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PRELIMINARY ANALYSIS OF THE RESPONSE OF TOBACCO HORMONAL PATHWAY SALICYLIC ACID AND JASMONIC ACID RESISTANCE-RELATED GENES TO *EXSEROHILUM TURCICUM*

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Abstract

Non-host disease resistance (NHR) is a broad-spectrum disease resistance in plants stimulated by non-adapted pathogenic with persistent disease resistance properties. In recent years, NHR has been considered as a new strategy for disease control. In this study, *Aalternaria alternata* and *E. turcicum* were inoculated into the main tobacco varieties Hong Hua Da Jin Yuan (HD) and K326. The expression of disease resistance genes (NtAOS, NtJAZ, NtMYC2) in the upstream and downstream of Jasmonic acid Pathway (JA) and disease resistance genes (NtMAPK, NtPR1, NtPR2, NtPR11) in the upstream and downstream of Salicylic acid Pathway (SA) were analyzed by RT-qPCR. The changes of SOD, POD, PAL enzyme activity and H_2O_2 content were detected. The results showed that the expression levels of NtMAPK, NtPR1, NtPR2 and NtPR11 were up regulated at 48h after inoculation with *A. alternata* and *E. turcicum*, the expression levels of HD and K326 were 1-13 times and 1-38 times higher than those without inoculation, respectively. The expression level of the gene after inoculation with *E. turcicum* was higher than inoculation with *A. alternata*; Similar to the SA pathway resistance-related genes, the expression levels of the JA pathway upstream resistance-related gene NtAOS and downstream related genes NtJAZ and NtMYC2 were higher than those in the control and *A. alternata* stress groups after 48h of *E. turcicum* stress. The peak of H_2O_2 content at 12h after *E. turcicum* stress was higher than that of control and *A. alternata* stress in the two varieties; Moreover, the activity levels of SOD, POD and PAL at 48h after *E. turcicum* stress were higher than those under *A. alternata* stress. In summary, *E. turcicum* can induce non-host disease resistance in tobacco, enhance the ability of tobacco to resist host pathogens, and reduces or protects tobacco from pathogens to a certain extent.

Key words: Systemic disease resistance, Non-host disease resistance, Tobacco brown spot disease.

Introduction

Tobacco brown spot is a common fungal disease caused by *Alternaria alternata* (Fries) Keisslar (*A. alternata*) infecting tobacco (Peng *et al*., 2021). It causes irreversible loss to tobacco once the disease develops. In severe cases, the loss rate of tobacco yield leaves reaches up to 28.56% and the output value loss rate reacheses up to 89.67% (Yu, 2011). The Chemical control is the most widely used method for preventing and controling brown spot (Yang, 2019). However, with the promotion of the development idea of green, ecology, health and civilization, pesticide residues in tobacco leaves have been taken as an important index to measure the safety in the international trade of tobacco. Green and sustainable control of diseases has become the focus of in the tobacco industry and the whole agriculture.

The long-term co-evolution of plants and pathogens has formed a multi-faceted defense system (Erik *et al*., 1998; Jose *et al*., 2018). Plants exhibit good defenses against a wide range of plant pathogens by generating induced resistance upon perception of pathogenic biosignals (Lee *et al*., 2017; Lee & Frank., 2018; Meena *et al*., 2022), and upon perception of non-host pathogenic biostimuli, the plant activates defense mechanisms and generates higher levels of immune functioning to protect the plant from the vast majority of pathogenic microorganisms, The resistance is called non-host resistance (NHR). The emergence of new pathogens and new strains is an important reason for the increasing incidence of outbreaks (Campos *et al*., 2021). In this case, using NHR to control diseases is a new idea. In NHR, the unaffinity interaction between pathogens and non-host plants usually induces different defense signal cascades responses in plants, including and not limited to gene signal transduction, changes in hormone levels, phenotypic changes, and accumulation of reactive oxygen species. The NHR mechanism has been investigated in some studies using *Arabidopsis thaliana* as a model plant. People have used Anthracnose and Asian soybean rust to explore the NHR of *Arabidopsis thaliana*, and showed that EDR1, PEN2, PEN3 genes and their related SA and JA were involved in the NHR of *Arabidopsis thaliana* (Marco *et al*., 2008; Ayumi & Yoshitaka, 2018). Maria (Maria *et al*., 2019) found that VMA isolated from *Streptomyces hygroscopicus* induced systemic resistance through SA, JA and ET signaling pathways, and positively affected Abscisic Acid (ABA) and Auxin (IAA) signaling pathways in Arabidopsis thaliana. At the same time, which showed that VMA improved disease resistance in the crops rice and wheat; another study demonstrated that the brassica pathogen Xcc was able to induce citrus non-host resistance and an associated set of defenses responses and protect the plant from citrus ulcer (Bian *et al*., 2020). Non-host disease resistance has made some progress in crops such as rice, wheat and citrus, but only partially in tobacco. Tong (Tong *et al*., 2021) reported that *Fusarium oxysporum* could induce the expression of resistance genes associated with tobacco root rot to enhance its resistance against root rot. Guo (Guo *et al*., 2020) showed that *Bacillus velezensis* could induce the activity of resistant components in tobacco plants to achieve black shank resistance. Xie (Xie *et al*.,

2021) reported that strain LZ88 had the ability to induce resistance to tobacco brown spot disease, as evidenced by an increase in the expression of the defense-related enzymes peroxidase (POD) and polyphenol oxidase (PPO) in the leaves. Wang (Wang *et al*., 2020a) indicated that *Verticillium dahliae* chromatin remodeling facilitates could repair damaged DNA and improve the ability to cope with ROS. Tobacco (*Nicotiana tabacum* L.) is a disease susceptible plant. According to the records at home and abroad, there are more than 116 tobacco diseases, and more than 68 infectious diseases on tobacco in China. Currently, research on tobacco disease resistance is limited to individual diseases caused by a single strain. As a broad-spectrum disease resistance, NHR is poorly understood in the various pathways of tobacco defense response and their relationships, and research at the molecular level is not complete.

Maize (*Zea mays* L.) and Tobacco are co-season crops, and the main diseases of the two crops do not infect each other. Northern Corn Leaf Blight caused by *Exserohilum turcicum* (*E. turcicum*) is one of the common diseases of maize (Kumar *et al*., 2020). In this study, by comparing the two pathogens of *E. turcicum* and *A. alternata*, infecting two varieties K326 and Hong Hua Da Jin Yuan (HD), the differences of resistance signal responses transmitted by tobacco in different pathways were analyzed, and the induction mechanism of *E. turcicum* on non-host resistance of tobacco was revealed, which provided a theoretical basis for the prevention and control of tobacco diseases by rational distribution of crop types.

Material and Methods

Tobacco variety: The tested tobacco varieties were HD and K326 which were provided by Yuxi Zhongyan Seed Co., Ltd.

