Comparative omics analysis of endophyte-infected and endophyte-free Achnatherum sibiricum in response to pathogenic fungi

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Abstract

Epichloë endophytes can improve the resistance of host grasses to pathogenic fungi in grasslands. However, little is known about the mechanisms involved. We investigated the mechanisms underlying the effect of *Epichloë sibirica* on the resistance of *Achnatherum sibiricum* to *Curvularia lunata* by metabolomics approaches. The results demonstrated that before and after pathogen inoculation, 58 and 157 differential metabolites (DMs) were respectively induced by endophytes. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of DMs showed that amino acids and phenols were mostly accumulated by endophytic infection. Integrated transcriptome and metabolomics KEGG analysis revealed that plant hormone signal transduction was significantly enriched. After measurement, we found that endophytic infection increased jasmonic acid (JA) concentration before pathogen inoculation and increased ethylene (ET) and pipecolic acid concentration after pathogen inoculation. Exogenous phytohormones treatment verified that endophytes improved the disease resistance of *A. sibiricum* by promoting JA and ET accumulation. In phenylpropanoid synthetic pathway, the endophytes promoted the accumulation of ferulic acid, p-coumaroylagmatine, and feruloylputrescine which was related to resistance against plant disease. Overall, our research suggests that *Epichloë* endophytes presumably trigger induced systemic resistance of the hosts to pathogenic fungi via activating JA/ET signaling pathways and promoted antimicrobial phenol accumulation in hosts.

Introduction

Plant diseases are the main factors affecting the health of natural grassland worldwide, which reduce the yield and quality of forages, and seriously restrict the sustainable utilization of grassland and the development of animal husbandry (Liu et al., 2016b; Zhang et al., 2020). In recent years, soil-borne microorganisms (such as saprophytic fungi *Trichoderma*) have been reported to stimulate plant growth (Ji et al., 2021), produce antibiotics (Liu et al., 2016a) and activate plant defense response to phytopathogens (Shoresh et al., 2005), and have been applied in the control of agricultural diseases as an environmentally friendly alternative to the massive use of chemical pesticides (Zin and Badaluddin, 2020). Besides soil-borne microbiota, endophytes widely live and reproduce in the plant kingdom without causing prominent diseases, protect host plants from environmental pathogenic microorganisms, and are essential potential resources for mining new biocontrol agents (Silva et al., 2019).

In natural grasslands, most cold-season grasses can form symbiotic relationships with endophytic fungi of the genus *Epichloë* (Clay, 1990). The fungal hyphae colonize the intercellular spaces of sheaths and leaves of the host (Clay and Schardl, 2002). Plants serve as hosts and provide nutrients to their endophytes, and the endophytic fungi protect them from biotic and abiotic stresses, such as drought (Ren et al., 2011), herbivores (Bastías et al., 2017), and pathogenic infections (Xia et al., 2018).

The positive effect of*Epichloë* infection on the disease resistance of the host grass has been reported not only in cultivated grasses such as *Lolium perenne* (Pańka et al., 2013a) and *Festuca arundinacea* (Chen et al., 2017); but also in natural grasses, such as *Festuca pratensis* (Sabzalian et al., 2012), *Leymus chinensis* (Wang et al., 2016), *Festuca rubra* (Niones and Takemoto, 2014) and*Achnatherum inebrians* (Xia et al., 2016). However, little is known about the involved mechanisms of resistance. It is well known that endophytic infections can enhance herbivore resistance of the host grasses due to the production of alkaloids (Bastías et al., 2017). However, alkaloids are not likely directly associated with fungal pathogenic resistance (Holzmann-Wirth et al., 2000). Several in vitro studies have identified that endophytes could produce inhibitory metabolites against plant pathogenic fungi, such as Epichlicin (Seto et al., 2007) and cyclosporin T (Song et al., 2015). Whether these inhibitory metabolites are also effective in endophyte-infected grasses has not been reported up to now.

