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HEAT SHOCK TREATMENT ENHANCES THE ABILITY OF CHINESE YAM TO RESIST *CURVULARIA LUNATA* INFECTION

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Abstract

This experiment explored the effects of different heat-shock temperatures on the disease resistance of yam, aiming to enhance resistance to *Curvularia lunata* infection and reduce pesticide usage without compromising yam quality. Yam seedlings were subjected to heat treatments for 1.5 hours before inoculating them with *Curvularia lunata.* Changes in yam leaves were observed using Diaminobenzidine and lactophenol-trypan blue staining methods. The expression of heat-shock genes and disease-resistance genes *PTI5* and *PR1* in yam plants was detected by qPCR. The Allantoin of yams was measured by HPLC, and the polysaccharide content was determined by the phenol-sulfuric acid method. The results showed that the leaves of yam subjected to the 45°C heat treatment exhibited minimal damage and heightened resistance to *Curvularia lunata*. Conversely, leaves from non-treated yams showed significant damage. qPCR analysis confirmed that 45°C heat treatment significantly upregulated heat shock and anti-fungal gene expression, aligning with phenotypic observations. Furthermore, urea and polysaccharide contents were increased in yams after the 45°C heat treatment. The study demonstrated a cross-response mechanism between yam heat-shock and disease resistance genes, offering an effective solution to fungal diseases and pesticide issues in yams, potentially improving crop quality. These findings may serve as a reference for yam disease resistance and breeding and could apply to other crops for developing eco-friendly disease resistance strategies.

Key words: Yam; Disease-resistant; Heat-shock; *PTI5*.

Introduction

Yam, recognized as (*Dioscorea opposita* Thunb.), is a perennial vine of the Dioscoreaceae family. Predominantly found in tropical and subtropical areas, its tubers are white, fleshy, and abundant in starch, protein, and crude fiber. Yam is a prevalent food crop globally, and the variety 'Xiaobaizui' investigated in this study is extensively cultivated in Hebei Province, China. Modern pharmacological research has revealed the multifaceted benefits of yam, such as its capacity to lower blood sugar and blood lipids, exhibited antioxidant properties, and its widespread application in the clinical management of conditions like diabetes, nephritis, and cancer. Due to its significant nutritional, medicinal, and economic value, yam is considered a promising crop with strong market potential and substantial prospects for development. Despite numerous advantages, yam plants frequently encounter a range of biotic and abiotic stresses throughout their growth (Yi *et al.*, 2020), significantly impacting yield and quality. Diseases and prevalent biotic stress are primary determinants of yam quality, leading to leaf loss, withering, and even plant mortality. While the primary focus of yam cultivation is on preventing and managing tuber diseases, it is essential to note that leaf diseases can also have a significant impact on photosynthesis, ultimately reducing both the yield and quality of yam tubers. The fungus *Curvularia lunata* used in this study can induce yam leaf blight, resulting in damage to leaf structures and potentially causing plant death, thereby severely compromising yield and quality. Previous studies have reported that certain plants exhibit heightened disease resistance following exposure to heat-shock treatment. For example, rice displayed a notable elevation in the expression of the HSF gene after heat-shock treatment, enhancing its resistance to diseases like white leaf blight and rice blast (Tanabe *et al*., 2016). Heat-shock genes can also regulate plant disease resistance by modulating the expression of disease resistance genes, enhancing the plant's overall disease resistance.

For most plants, defense mechanisms are divided into three types, including pathogen-associated molecular pattern (PAMP) triggered immunity (PTI), effector-triggered immunity (ETI), and RNAi-mediated defense. PTI offers broad-spectrum defense, whereas ETI is crucial in the secondary defense mediated by effectors. When pathogens infect plants, both PTI and ETI can be used to induce the expression of antimicrobial peptides (AMPs), pathogenesis-related (PR) proteins, RNA-interference (RIP) molecules, defensive secondary metabolites, and physiologically defensive compounds in plants. Among them, PR proteins accumulate in diseased plants and directly or indirectly participate in plant defense mechanism against pathogens (Muthamilarasan & Prasad, 2013). As a foundational element of the immune response, PTI is evolutionarily conservative, pivotal in plant pathogen resistance, and characterized by broad-spectrum and persistent characteristics. The *PTI5* gene, a significant member of the PTI disease-resistant gene family,