Pathogens: Tobacco brown spot pathogen was isolated directly from the diseased leaves of susceptible tobacco plants. *Exserohilum turcicum* was provided by Prof. Yueqiu He' s research group at Plant Protection College, Yunnan Agricultural University.

Plant material preparation and inoculation: The isolated and purified *Alternaria alternata* and *Exserohilum turcicum* were cultured in PDA medium. After sporulation, the surface of the colony was repeatedly brushed with sterilized cotton swabs. The brushed bacterial solution was introduced into a small beaker. The collected bacterial solution was filtered with 4 layers of gauze to make spore suspension, and the spore concentration of the spore suspension was adjusted to about 1×10^4 CFU/mL with sterilized distilled water.

HD and K326 were transplanted into flowerpots after seedling raising, with normal water and fertilizer management. When the tobacco seedlings grew to 6 leaves, 50 plants each of K326 and HD of the same growth and health were randomly selected. The spore suspension of *A. alternata* and *E. turcicum* was inoculated into the selected tobacco plants by spray inoculation method, and sterile distilled water was used as blank control.

RNA extraction: Tobacco samples were taken from the same site at 12h and 48h after inoculation, frozen in liquid nitrogen and stored, and the samples were homogenized in an ice bath and then subjected to RNA extraction by Trizol reagent.

cDNA: Used TAKARA 's PrimeScript RT reagent Kit with gDNA Eraser, 1ng of total RNA was used as a template to synthesize cDNA.

Primer design of related resistance genes: Based on the summarization of resistance genes in previous literature, the key genes of SA pathway and JA pathway NtPR1, NtPR2, NtPR11, NtAOS, NtJAZ, NtMAPK, NtMYC2 were selected. The sequences were searched and downloaded from the NCBI website. Primer 5.0 was used for primer specific design (Table 1). After verification of ordinary PCR products, it was used for subsequent RT-qPCR gene expression determination. The primer sequence was synthesized and returned by Yunnan Kunming Zhongmeiyun Co., Ltd.

Table 1. Primer sequence.

Primer name	Primer sequence	Anneal Temperature /℃
18s (reference gene)	Primer Forward: 5'- TTCCGTTAACGAACGAGACC -3' Primer Reverse: 5'- TGTCGGCCAAGGCTATAAAC -3'	58
NtAOS	Primer Forward: 5'- CAATACGGAAGAGCCAAACGC -3' Primer Reverse: 5'- AACTCATCGGGTCGGTCAAA -3'	60
NtJAZ	Primer Forward: 5'- GAGATTGTGGATTCCGGTCGAT -3' Primer Reverse: 5'- GCGGTGGATACCAAGGCTGA -3'	60
NtMAPK	Primer Forward: 5'- GAGATTGTGGATTCCGGTCGAT -3' Primer Reverse: 5'- GCGGTGGATACCAAGGCTGA -3'	58
NtMYC2	Primer Forward: 5'- TTCAACCAAGAAACCCTTCAACA -3' Primer Reverse: 5'- CCATCTCCCCAGCCCAACA -3'	60
NtPR1	Primer Forward: 5'- TGGTCAATACGGCGAAAACTTAGC -3' Primer Reverse: 5'- GTTACACTGAACCCTAGCACATCC -3'	58
NtPR2	Primer Forward: 5'- GCACGACATAACCTTCCACTCTTAG -3' Primer Reverse: 5'- TGATACCCTGCTGAATTTGTTCCTTG -3'	58
NtPR11	Primer Forward: 5'- GCGAATATCCACGGTCTTAGGG -3' Primer Reverse: 5'- AGAGTAACAATAATCTCCAACATTAGTAGC -3'	58

RT-qPCR: The RT-qPCR detection reagent was Vazyme ChamQ Universal SYBR qPCR Master Mix produced by Novizan. The reaction system was 20μL: 2×ChamQ Universal SYBR qPCR Master Mix 10.0μL, Primer Forward (10 μM) 0.4 μL, Primer Reverse (10μM) 0.4 μL. cDNA 1.0μL, ddH2O 8.2μL. A two-step amplification procedure was performed on CFX 96 Real-Time System (Bio-Rad Laboratories, C1000 Touch Thermal Cycler) PCR instrument. The amplification procedures were as follows: pre-denaturation at 95°C for 3 min; deformation 95°C for 10 s, annealing 58°C for 30s, and extension 60°C for 30s, running 40 cycles; at the end of the cycle, the temperature was increased to 90°C and held for 15s, 60°C and held for 60s. After the reaction, the melt curve was used to detect the specificity of PCR amplification, and the Ct value of the PCR reaction was derived. The relative expression of each gene was calculated using the $2^{-\triangle\triangle Ct}$ method.

Data analysis: IBM SPSS Statistics 27 was used for one-way test, Experimental data were tested for variance chi-square using Duncan and LSD multiple comparisons, and the a, b, c, d, and e indicate the significance of the differences between the different treatments at the 0.05 level. Charting with origin 2021.

Results and analysis

Expression of upstream and downstream genes in SA pathway during tobacco-fungus interaction: For HD, the expression of NtMAPK, the upstream gene of SA pathway, continued to increase with time under stress. The expression of NtMAPK gene showed an upward trend at 48h after stress by *A. alternata* and *E. turcicum*, and the expression of the two treatments was 1.2 times and 1.9 times that of the control. The expression of *E. turcicum* gene was 1.1 higher than that of *A. alternata*. The expression of NtMAPK gene was higher than that of *A. alternata* at 12h and 24h after inoculation with *E. turcicum*, and the highest gene expression was 2.91 at 48h (Fig. 1A). In K326, the expression of NtMAPK was increased with time after inoculation with *A. alternata* and *E. turcicum* for 48h, but the expression of NtMAPK was partially inhibited after inoculation with *A. alternata* for 12h and 48h, which was lower than that of control and *E. turcicum* treatment. The expression of NtMAPK in tobacco plants inoculated with *E. turcicum* was higher than that of control and *A. alternata* stress, and the expression level was still higher at 48h. The expression of NtMAPK gene in HD and K326 was increased at 48h after inoculation with *A. alternata* and *E. turcicum*, and the expression level of *E. turcicum* was higher than that of *A. alternata*. However, K326 was different from HD. Although the expression level increased after inoculation with *A. alternata*, it was significantly lower than that of control and inoculation with *E. turcicum* (Fig. 1B).

The expression of NtPR1, NtPR2 and NtPR11 genes was significantly up regulated at 48h after inoculation with *E. turcicum*, and the expression level was 13.1 times, 3.3 times and 1.1 times of the control. The NtPR1 gene was downregulated by nearly 2/3 at 48h after inoculation with *A. alternata* compared with 12h. The expression of NtPR2 and NtPR11 genes was consistent with that of *E. turcicum*. The expression of NtPR2 gene was 1.5 times that of the control, and the expression of NtPR11 gene was slightly lower than that of the control. The expression levels of these genes were higher in *E. turcicum* than in *A. alternata* at 12h and 48h after inoculation. In addition, the gene expression levels of NtPR2 and NtPR11 detected at the same time after 12h of stress by the host pathogenic fungus *A. alternata* were lower than those without inoculation (Fig. 1C, 1E, 1G).

