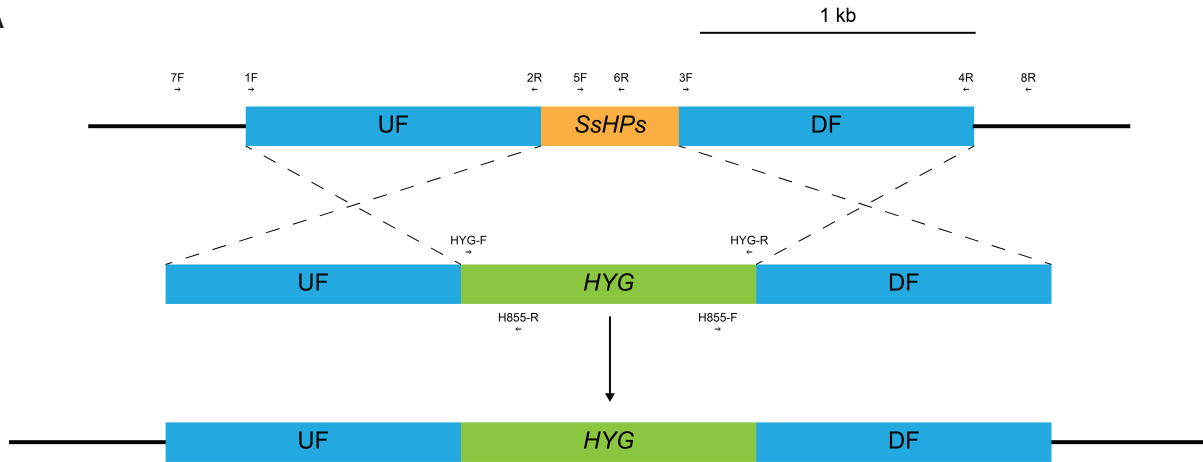
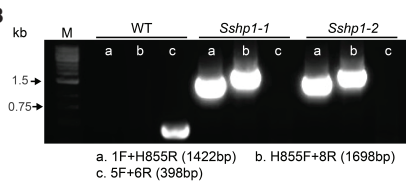
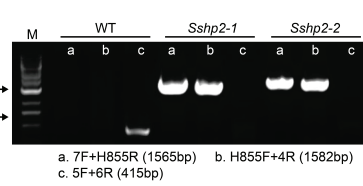
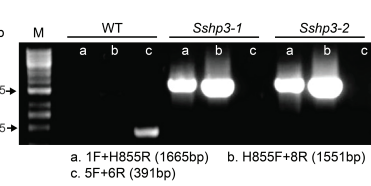
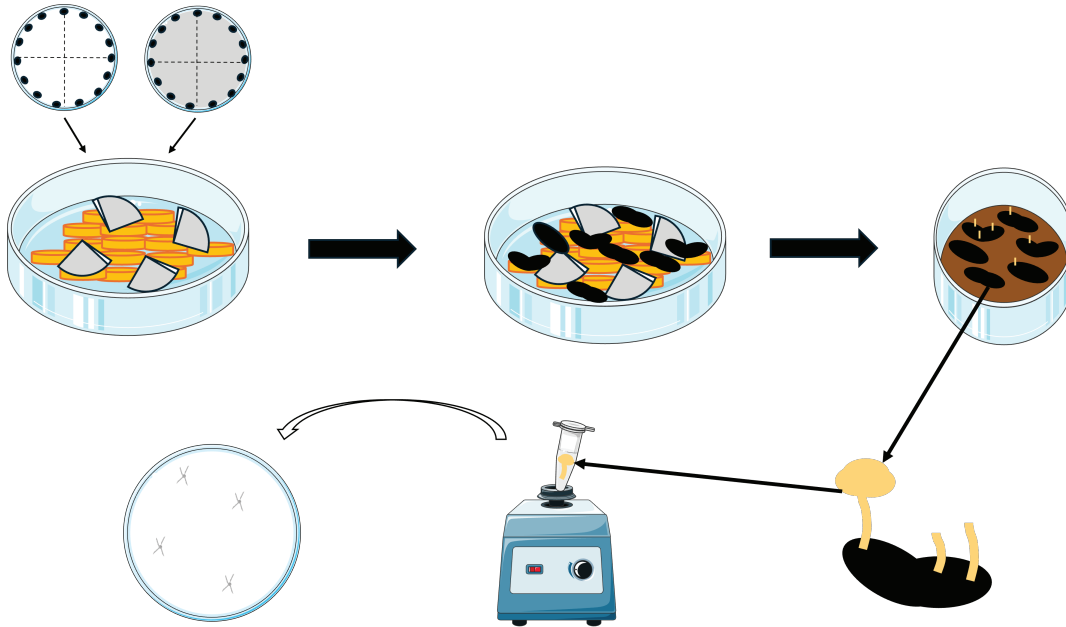
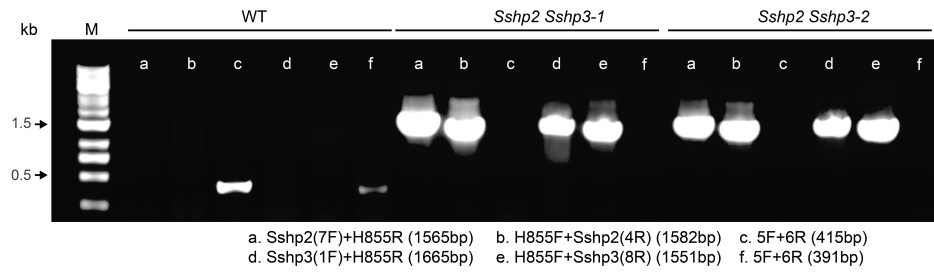