contributes to the defense mechanisms in peppers, enhancing disease resistance (Venkatesh *et al*., 2016). In tomatoes, *PTI5* and *PR1* genes significantly bolster resistance against *Botrytis cinerea* - induced gray mold (Wang *et al*., 2021; Zaid *et al*., 2022); Similarly, wheat exhibits increased *PR1* gene expression upon fungal infection, enhancing defense against pathogens (Ghorbel *et al*., 2021). While the molecular defense mechanisms of yam against fungal infections remain unexplored, the roles of *PTI5* and *PR1* genes in fungal resistance suggest their potential involvement in yam's defense against *Curvularia lunata*.

This study aims to enhance the expression of anti-fungal genes in yam plants through heat treatment to improve their resistance against *Curvularia lunata*. This approach diminishes the pesticides usage, thereby mitigating environmental pollution and aligning with sustainable, eco-friendly objectives. Additionally, this research investigates the impact of optimal heat treatment on the quality of yam, offering insights into eco-friendly cultivation and seed breeding strategies.

Material and Methods

Samples and reagents: In this study, the experimental subjects were young seedlings of var. 'Xiaobaizui' yam, germinated and cultured from uniformly sized bulbils acquired from Anguo City, Hebei Province, China. Heat-shock treatment was performed on yam seedlings at the two-leaf stage. The pathogen employed was *Curvularia lunata*, sourced from the China Agricultural Microbial Culture Collection Management Center. The total RNA extraction kit, cDNA reverse transcription kit, and qPCR kit were supplied by Nanjing Vazyme Company. The primer synthesis was primarily conducted by Sangon Biotech in Shanghai. Methanol and acetonitrile of chromatographic grade were utilized in the study. The urea reference material was sourced from Shanghai Yuanye Biotechnology Co., Ltd., and the D-glucose standard material was obtained from Beijing Solarbio Co., Ltd. Yam rhizomes from the optimal heat-shock treatment group and the normal temperature culture group were harvested in the autumn, dried, pulverized, and sieved. The analysis was performed using a Shimadzu LC-2030C high-performance liquid chromatograph. For the analysis of urea, the following chromatographic conditions were employed: Agilent ZORBAX Eclipse XDB C18 chromatographic column (4.6 mm × 250 mm, 5 μm); mobile phase: methanol-water (5:95); flow rate: 1 mL/min; injection volume: 10 μL/time; detection wavelength: 204 nm; column temperature: 30°C.

Heat-shock treatment: Heat-shock treatment at various temperatures was applied to yam seedlings, which resulted in changes to the visible morphology and gene expression in the leaves. The seedlings were treated at 40°C, 45°C, and 50°C for 1.5 hours in a constant-temperature incubator. After the heat-shock treatment, alterations in the gene expression of the yams were noted. Leaves were then rapidly cooled using liquid nitrogen and stored at a -80°C ultra-low-temperature freezer. Subsequently, fungal inoculation treatment was conducted on the yam plants in each group.

Pathogen treatment: White and transparent spores of *Curvularia lunata* were cultured on Potato Dextrose Agar (PDA) for 7 days. These spores were then suspended in sterile water and observed under a microscope, and their concentration was determined using a hemocytometer. Following this, the spores were diluted or transferred in sterile water (Liu *et al*., 2010). The yam seedlings underwent inoculation with these spores, which were evenly sprayed onto the leaves of the plants. Observations of the plants were continued for 7 days post-inoculation.

DAB staining: DAB staining solution was prepared, followed by immersion of leaves from both inoculated and non-inoculated yam groups in the solution. These samples were then stored in darkness at 25°C for 24 hours. The stained leaves were decolorized in an 80% ethanol boiling water bath for 10 minutes. The coloring of each group of leaves was observed and photographed.