NtPR2 and NtPR11 were up regulated at 48h after inoculation with *A. alternata*, and the expression level was 5 times and 4 times higher than that without pathogen treatment, while the expression of NtPR1 gene was 50% lower than that at 12h. At 48h after inoculation with *E. turcicum*, NtPR1, NtPR2 and NtPR11 were up regulated, and the expression level was 12.5 times, 38 times and 5.7 times higher than that without pathogen treatment. In contrast, the expression levels of NtPR1, NtPR2 and NtPR11 genes in tobacco plants inoculated with *E. turcicum* were higher than those inoculated with *A. alternata* and the response speed was faster at the same time point (Fig. 1D, 1F, 1H).

For HD and K326, the upstream and downstream genes of SA were up-regulated after 48h of inoculation with *E. turcicum*; in addition to NtPR1, NtMAPK, NtPR2 and NtPR11 genes were up-regulated in *A. alternata* treatment. In terms of up-regulated fold, the expression of downstream genes in SA pathway was significantly higher than that of upstream genes. as the whole, the expression trends of upstream and downstream genes in SA pathway of HD and K326 varieties were consistent after inoculation with *A. alternata* and *E. turcicum*.

Expression of upstream and downstream genes in JA during tobacco-fungus interaction: The expression of NtAOS gene in HD was up regulated at 48h after inoculation with A. *alternata* and *E. turcicum*. At 48h, the expression of *A. alternata* treatment was 81 times that of the control, and the expression of *E. turcicum* treatment was 141 times that of the control. The expression of genes without inoculation of pathogens was downregulated, and the inoculation of pathogens showed different trends compared with the treatment without inoculation of pathogens (Fig. 2A). the gene expression of K326 inoculated with pathogenic bacteria and uninoculated with pathogenic bacteria increased first and then decreased with time. At 12h, the expression of NtAOS in K326 inoculated with *E. turcicum* was 20 higher than that in control and inoculated with *A. alternata*. At 48h, the expression of NtAOS in K326 inoculated with *E. turcicum* was decreased by 91 %. The response speed of NtAOS expression treated with *E. turcicum* was faster and could return to the normal level faster. The expression of NtAOS gene in HD and K326 was different after inoculation with pathogenic fungi. The expression of HD was up-regulated, while the expression of K326 was down-regulated (Fig. 2B).

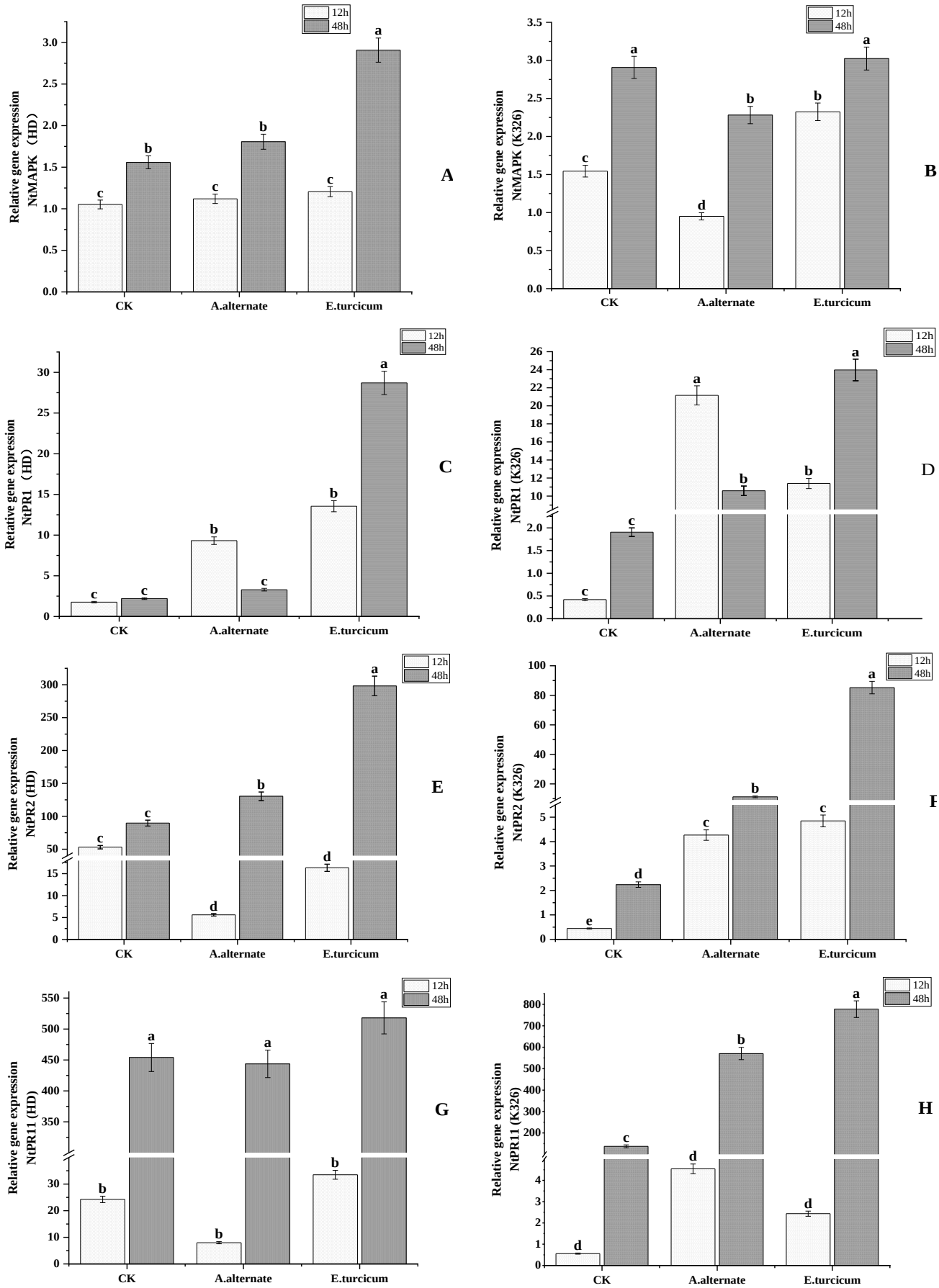

Fig. 1. The expression of upstream and downstream genes in SA of HD and K326 flue-cured tobacco varieties after inoculating with *A. alternata* and *E. turcicum* for 48h. Fig. 1A and 1B are the upstream genes of the SA, and Fig. 1C, 1D, 1E, 1F, 1G and 1H are the downstream genes of the SA.