In addition to metabolites produced by endophytes themselves, endophytic infections could reprogram the metabolism of the host grasses. Phenolic compounds are essential secondary metabolites involved in the regulation of the plant immune system against disease (Daayf et al., 2012). Researchers found that endophytes upregulated gene expression levels related to phenylpropanoid synthesis in the host *L. perenne* cv Samson (Dupont et al., 2015) and significantly induced total phenol accumulation in *L. perenne* (Rasmussen et al., 2008). Pańka et al. (2013a, 2013b) studied the disease resistance of tall fescue and perennial ryegrass and confirmed that endophytic infections might increase the resistance of the host by the accumulation of total phenols. However, there are numerous phenolic compounds in plants (Balasundram et al., 2006), which compounds that play a vital role in the endophytes effecting host disease resistance, are yet to be elucidated.

Plant immune responses to diseases include systemic acquired resistance (SAR) induced by pathogenic microorganisms and induced systemic resistance (ISR) enhanced by beneficial microorganisms (Vallad and Goodman, 2004). In general, SAR mainly relies on the activation of signal molecules such as salicylic acid (SA), pipecolic acid (Pip), and azelaic acid (Klessig et al., 2018); in comparison, ISR needs to rely on jasmonic acid (JA) and ethylene (ET), independent of SAR signal molecules to be triggered (Pieterse et al., 2014). A wide variety of mutualists, including *Bacillus* , *Trichoderma* , and*mycorrhiza* species, sensitize the plant immune system for activated ISR by JA/ET (Cameron et al., 2013; Gutjahr, 2014; Pieterse et al., 2014). As for *Epichloë* endophytes, Schmid et al. (2017) studied the transcriptome of *L. perenne* cv. Nui established that endophytes upregulated genes involved in SA, JA, and ET biosynthesis. However, whether endophytes activate ISR in grass hosts or not remains unknown.

Achnatherum sibiricum is a perennial, sparse bunchgrass widely distributed in northern China's natural grasslands, which harbors two different *Epichloë* species, *Epichloë gansuensis* and*Epichloë sibiricum* (Zhang et al., 2009). In the previous study, Niu et al. (2016) found that both species of endophytes could increase the resistance of *A. sibiricum* to common pathogens in grassland. In order to study the disease-resistance mechanism of endophytes to protect the host, the metabolome was used in this study. Herein, endophyte-infected (EI) and endophyte-free (EF) *A. sibiricum*plants were used as plant materials; *Curvularia lunata* was chosen as the pathogen, which is a common and severe disease that affects crops and forages worldwide (Chang et al., 2020; Nookongbut et al., 2020; Wonglom et al., 2019). Based on the evidence that*Epichloë* endophyte could significantly improve the resistance of*A. sibiricum* to *C. lunata* (Shi et al., 2020), the following two questions were investigated: 1) What are the significantly differential metabolites (DMs) in the host caused by endophytic infections before and after pathogen inoculation?; 2) What are the possible mechanisms by endophyte for enhancing host's disease resistance?

Materials and methods

Plants, fungi, and culture conditions

Seeds of*A. sibiricum* were collected from the experimental field of the Inner Mongolia Grassland Ecosystem Research Station of the Chinese Academy of Sciences (43° 38' N, 116deg 42' E). We separately prepared *E. sibiricum* -infected seeds and endophyte-free seeds according to the methods reported in a previous article (Shi *et al* ., 2020). Plump surface-sterilized seeds of EI and EF plants were sown in square pots (length x

width x height was 10x10x12 cm; one seed was sown in one pot) filled with vermiculite. After 30 days of cultivation, the endophytic infection status of each plant was inspected by trypan blue staining (Latch and Christensen, 1985). The endophytic infection rate in the EF plants was 0, and that in the EI plants was 100%. Identical sizes of EF and EI seedlings were selected and cultured for subsequent tests.

C. lunata was provided by the Agricultural Culture Collection of China (ACCC) and cultured on potato dextrose agar (PDA) culture medium at 25 degC for 15 days. To obtain a spore suspension of the pathogen, the hyphae of the pathogenic fungus were washed with sterile water (containing 0.02% Tween 20) and filtered through a double layer of sterile gauze. The concentration of the pathogen spore suspension quantified with a hemocytometer was 1.2 x 10^5spores/mL, and the spore germination rate was 80%.