Lactic acid-trypan blue staining: To prepare 100 mL of 0.5% trypan blue stock solution, lactic acid, glycerol, phenol, distilled water, and trypan blue were combined and dissolved. The stock solution was then diluted with an equal volume of ethanol to create the working solution, which was to be recycled after use. The leaves of both inoculated and non-inoculated groups of yam were immersed in the staining solution and boiled for 5-6 minutes. The decolorization was carried out using a chloral hydrate solution (2.5 g of chloral hydrate dissolved in 1 mL of distilled water) until there was no floating color of trypan blue on the leaves (the uncolored areas were almost colorless). The coloration of the leaves in each group was then observed and photographed (Zahid *et al*., 2021).

Quantitative PCR detection of gene expression: Experimental primers were designed based on yam genes listed in the NCBI database, with primer specificity analyzed for comparison (Tamiru *et al*., 2017; Mignouna *et al*., 2002). RNA was extracted from yam leaves and subsequently reverse transcribed to obtain cDNA, which served as a template for qPCR amplification reactions on samples subjected to various temperature heat treatments. The experiment comprised 3 biological replicates, each with 3 technical replicates. The $2^{-\triangle\triangle Ct}$ method was used to analyze the relative expression levels of genes, and statistical software SPSS was used for statistical analysis (Table 1).

Table 1. Primer sequence.

Primer	Forward primer	Reverse primer
Actin-like (ACT)	GCATGAGCAAGGAAATCACAGCAC	GGAAGCCAAGATAGAGCCACCAATC
Pathogenesis-related genes transcriptional activator PTI5-like (PTI5)	GAACGAGAATGACTCTCAGG	CCCTAATCTCAGCTGCATAC
Pathogenesis-related protein PR-1-like (PR1)	CTGTACACAAACACAGGGAC	GAGGTTCTCGCCATAGTTAC
Heat stress transcription factor A-2c (HSFA2c)	TCTCCAGGCTCTAGAAGTGA	AGGATCAGGCACTCTGTCTA
26.5 kDa heat shock protein (HSP26.5)	GGAGGAGTTCTCTCCTTCAT	CGGTCACTGTCAGTTGAGTA
Heat shock protein 90-6 (HSP90-6)	GAGATCCTCCAAGAAAGTCG	CCTCTTCTTGATCCAGTC`AC
Heat stress transcription factor B-2b-like (HSFB)	CACCTACAAACTCCAGTGAG	GCATCCTCCATTAGACTCAC

The results of allantoin content for yam: An Allantoin reference solution, accurately weighed at 1 mg, was introduced into a 5 mL volumetric flask, which was then filled to the mark with 20% methanol. After even shaking, a 0.2 mg/mL stock solution was prepared. The stock solution was further diluted to generate a range of concentrations for constructing an allantoin standard curve.

In a stoppered conical flask, precise amount of normal and heat-treated yam powder (0.25 g) was combined with 20% methanol (25 mL). The flask was sealed, shaken, and weighed. The mixture was sonicated for 0.5 h, cooled, and reweighed. The weight loss was compensated with 20% methanol. The mixture was then shaken, centrifuged at 13,000 rpm for 10 minutes, and the supernatant was collected for allantoin content analysis. The allantoin content in different groups was calculated using the external standard method.

Determination of polysaccharide content: To prepare the glucose standard solution, accurately weighed 10 mg of anhydrous glucose was dried at 105℃ to constant weight, dissolved in distilled water to make up to 100 mL, and mixed thoroughly to achieve a 0.1 mg/mL glucose standard solution. To prepare a 6% phenol solution, 6g of phenol was precisely weighed, dissolved in distilled water, and diluted to a total volume of 100 mL. The solution was thoroughly mixed and stored in a refrigerator for subsequent use.