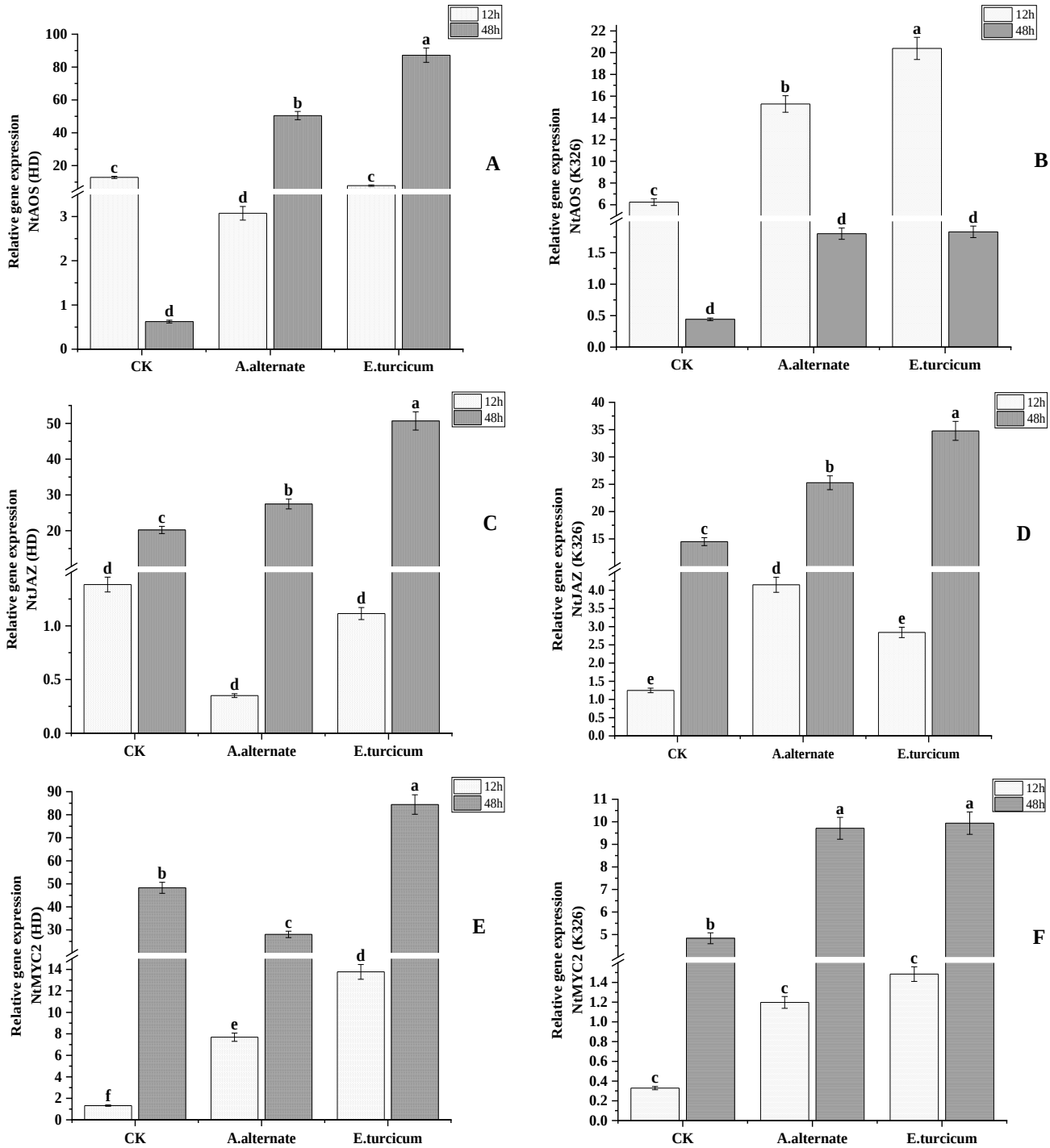

Fig. 2. The expression of upstream and downstream genes in JA of HD and K326 48h after inoculating with *A. alternata* and *E. turcicum.* Fig. 2A and 2B are upstream genes of JA; Fig. 2C, 2D, 2E, 2F are downstream genes of JA.

The expression level of NtJAZ gene in HD cultivar was lower than that in the control at 12h after inoculation with *A. alternata* and increased at 48h and higher than that in the control. The expression level of NtJAZ gene in *E. turcicum* treatment was higher than that in A. *alternata* treatment at each time point. At 48h, the corresponding amount of NtJAZ gene was the highest, which was higher than that of control and *A. alternata* treatment, reaching 50.68, which was nearly twice that of *A. alternata* treatment. The expression level of NtJAZ in *E. turcicum* treatment increased with time, and the response time of NtJAZ was longer than that of *A. alternata* treatment (Fig. 2C). the expression of NtJAZ gene in K326 was up regulated in all treatments. The expression of NtJAZ gene in the treatment of inoculation with pathogenic fungi was higher than that in the control group at 12h and 48h. The expression of NtJAZ gene in the treatment of inoculation with *A. alternata* was higher than that of *E. turcicum* at 12h and lower than that of *E. turcicum* at 48h. The expression of NtJAZ gene was increased fastest at 48h after inoculation with *E. turcicum*, which was 12 times higher than that at 12h, and the peak was higher than that of inoculation with *A. alternata*. The gene expression levels of HD and K326 were up-regulated regardless of the inoculation of pathogenic fungi, but the up-regulation peak was higher after

inoculation of pathogenic fungi, and the inoculation of *E. turcicum* was higher than that of uninoculated pathogens and *A. alternata* treatment (Fig. 2D).

The expression trend of NtMYC2 gene after inoculation of pathogenic fungi in HD was consistent compared to control. The expression level of NtMYC2 gene at 48h was higher than that at 24h. The expression level of NtMYC2 gene at 12h after inoculation with *A. alternata* was 5.8 times that of the control, and the expression level at 48h did not reach the control level, which was only 58 % of the control; The gene expression of *E. turcicum* treatment was higher than that of the control, which was 12 times and 1.8 times higher than that of the control at 12h and 48h. The gene expression of *E. turcicum* treatment was more than that of *A. alternata* treatment and the response speed was faster in a short time (Fig. 2E). the expression of NtMYC2 gene in K326 variety inoculated with pathogenic bacteria was up regulated, showing the same trend as that of the control. The expression of NtMYC2 gene at 48h after inoculation with *A. alternata* was 8 times higher that of the previous time point, and the expression of NtJAZ gene at 48h after inoculation with *E. turcicum* was 6 times higher that of the previous time point. The expression levels of the two treatment genes inoculated at 48h were similar, but the response speed of NtJAZ treated with *E. turcicum* was faster at 12h, and the expression level was higher than that of the control and inoculated with *A. alternata*. The change trend of NtJAZ gene expression in HD and K326 varieties was consistent, but the peak value of gene expression in *E. turcicum* treatment was higher and the response speed was faster in a short time (Fig. 2F).