Experimental design

The present study consisted of two main experimental parts. First, we used metabolomics and transcriptome technology to compare the metabolite differences between the EF and EI leaves that were either inoculated with the pathogen or not. We discovered that the JA, ET, and Pip were involved in endophyte-associated disease resistance in the host. Afterward, exogenous phytohormone treatments were used to verify the effects of plant hormones.

Metabolomics and transcriptome experiment: two factors were considered under a randomized block design. The first factor was the status of the endophyte (EI or EF). The second factor was pathogen inoculation (control or inoculated). Each treatment was replicated 9 times, with two pots as one replicate; a total of 72 pots were prepared. After 90 days of EF and EI plants cultivation, a spore suspension of *C. lunata*was uniformly sprayed on leaves until the liquid dripped off, and the control leaves were sprayed with sterile water containing 0.02% tween 20. On the fifth day post pathogen inoculation, 24 pots of each EF and EI plant were sampled for metabolite extraction and phytohormones determination, while 12 pots of each EF and EI plant were sampled for RNA extraction: the second and third leaves from the newborn leaf in each tiller were collected and frozen in liquid nitrogen and stored at -80 °C. The experiment began in August 2020 and was conducted in the campus experimental field at Nankai University, Tianjin, China. During the experiment, the position of the pots was randomly changed every week to eliminate the position effect. Each pot was watered and fertilized as needed.

Verification experiment: The experiment was carried out on 110-old days EF and EI plants. A total of 2 x 5 treatments were applied (control, MeJA, ETH, SA, or Pip). Five biological replicates were set for each treatment, resulting in a total of 50 pots. The method of screening optimum concentrations are described in Method S1. A 25 mL aliquot of the solutions (containing 0.5% (V/V) dimethyl sulfoxide (DMSO)) of MeJA (0.2 mM), ETH (0.2 mM), SA (0.2 mM), and Pip (1mM) was sprayed on each plant. Ultrapure water containing 0.5% (V/V) DMSO was used as a control (Fig. S1). After 24 hours, we performed pathogenic inoculation as described above. Five days later, the disease index (DI) was calculated.

Metabolomics and transcriptomics

Briefly, each sample (50 mg) was grounded in liquid nitrogen and homogenized with 40 μl of methanol/water (4:1, v/v) containing L-2-chloro-phenylalanine (0.3 mg/mL) as internal standard. The extraction was evaporation-dried and redissolved in 200 μL methanol for analysis. LC-MS instrumentation, metabolite identification, and data analysis are described in Method S2.

Total RNA was extracted from ultralow-temperature frozen leaves (approximately 0.1 g) using a plant RNA purification kit (Invitrogen, Carlsbad, CA, USA). cDNA libraries were constructed using the TruseqTM RNA sample prep Kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA sequencing, de novo assembly, functional annotation of unigenes, and data analysis are described in Method S3.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to analyze the enrichment pathways in MBROLE (http://Csbg.cnb.csic.es/mbrole2/) to obtain the KEGG ID of the significant differential metabolites (DMs) and differentially expressed genes (DEGs). The statistical significance of pathway enrichments was identified using Fisher's exact test, and$P$ values from the KEGG enrichment analyses were corrected by

Benjamini and Hochberg as well. The corrected P values (false discovery rate, FDR) [?]0.05 were considered significantly enriched. Based on the results of KEGG pathway enrichment analysis of integrated transcriptome and metabolome, we collected the key DEGs groups involved in the biosynthesis of plant signaling molecules and phenylpropanoids (Table S1).

Quantitative determination of phytohormones

Five of the six biological replicates were randomly selected in each treatment, with a total of 20 samples measured. 0.3g (fresh weight) of frozen-dried leaf samples were grounded and extracted with 1 mL methanol/water solution (80:20, V: V) two times. The extracted solution was subsequently taken for quantification of JA, ET, and Pip. While the concentrations of JA and ET were quantified with enzyme-linked immune assays (ELISAs) (JA/ET ELISA kit, Fankewei, China), Pip concentration was determined by the LC-MS method (Chen et al., 2018).