Supplemental Figure S1. SsHP1 is class I HP, while SsHP2 and SsHP3 belong to class II.

A. Protein structures of SsHP1, SsHP2, and SsHP3 as predicted by AlphaFold3. SignalP region and HP domain are highlighted in orange and green boxes respectively with amino acid (aa) positions labeled below.

B. Hydropathy plot of SsHP1, generated using ExpPASy ProtScale (<https://web.expasy.org/protscale/>) based on the Kyte and Doolittle algorithm, was compared with class I HP MoMPG1 from *M. oryzae*. Hydrophobic regions are displayed above the x-axis, while hydrophilic regions appear below.

C. Hydropathy plot of SsHP2 and SsHP3, generated using ExpPASy ProtScale based on the Kyte and Doolittle algorithm, were compared with class II HP MoMHP1 from *M. oryzae*. Hydrophobic regions are displayed above the x-axis, while hydrophilic regions appear below.

A**B****C****D****E****F**

Supplemental Figure S2. Two independent deletion alleles of *Sshp1*, *Sshp2*, and *Sshp3* were obtained through homologous recombination.

A. The *SsHP* genes and *HYG* gene are presented as orange and green rectangles, respectively. The primers labelled in the diagram were used for mutant screening. The scale is shown at the bottom.

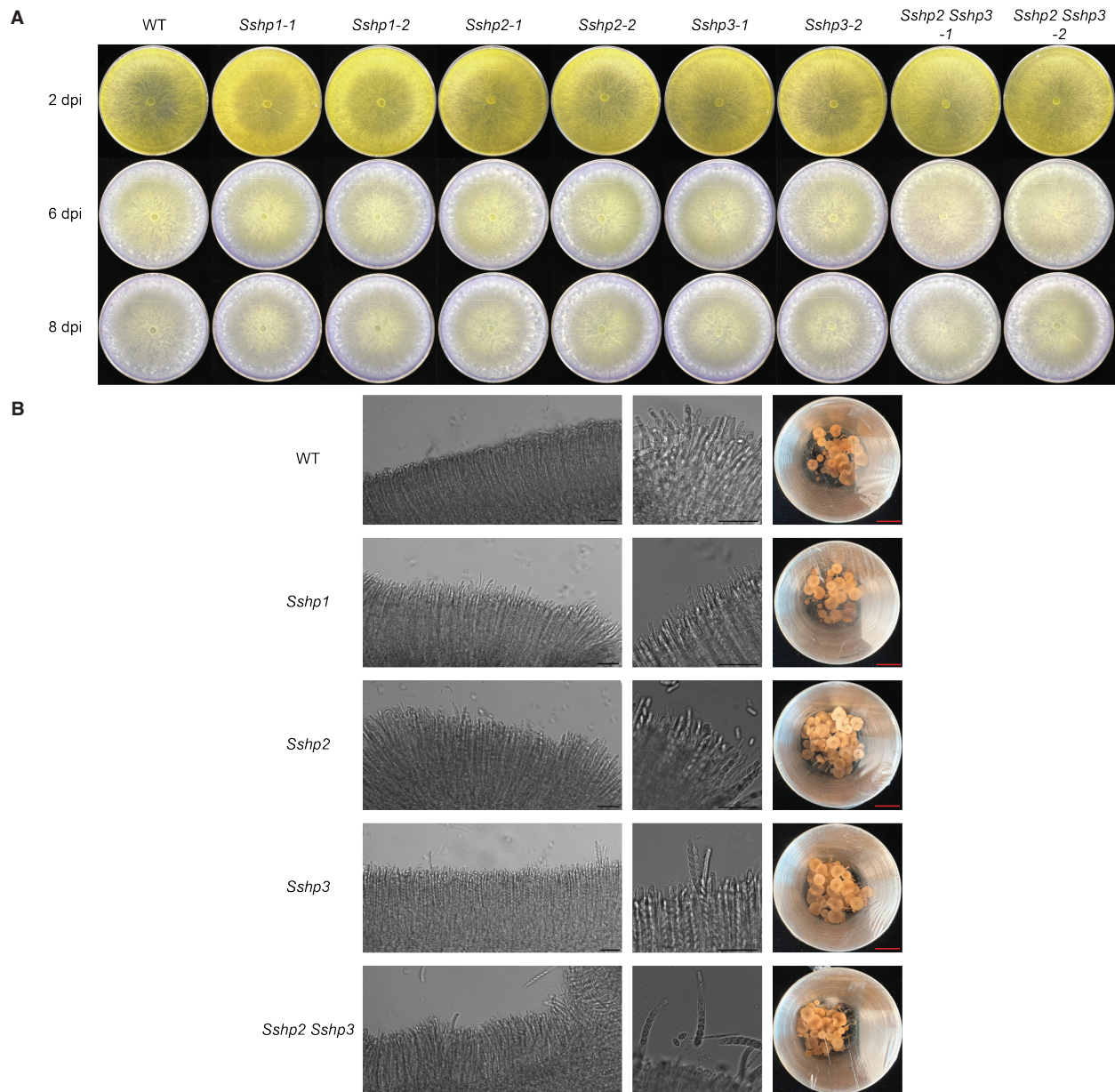