H_2O_2 content in tobacco leaves after pathogen infection: The content of H_2O_2 in HD inoculated with *A. alternata* was higher at 12h, decreased to 9μmol/g at 24h, and increased to 19μmol/g at 48h, which showed a trend of first expression, then decreased and then increased afterward, which was the same as the control. The content of *E. turcicum* reached a peak of 27μmol/g at 12h after inoculation, and the low level was restored at the subsequent time. The three treatments showed the same trend, but the inoculation of *E. turcicum* rapidly accumulated H_2O_2 in a short period of time and could quickly reduce to normal levels. The peak value of H_2O_2 content in *E. turcicum* was significantly higher than that in *A. alternata* group, and the scavenging efficiency was faster (Fig. 3A).

The content of H_2O_2 detected in K326 was almost the same at 12h and 24h after *A. alternata* stress but increased significantly to 19μmol/g at 48h. K326 reached a peak of 21μmol/g at 12h under *E. turcicum* stress, and then the H_2O_2 content decreased significantly. The trend of inoculation of *E. turcicum* and inoculation of *A. alternata* at the same time was inconsistent. The reaction of inoculation of *E. turcicum* was faster, H_2O_2 bursted rapidly and reached the peak in a short time, while the reaction of inoculation of *A. alternata* was slower and reached the peak at 48h (Fig. 3B).

The content of H_2O_2 in HD and K326 was the highest in all treatments at 12h after inoculation with *E. turcicum*, but it was no longer maintained a high level after 12h. The content of H_2O_2 in HD and K326 tobacco plants inoculated with *A. alternata* for 48h was the highest among all treatments. The accumulation of H_2O_2 in HD and K326 was similar after pathogen stress.

SOD activity in tobacco leaves after pathogen infection: The SOD enzyme activity of HD showed a continuous increase after *A. alternata* and *E. turcicum* stress, especially the SOD enzyme activity of HD increased rapidly at 48h after inoculation with *E. turcicum*. Compared with the control group, the SOD enzyme activity of HD did not change from 24h to 48h after inoculation with *A. alternata*, which was 560 U/g, lower than that of the control group at 24h (622 U/g), and similar to that of the control group at 48h ; after inoculation with non-host pathogen *E. turcicum*, the SOD enzyme activity continued to increase significantly at 12h, 24h and 48h, and reached 805 U/g at 48h, which was significantly higher than that of the group at 12h and 24h. The enzyme activity was more than twice the 360 U/g at 12h, and significantly higher than the SOD enzyme activity of the control group and the inoculated *A. alternata* at 48h (Fig. 4A). After K326 was stressed by *A. alternata* and *E. turcicum*, the SOD activity of the two groups was inhibited at 12h, but the SOD activity was increased significantly at 24h and 48h after inoculation. After K326 was inoculated with *A. alternata* and *E. turcicum*, the SOD activity was decreased first and then increased continuously, which was inconsistent with the trend of non-inoculation treatment. Compared with the control group, the enzyme activity of *A. alternata* inoculated with K326 decreased significantly at 12h, but the subsequent enzyme activity was increased continuously. At 24h, the enzyme activity was the same as that of the control, and at 48h, the enzyme activity was 793 U/g higher than that of the control group 712 U/g; in addition, the SOD activity of *E. turcicum* was lower than that of *A. alternata* and far lower than that of the control group at 12h after inoculation. The SOD activity continued to increase significantly with the increase of inoculation time, and reached 800 U/g at 48h, which was significantly higher than that of the control group and the *A. alternata* group (Fig. 4B).

POD enzyme activity in tobacco leaves after pathogen infection: POD enzyme activity in HD tobacco plants showed a continuous increase from 12h to 48h after A. *alternata* stress. From 12h to 48h after *E. turcicum* stress, POD enzyme activity first showed a decrease, and then continued to increase. At 48h, it reached 158 U/g, nearly twice the level of this group at 12h, significantly higher than the enzyme activity at 12h and 24h in this group, and also significantly higher than the POD enzyme activity of the control group and inoculated *A. alternata* at 48h (Fig. 5A). The activity of *A. alternata* and *E. turcicum* increased after 12h and increased significantly until 48h. The POD enzyme activity of *E. turcicum* inoculated with non-host pathogen showed an increasing trend at 12h, and it showed 93 U/g at 12h, which was higher than that of the control 68 U/g at 12h. At the follow-up time points of 24h and 48h, the POD enzyme activity exhibited a very significant difference, which was increased to 203 U/g and 265 U/g, and 48h was the activity peak at three time points, far exceeding the enzyme activity at 12h. It was also significantly higher than the control group and the POD enzyme activity of *A. alternata* inoculated at 48h (Fig. 5B).

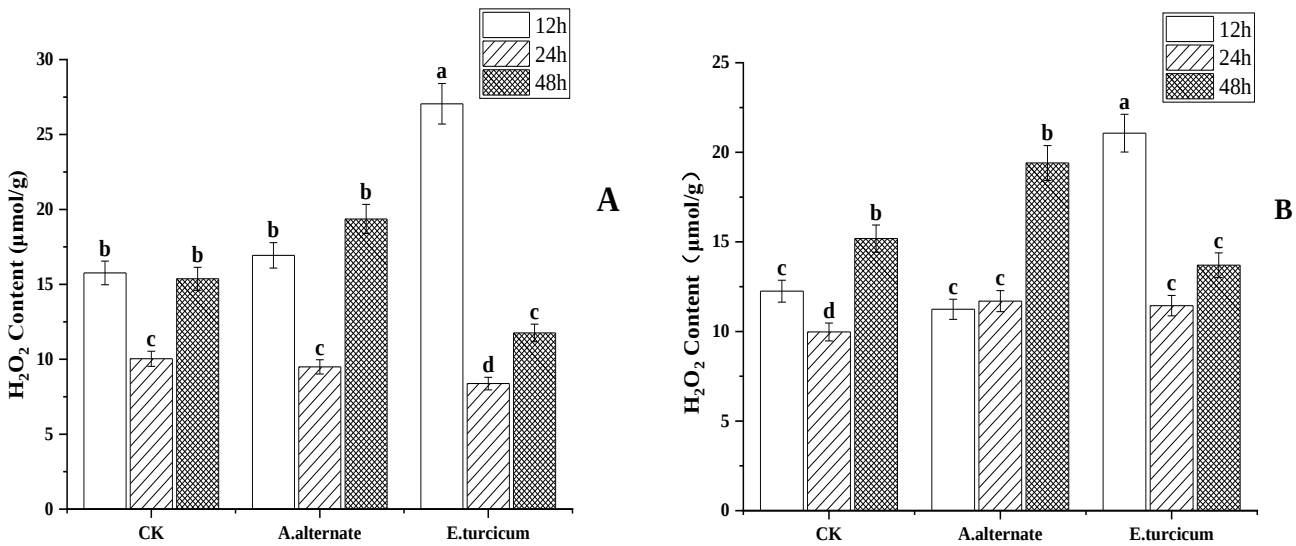

Fig. 3. Changes of H_2O_2 content in HD and K326 after 48h inoculation with *A. alternata* and *E. turcicum* (Fig. 3A-HD, Fig. 3B-K326). Different lowercase letters in the same group indicate that there is a significant difference between the control and the treatment at the 5% level; CK: control group, *A. alternata*: inoculated with *A. alternata*, *E. turcicum*: inoculated with *E. turcicum*, the same below.