For the standard curve, varying volumes (0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL, and 1.2 mL) of a 0.1 mg/mL glucose standard solution were transferred using a micropipette into separate tubes and adjusted with water to a total volume of 2.0 mL each. To each tube, 1.0 mL of the 6% phenol solution and 5.0 mL of concentrated sulfuric acid were added, mixed immediately, and allowed to stand for 20 minutes at room temperature, away from light. The absorbance was measured at 490 nm to construct the standard curve. After that 3 g of normal and 45℃ heat-treated yam root and stem powder were weighed and subjected to reflux extraction with fivefold 80% ethanol for 1 hour at 90℃ in a water bath. The residue was collected, dried, and re-extracted twice with thirtyfold distilled water by refluxing for 2 hours at 90℃. After filtration, the extracts were combined and concentrated. 5-fold 95% ethanol was added while stirring rapidly and poured slowly, followed by standing at 4℃ for 12 hours, then centrifugation at 4200 rpm/min and drying. The dried sample was dissolved in water, stored at -80℃, and freeze-dried after 3 days to yield yam polysaccharide. A 0.1 mg/mL solution of yam polysaccharide was prepared for analysis. A precise volume of 1.0 mL of the solution was pipetted for sample analysis, diluted to 2.0 mL with water, and the absorbance was measured at 490 nm. The content of yam polysaccharide was calculated based on the standard curve.

Results

Phenotypic results of yam seedling

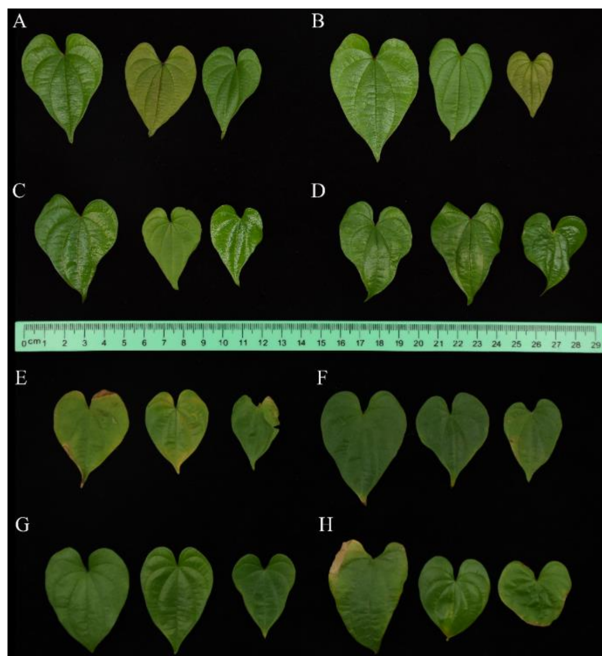

Fig. 1. Phenotypic variations in yam leaves under different treatments.
A: Phenotypes of normal yam leaves; B: Phenotypes of yam leaves after 40℃ heat-shock treatment; C: Phenotypes of yam leaves after 45℃ heat-shock treatment; D: Phenotypes of yam leaves after 50℃ heat-shock treatment; E: Phenotypes of yam leaves after pathogens inoculation; F: Phenotypes of yam leaves after pathogens inoculation with 40℃ heat-shock treatment; G: Phenotypes of yam leaves after pathogens inoculation with 45℃heat-shock treatment; H: Phenotypes of yam leaves after pathogens inoculation with 50℃ heat-shock treatment).