B. PCR verification of *sscle_12g090620* (*SsHP1*) gene disruption. Genomic DNA isolated from WT and independent deletion mutant alleles *Sshp1-1* and *Sshp1-2* were used as PCR templates. Three pairs of primers were designed to verify the upstream and downstream of the hygromycin resistance gene (1F + H855R and H855F + 8R) and *SsHP1* deletion (5F + 6R). The amplicon sizes are indicated in brackets. M shows the lane for DNA marker.

C. PCR verification of *sscle_01g010490* (*SsHP2*) gene disruption. Genomic DNA isolated from WT and independent deletion mutant alleles *Sshp2-1* and *Sshp2-2* were used as PCR templates. Three pairs of primers were designed to verify the upstream and downstream of the hygromycin resistance gene (7F + H855R and H855F + 4R) and *SsHP2* deletion (5F + 6R). The amplicon sizes are indicated in brackets. M shows the lane for DNA marker.

D. PCR verification of *sscle_15g106410* (*SsHP3*) gene disruption. Genomic DNA isolated from WT and independent deletion mutant alleles *Sshp3-1* and *Sshp3-2* were used as PCR templates. Three pairs of primers were designed to verify the upstream and downstream of the hygromycin resistance gene (1F + H855R and H855F + 8R) and *SsHP2* deletion (5F + 6R). The amplicon sizes are indicated in brackets. M shows the lane for DNA marker.