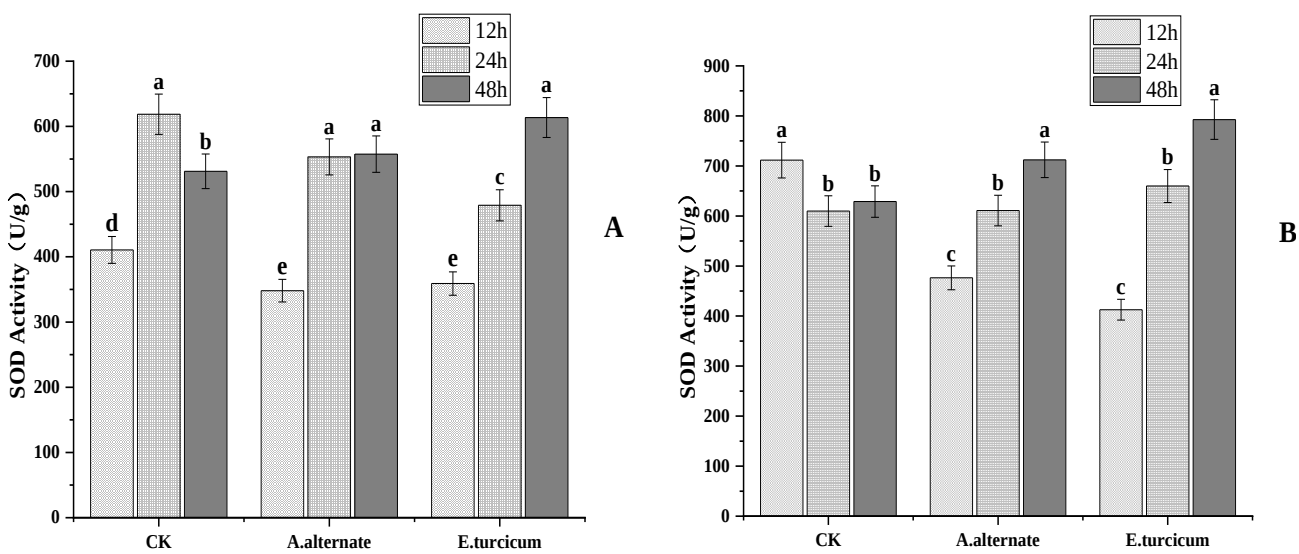

Fig. 4. Changes of SOD activity in HD and K326 after inoculating with *A. alternata* and *E. turcicum* for 48h (Fig. 4A-HD, Fig. 4B-K326).

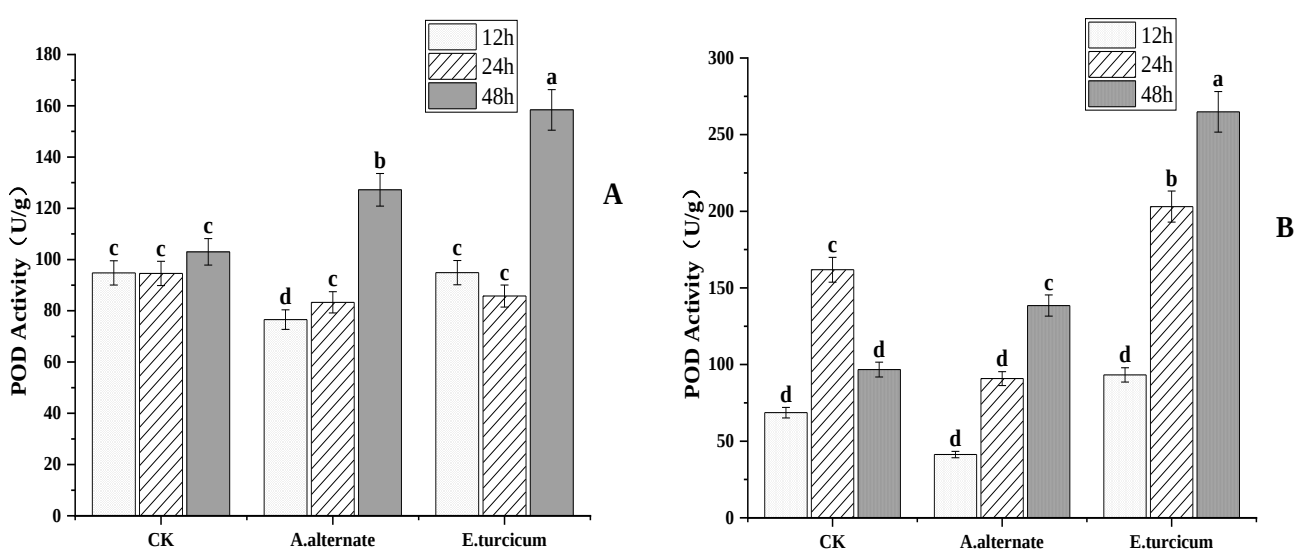

Fig. 5. Changes of POD activity in HD and K326 after inoculating with *A. alternata* and *E. turcicum* for 48h (Fig. 5A-HD, Fig. 5B-K326).

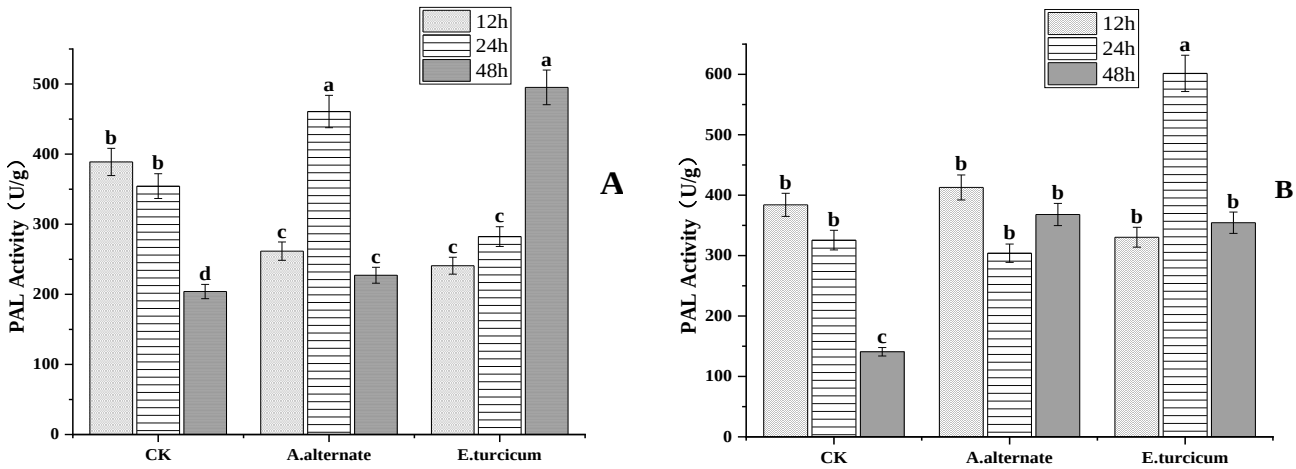

Fig. 6. Changes of PAL activity in HD and K326 after inoculation with *A. alternata* and *E. turcicum* for 48h (Fig. 6A-HD, Fig. 6B-K326).