The results of accumulation of H_2O_2 by yam

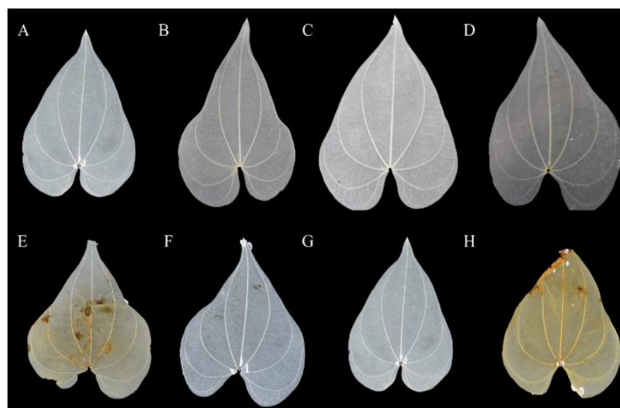

Fig. 2. The blade phenotype changes were treated by different temperatures and DAB staining.
(Note: A, E: Phenotypes of yam leaves under normal temperature cultivation with and without pathogens inoculation, respectively; B, F: Phenotypes of yam leaves under 40℃ heat-shock treatment with and without pathogens inoculation, respectively; C, G: Phenotypes of yam leaves under 45℃ heat-shock treatment with and without pathogens inoculation, respectively; D, H: Phenotypes of yam leaves under 50℃ heat-shock treatment with and without pathogens inoculation, respectively).

'Xiaobaizui' yam bulbils were used to culture seedlings, which underwent different temperature heat treatments upon reaching the two-leaf stage. No significant changes were observed in yam leaves after 40°C and 45°C heat treatments (Fig. 1B, C), whereas leaves exhibited curling and wrinkling post 50°C treatment (Fig. 1D). After 3 days of treatment, the yam leaves were inoculated with *Curvularia lunata*. Leaves without heat treatment showed the most severe damage post-inoculation (Fig. 1E), while those treated at 40°C exhibited less damage (Fig. 1F). Yam leaves subjected to heat treatment at 50°C exhibited significant damage (Fig. 1H), possibly due to the excessively high heat-shock temperature, leading to cell death in the leaves. Conversely, leaves treated at 45°C showed comparatively less damage (Fig. 1G). Phenotypic analysis revealed that yam leaves treated at 45°C experienced reduced damage and enhanced resistance against *Curvularia lunata* infection.

DAB staining was employed to assess leaf damage and validate the phenotypic observations of yam leaves. DAB staining analysis revealed that leaves subjected to 50℃ heat treatment accumulated more H_2O_2 (Fig. 2D) than those under 40℃ and 45℃ treatments (Fig. 2B, C), with normal leaves showing minimal H_2O_2 accumulation. After inoculation, the highest H_2O_2 accumulation was observed in non-heat-treated leaves (Fig. 2E), whereas leaves treated at 45℃ exhibited the lowest H_2O_2 accumulation in the inoculated group (Fig. 2G). H_2O_2 accumulation was greater in leaves treated at 40℃ and 50℃ compared to those at 45℃ in the inoculated group (Fig. 2F, H), especially under the 50℃ condition, suggesting potential high-temperature stress effects on the plants. Further investigations are warranted to delve deeper into this phenomenon. Initial findings indicated that the 45℃ heat treatment enhances yam plant resistance against *Curvularia lunata* infection.

Microscopic observations showed? cellular-level changes in yam leaves across different groups, complementing the staining results. Observations revealed that, in the absence of *Curvularia lunata* inoculation, leaves exposed to 50℃ heat treatment exhibited the most significant H_2O_2 (Fig. 3S, T, U). After inoculation with the fungus, the yam leaves under the 45℃ heat-treatment condition in the inoculated group showed the lowest accumulation of H_2O_2 (Fig. 3P, Q, R), whereas the highest H_2O_2 levels were noted in the non-heat-treated leaves of the inoculated group (Fig. 3D, E, F). Combining phenotypic and microscopic analyses indicated that yam plants subjected to 45℃ heat treatment experienced less damage and demonstrated enhanced resistance against *Curvularia lunata* infection.