E. Flow chart of *Sshp2 Sshp3* double KO mutant generation through mycelial fusion. *Sshp2* and *Sshp3* single KO strains were placed face-to-face in direct contact and co-inoculated onto carrot medium. The large sclerotia produced on the carrot medium were collected, washed, sterilized, and then induced to generate apothecia. Ascospores from individual apothecium were vortexed for collection and plated onto PDA plates with hygromycin B. The genotype of single colonies was confirmed by PCR. Images adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

F. PCR verification of *SsHP2* and *SsHP3* genes disruption. Genomic DNA isolated from WT and deletion mutant alleles *Sshp2 Sshp3-1* and *Sshp2 Sshp3-2* were used as PCR templates. Six pairs of primers were used to verify the upstream and downstream of the hygromycin resistance gene and targeted genes deletion. The amplicon sizes are indicated in brackets. M shows the lane for DNA marker.



Supplemental Figure S3. Oxalic acid accumulation, apothecia formation, and asci development in HP mutants

A. WT and HP mutants were inoculated on PDA medium supplemented with 50 mg/L bromophenol blue. Acidification, indicating oxalic acid production, is shown by the color change from violet to yellow. Photos were taken at 2, 6 and 8 dpi, respectively.

B. Apothecia induction and asci development in WT and HP mutants. Black scale bar = 50 µm; red scale bar = 1 cm.

Supplemental Table S1. List of primers used in this study.

Primer name	DNA sequence (5' to 3')	Purpose
<i>SsHP1</i> -1F	GTTTTCTCGCTACCACCAAG	<i>SsHP1</i> knockout
<i>SsHP1</i> -2R	cgactctagaggatcccggGTGTTGTGTTTGAAGCGAAG	
<i>SsHP1</i> -3F	gttggtgtcgatgtcagctcGGTACACAGGGAGGAATCAA	
<i>SsHP1</i> -4R	TCCCAGCACCGAGTAATAAT	
<i>SsHP1</i> -5F	ACCACCCTCCTTACAATCAA	
<i>SsHP1</i> -6R	TAGGGAAGACTTACGCTGGT	
<i>SsHP1</i> -7F	GAAAATGGACTTGGCTGCAGC	
<i>SsHP1</i> -8R	CTACTCAATCCCTCTCTAAGC	
Hygro-F	CCGGGATCCTCTAGAGTCG	
Hygro-R	GAGCTGACATCGACACCAAC	
<i>SsHP2</i> -1F	CTAGTCTCTTTCGCGTTTCG	<i>SsHP2</i> and <i>SsHP3</i> knockout
<i>SsHP2</i> -2R	cgggtaccgagctctttgaaggttgGATTGAGTGATGGTGTAG	
<i>SsHP2</i> -3F	gccgaccgggatccacttaacgttacCTTGTGCAAATCCGCGTCAG	
<i>SsHP2</i> -4R	GGTGGAGTTGAACGATCAAG	
<i>SsHP2</i> -5F	CACACAACAGACTCCCATC	
<i>SsHP2</i> -6R	CTGGAGAATTGCAGAGGAGAC	
<i>SsHP2</i> -7F	CTGTCTAAAGAGCTAGCTAC	
<i>SsHP2</i> -8R	GATGGTATGTAATCATGCGC	
HYG-F	CACAACCTTCAAAGAGCTCGGTACCCG	
HYG-R	GTAACGTTAAGTGGATCCCGGTCGGC	
<i>SsHP3</i> -1F	GAGGCTGAAGAACAGTAACC	
<i>SsHP3</i> -2R	cgggtaccgagctctttgaaggttgGTTGGTGGGTTGGAAGTGATG	
<i>SsHP3</i> -3F	gccgaccgggatccacttaacgttacGAATGGGAATTAGTGCGGC	
<i>SsHP3</i> -4R	GTCGAAGCGCACTAATTATC	
<i>SsHP3</i> -5F	GCAATTCACAACCGCAACAC	
<i>SsHP3</i> -6R	CCATTCTAATCTCAGGCTTC	
<i>SsHP3</i> -7F	CATATGTGCGAAGAAGAGG	
<i>SsHP3</i> -8R	GCGAATACTTCACAACCTATC	
H855F	GTCGATGCGACGCAATCGT	Check <i>HYG</i> gene insertion
H855R	GAACCATCTTGTCAAACGAC	