Calculation of disease index (DI)

DI severity was classified into nine different levels according to the percentage of the leaf area affected by the disease in individual leaves (James, 1971). Levels 0 to 9 respectively were 0, 0˜5, 6˜10, 11˜20, 21˜30, 31˜40, 41˜60, 61˜80, and 81˜100 %, respectively. DI was calculated using the following formula:

$$DI = \frac{\sum (x * t)}{s * \sum t} \times 100$$

where x is the degree of disease severity, t is the total number of leaves showing each degree of disease severity, and s is the highest degree of disease severity observed.

Statisticalanalysis

The plant DI, phytohormones concentration and colony diameter, were analyzed with the SPSS software (Version22.0, IBM, Chicago, USA). Statistically significant differences were determined by ANOVA followed by Duncan's post hoc tests. A value of $P < 0.05$ was considered to be statistically significant.

Results

Metabolomic changes of *A. sibiricum* in response to endophytes

Compared to the control, the endophytic infection resulted in 58 DMs before pathogen inoculation (Fig. S2a) and 157 DMs after pathogen inoculation (Fig. S2b). There were 8 common DMs (Fig. S2c). Among them, the contents of arginyl-proline, 4-methyl-5-thiazoleethanol, and aspergillomarasmine A were considerably higher in the EI leaves compared to those in the EF (Fig. 1).

KEGG metabolic pathway analysis

Next, differential metabolites of EI vs. EF and EICL vs. EFCL were performed using the KEGG website for online analysis using Metabo Analyst, which provided the following enrichment analysis (P ¡0.05). The results established that 23 DMs caused by endophytic infection were significantly enriched into 5 and 11 KEGG pathways before and after pathogen inoculation, respectively (Table 1; Fig. S3).

The effects of endophytes on the contents of lipids, flavonoids, and isoflavones in the host showed a decreasing trend (Table 1). On the contrary, the effects of endophytes on the contents of amino acids and phenols showed an increasing trend. For the other 8 DMs, the increase in the contents of 1-aminocyclopropane-1-carboxylic acid (ACC), 3-indoleacetic acid, and 4-methyl-5-thiazole was the most pronounced, and their contents in EI plants were much higher than those in EF, regardless of pathogen inoculation; gentisic acid, tyramine, and D-pipecolic acid were significantly accumulated in EI after pathogen inoculation.

Integrated transcriptome and metabolomics analysis

KEGG pathway analysis of transcriptome and metabolome

The DEGs and DMs of the two groups under comparison (EI vs. EF and EICL vs. EFCL) were performed for the KEGG pathway enrichment analysis. The results showed that there were five common pathways in DEGs and DMs (EI vs. EF) before pathogen inoculation, in which "arginine and proline metabolism" and "cysteine and methionine metabolism" were both significantly enriched (Fig. 2a). After pathogen inoculation, there were 24 common KEGG pathways (EICL vs. EIFL) in the host, but only "plant hormone signal transduction" was significantly enriched (Fig. 2b). These results suggested that plant hormone signaling might be an essential pathway for endophytes to enhance the host's disease resistance.

Gene expressions, biosynthesis and roles verification of phytohormones

Both endophyte and pathogen significantly affected the content of JA, ET and Pip in *A. sibiricum* , and their interaction had significant effects on the content of ET and Pip (Table 2). In the JA synthesis pathway, endophytes induced 8 gene expressions before pathogen inoculation (Fig. 3) and significantly increased JA content ignore pathogen inoculation (Fig. 4a). In the ET synthesis pathway, endophytes upregulated *ACS* and *ACO* expression levels and significantly promoted 1-aminocyclopropane-1-carboxylate (ACC) and ET synthesis after pathogen inoculation (Table 1; Fig. 3 and 4b). Furthermore, endophytes induced *ALD1* and *FMO1* expression in the Pip synthesis pathway and promoted L-lysine and D-Pip synthesis after pathogen inoculation (Table 1; Fig. 3 and 4c).

The exogenous chemicals, pathogens, and their interaction significantly all affected DI (Table 2) The results of the phytohormones verification test show that compared to the control, ETH treatment significantly reduced the DI in EI and EF, while MeJA treatment significantly reduced disease symptoms in EF but had no significant effects in EI (Fig. 4d). Conversely, Pip treatment significantly reduced disease symptoms in EI but had no significant effects in EF. Moreover, SA treatment had no significant effect on the disease symptoms in EI and EF. These results suggested that endophytes might enhance the disease resistance of the host by promoting ET and JA accumulation.