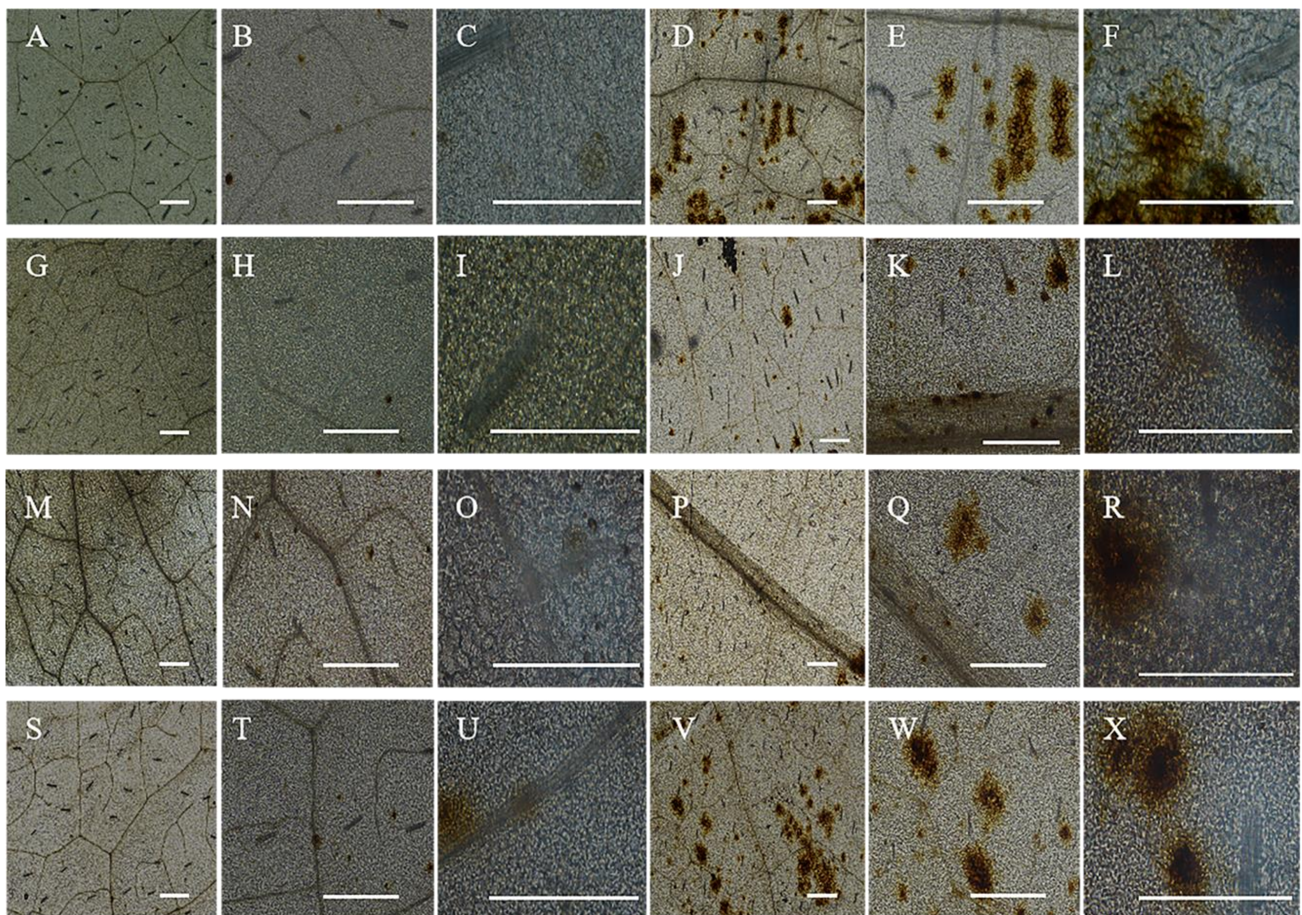

Fig. 3. The blade microscopic changes were treated by different temperatures and DAB staining.
(Note: A, B, C: Micrographs of yam leaves without pathogens inoculation and without heat-shock treatment; D, E, F: Micrographs of yam leaves with pathogens inoculation and without heat-shock treatment; G, H, I: Micrographs of yam leaves under 40°C heat-shock treatment without pathogens inoculation; J, K, L: Micrographs of yam leaves under 40°C heat-shock treatment with pathogens inoculation; M, N, O: Micrographs of yam leaves under 45°C heat-shock treatment without pathogens inoculation; P, Q, R: Micrographs of yam leaves under 45°C heat-shock treatment with pathogens inoculation; S, T, U: Micrographs of yam leaves under 50°C heat-shock treatment without pathogens inoculation; V, W, X: Micrographs of yam leaves under 50°C heat-shock treatment with pathogens inoculation. Bars= 500 μm).