PAL enzyme activity in tobacco leaves after pathogen infection: The changes of PAL enzyme activity at 12h, 24h and 48h after inoculation with *A. alternata* and *E. turcicum* showed that the PAL enzyme activity of HD was decreased at 12h and 24h after inoculation with *A. alternata* and *E. turcicum*, and increased at 48h. Compared with the control group, *A. alternata* inoculated with HD showed a decrease at 12h and 48h time points, but a certain increase at 48h compared with the control. Further analysis of the changes in the group, the inoculation of *A. alternata* 24h was significantly higher than the previous time point, but 48h was significantly inhibited; compared with the control group, the inoculation of *E. turcicum* showed significant inhibition from 12h to 24h, and the PAL enzyme activity was higher than that of CK control group and *A. alternata* group at 48h. Under the stress of the host pathogen *A. alternata*, the PAL activity was increased first and then decreased from 12h, while the PAL activity continued to increase under the stress of the host pathogen *E. turcicum*. In particular, the PAL activity of HD tobacco plant reached 513 U/g at 48h, which was about 3 times that at 12h (Fig. 6A).

Compared with the control group, K326 was significantly reduced by pathogenic fungi *A. alternata* at 24h compared with the previous time point, and there was a slow recovery trend at 48h and the enzyme activity was higher than the control. Further analysis of the changes within the group, the PAL enzyme activity of K326 tobacco plants showed a downward trend from 12h to 48h after *E. turcicum* stress, but the PAL enzyme activity in the whole tobacco plant was higher than 330 U/g, while the PAL enzyme activity in CK was 173 U/g at 48h, which was significantly lower than that at 24h. Under the stress of non-host fungus *E. turcicum*, the PAL enzyme activity of tobacco plants was higher than that of host pathogenic fungus *A. alternata*. In addition, by comparing the results of HD and K326 inoculated with *A. alternata* and *E. turcicum* 48h after inoculation, it showing that the PAL enzyme activity was increased more after inoculation with *E. turcicum* (Fig. 6B).

Discussion

The study of tobacco disease resistance related genes can help us to understand the defense mechanism of tobacco against pests and diseases, which is also of great significance to the green cultivation of tobacco. MAPK is a key pathway for plant defense. Also is progressively phosphorylated and then acts on the downstream transcription factors or target genes thus exerting a role in defending against the pathogens, also participate in stomatal regulation through cascade networks to resist biotic and abiotic stresses, and some genes act as regulatory genes for natural defense (Kwang *et al*., 2001; Yuree *et al*., 2016). In tobacco, the MPK6 ortholog SIPK (Salicylic acid-induced protein kinase) is activated by various biotic and abiotic treatments. The co-expression of SIPK and WRKY1 in tobacco enhances the induction of cell death, suggesting that SIPK mediates HR cell death by regulating WRKY1 (Menke *et al*., 2005; Adachi *et al*., 2016). In this experiment, NtMAPK, the upstream gene of SA pathway, was up regulated under the stress of *A. alternata* and *E. turcicum*, and the expression of NtMAPK gene under the stress of *E. turcicum* was higher than that of control and *A. alternata*. It indicated that NtMAPK responded to the immune mechanism and reacted more rapidly to non-host pathogens, resulting in plants showing stronger resistance as a whole. NPR family genes are key factors in SA signal transduction, which are currently studied and is found that the NPR1, NPR2, NPR3, NPR4, and NPR6 family of genes interact with SA to play a regulatory role. (Fu *et al*., 2012; Wang *et al*., 2020b). In this study, it was found that the expression levels of NtPR1, NtPR2 and NtPR11 genes under *E. turcicum* stress were higher than those under *A. alternata* stress and control, while the genes NtPR2 and NtPR11 were inhibited at 12h under *A. alternata* stress. The results showed that some genes of the NPR family had a higher signal response to *E. turcicum*, but were not sensitive to *A. alternata*, resulting in lower gene expression, indicating that non-host pathogens can induce host resistance gene expression and increase overall resistance to a certain extent.

For specific stress stimulation, the content of Jasmonic acid in plants increases rapidly, and causes genome-wide changes in gene transcript expression profiles. AOS, JAZ and MYC2 transcription factors can regulate the Jasmonic acid pathway. AOS is related to JA accumulation (Edward *et al*., 2010). JAZ and MYC2 directly regulate the opening and closing of the JA pathway (Li *et al*., 2021), so that plants can effectively resist various stresses. The expression of NtAOS gene in HD was up-regulated after pathogen stress, but the expression level of *E. turcicum* was higher than that

of *A. alternata* at each time point, indicating that JA pathway responded faster to non-host pathogen *E. turcicum*. The expression levels of NtJAZ and NtMYC2 genes in HD were higher than those in *A. alternata* stress and control at 48h after non-host pathogenic fungus *E. turcicum* stress. The NtJAZ gene showed a trend of first inhibition and then burst after inoculation with pathogenic fungi, and the response of inoculation with *E. turcicum* was greater, indicating that non-host pathogens had stronger ability to induce systemic resistance. The trend of NtAOS and NtJAZ gene expression in K326 was different from that of HD. The expression of NtAOS was lower at 48h than that at 12h. Although the expression of NtJAZ was increased, the expression of *E. turcicum* was not higher than that of *A. alternata* at 12h, which may be related to the characteristics of the variety.