Transcription and synthesis of phenols in the phenylpropanoid synthesis pathway.

The KEGG pathway analysis of DMs in EICL vs. EFCL revealed that "phenylpropanoid biosynthesis" and "tyrosine metabolism" were significantly enriched. Most phenylpropanoids took L-phenylalanine and L-tyrosine as raw materials and synthesized corresponding metabolites through deamination, hydroxylation, and coupling reactions. Thus, we constructed the transcription and synthesis roadmap of phenols in the phenylpropanoid synthetic pathway.

In the L-phenylalanine metabolic pathway, endophytes had no significant effect on the contents of L-phenylalanine, cinnamic acid, and 2-hydroxy-cinnamate in the phenolic synthesis pathway of L-phenylalanine, regardless of whether the pathogen was inoculated (Fig. 5). After pathogen inoculation, endophytes significantly inhibited*PAL* and *C4H* expression (Fig. 5). In the L-tyrosine metabolic pathway, endophytes promoted the accumulation of ferulic acid, p-coumaroylagmatine, feruloylputrescine, and sinapic acid before pathogen inoculation (Table 1; Fig. 5). After pathogen inoculation, the endophyte significantly induced the *PTAL* expression, and the contents of p-coumaroylagmatine and feruloylputrescine were higher in EI than that in EF. These results suggest that endophytes may promote phenols synthesis through the L-tyrosine metabolism pathway.

Discussion

Jasmonic acid and ethylene are the key signaling molecules of*E. sibirica* -mediated induced systemic resistance

Jasmonic acid has been reported to be a fundamental signaling molecule of ISR, which emerged as a crucial mechanism in priming the whole plant body for enhanced defense against a broad range of pathogens by plant growth-promoting microorganisms (Pieterse et al., 2014), such as arbuscular mycorrhizal fungi (Nair et al., 2015) and *Trichoderma*(Ji et al., 2021). In addition, ET can also cooperate with JA to trigger ISR regulated by some *Trichoderma* species (Shoresh et al., 2005; Yuan et al., 2019). As for endophytes, many*Epichloë* species can promote the growth of the host grasses and improve the disease resistance of hosts

(Bastías et al., 2021; Pérez et al., 2020), whether it generates ISR or not remains unclear. Guo et al. (2019) found that*Epichloë festucae* var. *lolli* infection improved the disease resistance of *L. perenne* while had a null effect on JA content. In another study, *E. gansuensis* enhanced the resistance of *A. inebrians* to pathogens but significantly decreased JA content in hosts (Kou et al., 2021). As for ET, there is currently no available research on the influence of endophytic infection on the ET content of the host grasses.

In this study, the result of integrated transcriptome and metabolomics KEGG enriched analysis unveiled that plant hormone signal transduction was significantly enriched after pathogen inoculation, suggesting that plant hormone signaling might be a critical pathway for enhancing the host's disease resistance by endophytes. Moreover, endophytes significantly increased JA and ET contents and upregulated gene expression levels in the JA/ET signaling pathway. Exogenous hormone treatment further corroborated the positive effect of JA/ET on *A. sibiricum* resistance to *C. lunata* . Thus, we hypothesize that endophytes activate the ISR of the host by inducing JA accumulation before pathogen inoculation and promote JA and ET synthesis after pathogen inoculation.

Akin to SA, Pip is also a key signal molecule of SAR in plants (Klessig et al., 2018). Herein, endophytes had no significant effect on SA content, although they promoted Pip accumulation after pathogen inoculation. Pip was reported to be a critical regulator of SAR to biotrophic pathogens (Chen et al., 2018). Here, exogenous chemicals treatment did not verify the positive effect of Pip on necrotrophic*C. lunata* . In previous research, endophytes could also enhance the resistance of *A. sibiricum* to biotrophs but had no significant effect on SA content (Shi et al., 2020), which suggests that endophytes may improve the resistance of the host to biotrophs via the Pip signaling pathway.