Lactic acid-trypan blue staining results

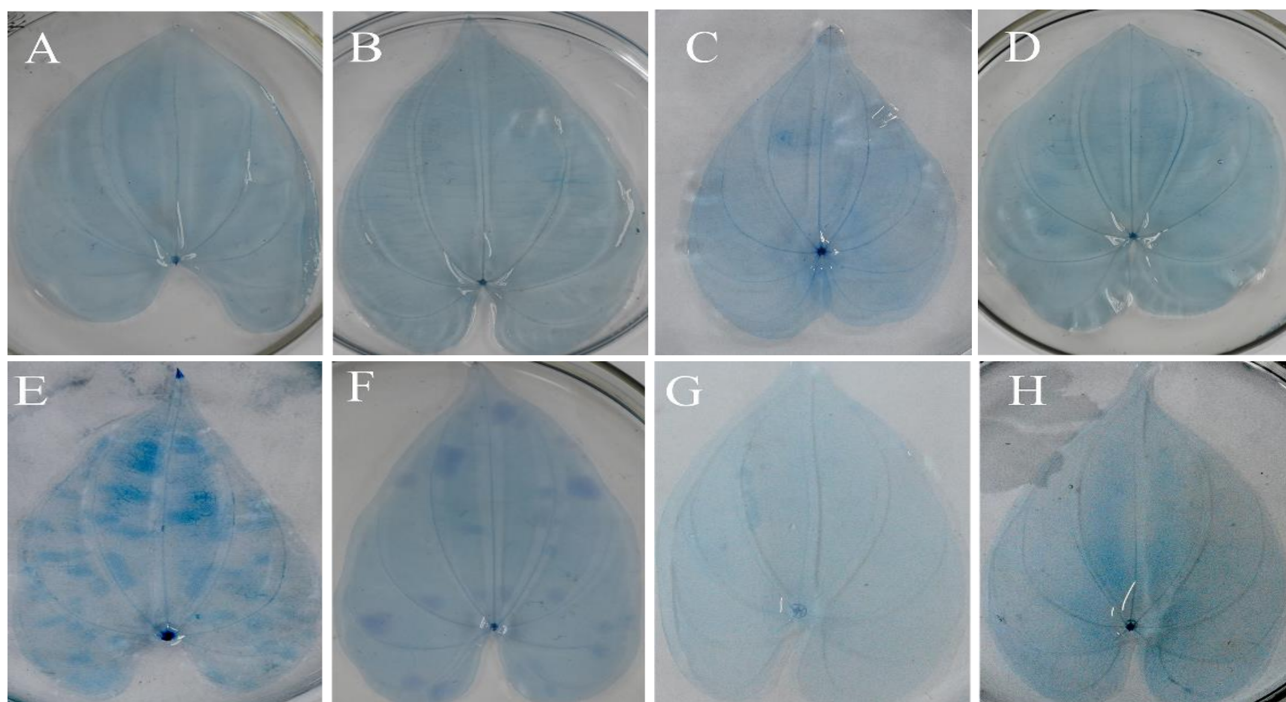

Fig. 4. Leaf phenotype changes of yam treated with different temperatures and trypan blue.
(Note: A, E: Phenotypes of yam leaves under normal temperature cultivation without and with pathogens inoculation, respectively; B, F: Phenotypes of yam leaves under 40°C heat-shock treatment with and without pathogens inoculation, respectively; C, G: Phenotypes of yam leaves under 45°C heat-shock treatment with and without pathogens inoculation, respectively; D, H: Phenotypes of yam leaves under 50°C heat-shock treatment with and without pathogens inoculation, respectively).