Reactive oxygen species (ROS) are involved in both plant defense processes against pathogens HR and NHR, and are key signal messengers in plant defense responses, leading to stomatal closure, cell wall enhancement (Including callose deposition), accumulation of phenolic compounds, and induction of defense gene expression in infected cells (O'Brien *et al*., 2012; Qi *et al*., 2017; Singh *et al*., 2017). The antioxidant system present in plants initiates scavenging process in the presence of excessive ROS (Dumanovic *et al*., 2021). In this experiment, the content of H_2O_2 in HD and K326 inoculated with *E. turcicum* increased first and then decreased rapidly to maintain the conventional level. Studies have shown that H_2O_2 excitation is instantaneous when plants encounter stress, and H_2O_2 can accumulate rapidly in a short time. This study is consistent with its research results. The HD and K326 inoculated with *A. alternata* eventually showed an upward trend, which may be related to the reaction time of the active oxygen antioxidant system, and the active oxygen of the tobacco plant can be quickly removed after the infection of the non-host pathogen. There is a dynamic and well-integrated biological signal mechanism in plant cells to control the defense response of plants to pathogens. After the host is infected or induced by the pathogen, it will induce some disease resistance-related enzymes. SOD, POD and PAL enzymes are important defense enzymes (Manikandan *et al*., 2021). Compared with the control, the SOD activity of HD inoculated with *A. alternata* did not change much, while the SOD activity of *E. turcicum* inoculated for 48h was significantly increased. The SOD enzyme activity of the two varieties showed different trends, which may be related to the resistance of the varieties themselves, but the enzyme activity of the two varieties was the highest at 48h after inoculation with *E. turcicum*, indicating that *E. turcicum* induced SOD enzyme activity to increase more significantly, and SOD enzyme was more prominent in response to non-host pathogens. The POD enzyme activity of K326 showed an upward trend after inoculation with two pathogens, indicating that the POD enzymes of HD and K326 varieties were less affected by the pathogen types when dealing with host and non-host pathogens. The PAL enzyme activity of *A. alternata* inoculated was consistent with the trend of the control group, while the PAL enzyme activity increased when inoculated with *E. turcicum*, indicating that the PAL enzyme did not respond to host pathogen infection. The recognition reaction of non-host pathogens promotes the increase of enzyme activity, which may be caused by *A. alternata* 's "silent response" (Li *et al*., 2022). After K326 was inoculated with these two pathogens, the PAL activity of *E. turcicum* was higher than that of the control and *A. alternata* group, reflecting that the PAL enzyme had a stronger effect on *E. turcicum* in K326 varieties. PAL enzyme is closely related to fungal infection in plants. PAL enzyme is related to lignin formation. Lignin accumulation enhances the ability of cells to resist fungi. In this experiment, non-host pathogen *E. turcicum* induces an increase in PAL enzyme activity. In a sense, it can induce plants to resist fungi through non-host pathogens. Bednarek (Bednarek *et al*. 2005) published the differential expression of host and non-host pathogens infecting *Arabidopsis thaliana*, showing the mechanical and genetic defense levels of *Arabidopsis thaliana* plants against host and non-host pathogens, and it has been observed that the defense differences against non-host pathogens will be more prominent. This experiment has similar conclusions with this study in terms of gene expression level and defense enzyme activity, showing that tobacco plants infected by non-host pathogens have stronger defense response and higher resistance level.

The defense level of plants against host pathogens and non-host pathogens is different. Nina (Nina *et al*., 2010) showed that plants activate similar processes, but strong transcriptional activation or inhibition occurs earlier in non-host interactions by comparing and analyzing transcriptional profiles after inoculation with adaptive and non-adaptive pathogens. The transcription of the test gene was activated, and the upstream gene NtMAPK of the SA of HD and K326 positively regulated the downstream NtPR1, NtPR2, and NtPR11 genes, which showed that the upstream genes in the SA pathway were highly expressed and the downstream genes were also highly expressed. Changes in defense-related gene expression and transcription factor activity can amplify defense signals in a positive feedback loop, thereby enhancing NHR. However, various germplasms of non-host species show different responses to the same non-adaptive pathogen or its effectors (Mellersh & Heath, 2001; Wroblewski *et al*., 2009). The upstream gene NtAOS of JA pathway in HD positively regulated the downstream NtJAZ and NtMYC2 genes, which showed that the upstream and downstream genes were highly expressed at the same time point. The upstream gene NtAOS in the K326 variety showed negative regulation on the downstream genes NtJAZ and NtMYC2. It may also be that the higher expression of the downstream gene showed a reverse regulation of the upstream gene. The expression of the upstream gene was higher and the expression of the downstream gene was lower at 12h, and the expression of the upstream gene was decreased and the expression of the downstream gene was increased at 24h. The concentration of reactive oxygen species in plant cells under biotic and abiotic stresses increases, and then reactive oxygen species are metabolized to H_2O_2. In recent years, studies have found that H_2O_2 acts as an auxiliary messenger in the signal transduction network and participates in plant stress response (Ina *et al*., 2016). As a defense line of tobacco disease resistance, defense enzymes have been widely studied in recent years (Baohai *et al*., 2004; Fu *et al*., 2013; Yuanchan *et al*., 2021). In this experiment, SOD and POD enzymes showed the same trend after being stressed by pathogens, suggesting that there was a certain signal transmission

between SOD and POD to induce plant resistance. Through this experimental study and previous research results (Lijing *et al*., 2016; Ning *et al*., 2019; Qiuying *et al*., 2019; Yuanyuan *et al*., 2019; Yanan *et al*., 2020; Xianping *et al*., 2021), speculated that there may be a defense pathway in tobacco (Fig. 7). NHR and HR are homologous and partially overlapped. Salicylic acid (SA) and Jasmonic acid (JA) are the main endogenous signals to mediate plant HR and NHR resistance, accompanied by the expression of molecules in plants (Baruah *et al*., 2020). At the same time, some related enzymes also play an important regulatory role, such as superoxide dismutase (SOD). Catalase (CAT) and peroxidase (POD). (Ruifang *et al*., 2007).

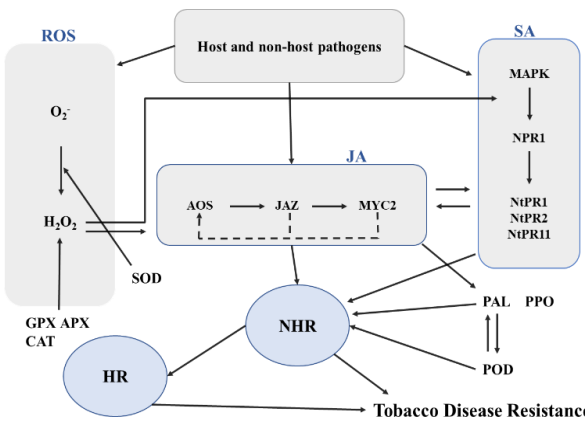

Fig. 7. Non-host disease resistance network of tobacco induced by pathogenic fungus.

Conclusion

The stress of non-host pathogenic fungi (*E. turcicum*) on HD and K326 resulted in higher expression of resistance genes in the salicylic acid (SA) pathway and jasmonic acid (JA) pathway; In the defense enzyme system, the defense enzyme activity induced by *E. turcicum* had the same defense tendency but a higher level of defense compared with that of the host pathogenic fungus (*A. alternata*), and both the results indicate that *E. turcicum* is able to induce improved non-host disease resistance in tobacco.

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