Endophyte induced remodeling of genes expression and metabolites accumulation inphenylpropanoid biosynthesis pathway in the host

Plants can resist the invasion of pathologic microorganisms by synthesizing phenolic compounds (Balasundram et al., 2006). Pańka et al. (2013a, 2013b) found that endophytes might enhance disease resistance by increasing the total phenolic content in grasses, but changes in phenolic metabolites were not evaluated. In this research, endophytes activated the transcription of *PTAL* and *4CL* genes involved in the synthesis of phenolic substances and promoted the accumulation of ferulic acid, p-coumaroylagmatine, and feruloylputrescine in the tyrosine metabolism pathway in the host. Ferulic acid (Reem *et al* ., 2016), p-coumaroylagmatine (Ube et al., 2017), and feruloylputrescine (Valette et al., 2020) can be synthesized in large quantities in response to plant diseases and have significant antimicrobial activities. Therefore, our results suggest that endophytes may enhance the host's disease resistance by promoting the accumulation of ferulic acid, p-coumaroylagmatine, and feruloylputrescine through the l-tyrosine metabolism pathway.

Other potential antimicrobial bioactivity metabolites

According to the Green et al. (2020) study on *E. festucae -L. perenne* symbiont, Epichloëcyclins (A-E) were the most abundant class of DMs between symbionts and non-symbionts. Epichloëcyclins (cyclic peptides) were composed of lysine, tyrosine, proline, methionine, phenylalanine, and arginine and might have a bioactive role (Johnson et al. 2015). As epichloëcyclins information was not obtained in our metabolite database, we did not detect epichloëcyclins in EI plants in this study. However, endophytes induced the accumulation of lysine, arginine, and proline, the main constitutes of epichloëcyclins. Therefore, Epichloëcyclins might occur in *A. sibiricum* and possess antimicrobial activities. Moreover, Aspergillomarasmine A and 4-Methyl-5-thiazoleethanol were highly accumulated in the endophyte-infected host. Aspergillomarasmine A is a rapid and effective enzyme inhibitor that restores the sensitivity of carbapenems to resist bacteria (Meziane-Cherif and Courvalin, 2014). Thiazoles with fungicidal effects have been widely used in disease prevention and crops control (Cui et al., 2012). Therefore, Aspergillomarasmine A and 4-methyl-5-thiazoleethanol might play a role in the endophyte-associated pathogen resistance of the host.

In summary, We first reported that*E. sibirica* endophytes activated the ISR in the host by JA and ET signaling pathways which might play an essential role in enhancing host disease resistance. In addition, we discovered that the antimicrobial phenolic metabolites and potential bioactive metabolites were accumulated

in large quantities in endophyte-infected leaves. So far, achievements have been made in breeding *Epichloë* - grass symbionts that do not produce toxic alkaloids to mammals but have toxic alkaloids to insect herbivores (Bharadwaj et al., 2020). Once the metabolites responsible for pathogen resistance are further tested, the endophyte-grass symbionts will become crucial resources of biological pesticides.

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Table 1 Differential metabolites significantly enriched to KEGG pathways

Metabolites	**Formula**	**Log_2(Fold change)**	**Log_2(Fold change)**
		EI vs. EF	**EICL vs. EFCL**
Sphingolipids			
Phytosphingosine	$C_{18}H_{39}NO_3$	-1.796**	-0.548
Fatty Acyls			
8,11,14-Eicosatrienoic acid	$C_{20}H_{34}O_2$	-0.561	-5.796*
11,14,17-Eicosatrienoic acid	$C_{20}H_{34}O_2$	1.354	1.857*
13S-hydroxyoctadecadienoic acid	$C_{18}H_{28}O_2$	-1.012*	0.010
Amino acids			
L-Lysine	$C_{11}H_{12}N_2O_2$	0.148	2.024**
L-Glutamine	$C_6H_{14}N_2O_2$	1.080	1.594*
L-Proline	$C_5H_{10}N_2O_3$	0.289	1.329*
L-Arginine	$C_5H_9NO_2$	1.780*	2.521**
Phenols			
P-Coumaroylagmatine	$C_{14}H_{20}N_4O_2$	3.124**	0.333
Ferulic acid	$C_{11}H_{14}O_4$	0.979*	-0.456
Sinapic acid	$C_9H_8O_3$	0.670	-1.133*
Feruloylputrescine	$C_{21}H_{20}O_{11}$	1.953*	1.227*
Flavonoids and isoflavones			
6"-O-Malonyldaidzin	$C_{14}H_{20}N_4O_2$	-0.943*	-0.458
Biochanin A 7-(6-malonylglucoside)	$C_{21}H_{20}O_{11}$	-0.589*	-0.533
Ononin	$C_{25}H_{24}O_{13}$	0.622	-1.905*
Other metabolites			
S-Adenosylhomocysteine	$C_7H_6O_4$	-1.761*	-1.241*
Gentisic acid	$C_8H_{11}NO$	-0.894*	0.629
Tyramine	$C_{11}H_{12}N_2O_4$	-1.599*	1.364*

Metabolites	**Formula**	**Log_2(Fold change)**	**Log_2(Fold change)**
5'-Methylthioadenosine	C_6H_9NOS	0.653*	-0.019
1-Aminocyclopropane-1-carboxylic acid	$C_{14}H_{20}N_6O_5S$	0.408*	1.989**
3-Indoleacetic acid	$C_{10}H_9NO_2$	0.847	1.357*
D-Pipecolic acid	$C_6H_{11}NO_2$	-1.038	1.489**
4-Methyl-5-thiazoleethanol	$C_4H_7NO_2$	6.040**	6.138**

Note: The fold change in each substance was obtained from the relative quantitative value of the metabolites in one treatment divided by the relative quantitative value of the metabolites in the other. The significant values were confirmed by Student's t-tests. *A significant difference ($0.001< p < 0.05$); **A extremely significant difference ($p < 0.001$). EF, endophyte-free plants; EI, endophyte-infected plants; EFCL, EF plants inoculated with*Curvularia lunata* ; EICL, EI plants inoculated with *C. lunata.*

Table 2 Two-way ANOVA of the effects of the endophyte (E), pathogen (P) and exogenous chemicals (C) on the disease index and concentration of phytohormones (jasmonic acid, ethylene and pipecolic acid) in *Achnatherum sibiricum* .

Treatment	**Disease index**	**Disease index**	**Jasmonic acid**	**Jasmonic acid**	**Ethylene**	**Ethylen**
	F	P	P	F	P	P
Endophyte (E)	**257.03**	**¡0.001**	**47.33**	**¡0.001**	**32.42**	**¡0.001**
Pathogen (P)			**43.12**	**¡0.001**	**190.3**	**¡0.001**
Exogenous chemicals (C)	**24.55**	**¡0.001**				
E×P			0.41	0.530	**19.91**	**¡0.001**
E×C	**6.90**	**¡0.001**				

Significant P -values ($P < 0.05$) are shown in bold.

Figures Captions

Fig. 1 The key common differential metabolites of EI vs. EF and EICL vs. EFCL. Box-and-whisker plots for selected metabolites comparing normalized intensities. The data of six biological replicates were normalized by summing and Pareto scaling. The significant values were confirmed by Student's t -tests. *A significant difference ($0.001< P < 0.05$); **A extremely significant difference ($P < 0.001$). EF, endophyte-free plants; EI, endophyte-infected plants; EFCL, EF plants inoculated with*Curvularia lunata* ; EICL, EI plants inoculated with *C. lunata.*

Fig. 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways of differentially expressed genes and identified metabolites in EI vs. EF **(a)** and EICL vs. EFCL **(b)** . The statistical significance of pathway enrichments was identified using Fisher's exact test, and$P$ values from the KEGG enrichment analyses were corrected by Benjamini and Hochberg as well. The corrected P values (false discovery rate, FDR) [?]0.05 were considered significantly enriched. Bars across the dashed lines indicated the KEGG pathways were significantly enriched. EF, endophyte-free plants; EI, endophyte-infected plants; EFCL, EF plants inoculated with *Curvularia lunata* ; EICL, EI plants inoculated with *C. lunata.*

Fig. 3 Transcription and metabolism roadmap in plant signal molecular JA. ET and Pip synthesis pathways. Heatmaps show the values of relative expression of each treatment group compared to the EF (values = Log_2 (fold change)). Fold change calculation was based on fragments per kilo base of exon per million fragments mapped (FPKM) values (the information of genes were shown in Table S1). Box-and-whisker plots for selected metabolites comparing normalized intensities. The metabolite data of six biological replicates were normalized by summing and Pareto scaling. The significant values were confirmed by Student's t -tests. Ns means no significance; *A significant difference ($0.001< P < 0.05$); **A extremely significant

difference ($P < 0.001$). The red font represents the upregulated gene expression or increased metabolite content in EI vs. EF or EICL vs. EFCL, and the black font represents no significant change. LOX, lipoxygenase; AOS, allene oxide synthase; OPR, 12-oxo-phytodienoate reductase; OPCL1, OPC-8:0 CoA ligase 1; ACOX, acyl-coenzyme A oxidase; ACAA, 3-ketoacyl-CoA thiolase; SAMS, S-adenosylmethionine synthase; ACS, 1-aminocyclopropane-1-carboxylate synthase; ACO, 1-aminocyclopropane-1-carboxylate oxidase; ALD1, aminotransferase; SARD4, systemic acquired resistance-deficient4; FMO1, flavin-dependent-monooxygenase 1. EF, endophyte-free plants; EI, endophyte-infected plants; EFCL, EF plants inoculated with *Curvularia lunata* ; EICL, EI plants inoculated with *C. lunata.*

Fig. 4 Endogenous levels and roles verification of plant signaling molecules. The concentration of jasmonic acid **(a)** , internal ethylene **(b)** , and pipecolic acid **(c)** were quantified in EF and EI *Achnatherum sibiricum* before and after *Curvularia lunata* inoculation. The disease index **(d)** of *A. sibiricum* treated with water (control), salicylic acid (SA, 0.2 mM), methyl jasmonate (MeJA, 0.2 mM), ethephon (ETH, 0.2 mM), or pipecolic acid (Pip, 1 mM) were measured at five days post pathogen inoculation. Data are represented as means ± SEM of five biological replicates. Different letters above the bars denote statistically significant differences between different treatments ($P< 0.05$, Two-way ANOVA and post hoc Duncan's test). EF, endophyte-free plants; EI, endophyte-infected plants; EFCL, EF plants inoculated with *Curvularia lunata* ; EICL, EI plants inoculated with *C. lunata.*

Fig. 5 Transcription and metabolism roadmap of phenols in the phenylpropanoid synthetic pathway. Heatmaps show the values of relative expression of each treatment group compared to the EF (values = Log_2 (fold change)). Fold change calculation was based on fragments per kilo base of exon per million fragments mapped (FPKM) values (the information of genes were shown in Table S1). Box-and-whisker plots for selected metabolites comparing normalized intensities. The metabolite data of six biological replicates were normalized by summing and Pareto scaling. The significant values were confirmed by Student's t-tests. Ns means no significance; *A significant difference ($0.001< P < 0.05$). The red font represents the upregulated gene expression or increased metabolite content in EI vs. EF or EICL vs. EFCL; the green font represents the decreased gene expression or metabolites content, and the black font represents no significant change. Solid arrows represent the direct synthesis pathway, and dashed arrows represent the indirect synthesis pathway. PAL, phenylalanine ammonia-lyase; PTAL, phenylalanine/tyrosine ammonia-lyase; C4H, trans-cinnamate 4-monooxygenase, 4CL, 4-coumarate-CoA ligase; F5H, ferulate 5-hydroxylase. EF, endophyte-free plants; EI, endophyte-infected plants; EFCL, EF plants inoculated with *Curvularia lunata* ; EICL, EI plants inoculated with *C. lunata.*

Supplementary Material

Fig. S1 Hyphal growth of *Curvularia lunata* on nutrient agar medium containing chemicals.

Fig. S2 Metabolite profiling of *Achnatherum sibiricum* .

Fig. S3 Enrichment analysis of KEGG pathway in EI vs. EF **(a)** and EICL vs. EFCL **(b)** .

Table S1 Identificated key differentially expressed genes involved in the biosynthesis of plant signaling molecules and phenylpropanoids.

Method S1 The method of screening optimum concentrations for verification experiment.

Method S2 LC-MS instrumentation, metabolite identification, and data analysis.

Method S3 RNA sequencing, de novo assembly, functional annotation of unigenes, and data analysis.