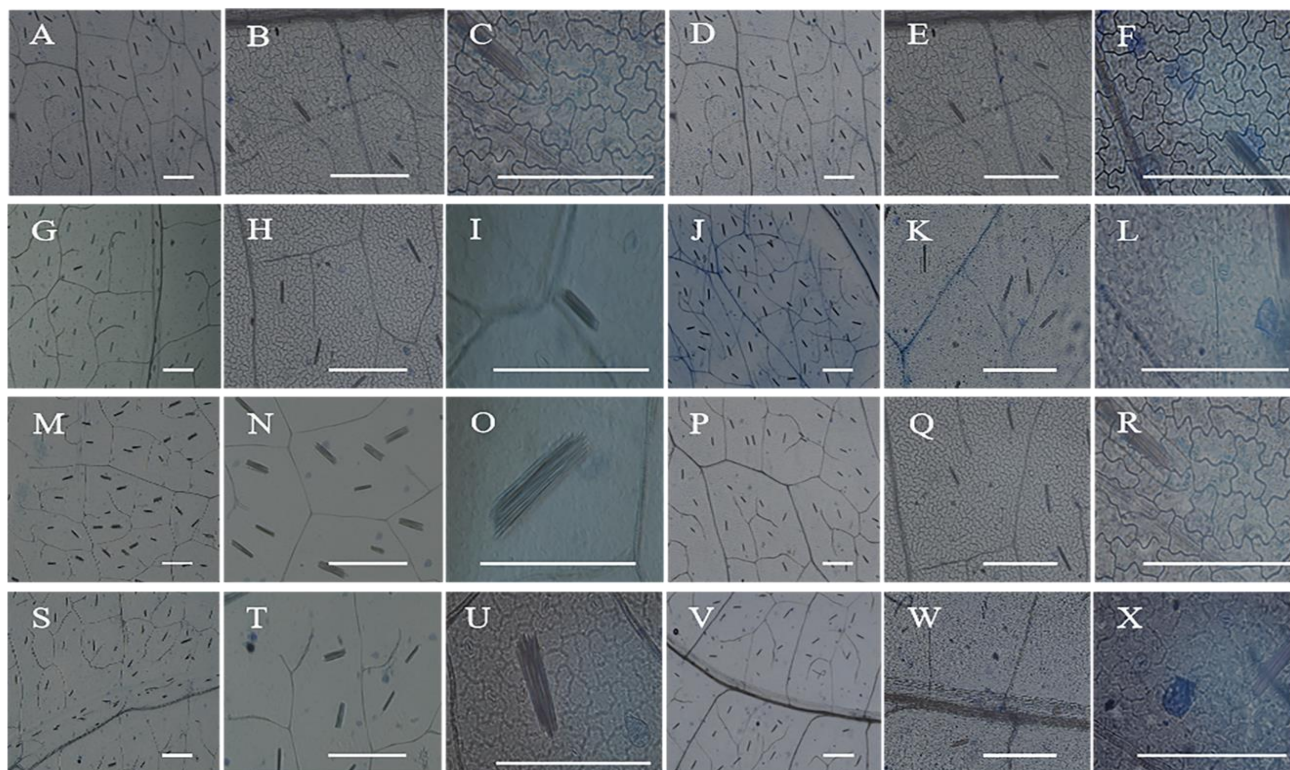

Fig. 5. The microscopic changes of yam leaves under different temperatures and trypan blue treatments.
(Note: A, B, C: Micrographs of yam leaves without pathogens inoculation and without heat-shock treatment; D, E, F: Micrographs of yam leaves with pathogens inoculation and without heat-shock treatment; G, H, I: Micrographs of yam leaves under 40°C heat-shock treatment without pathogens inoculation; J, K, L: Micrographs of yam leaves under 40°C heat-shock treatment with pathogens inoculation; M, N, O: Micrographs of yam leaves under 45°C heat-shock treatment without pathogens inoculation; P, Q, R: Micrographs of yam leaves under 45°C heat-shock treatment with pathogens inoculation; S,T,U: Micrographs of yam leaves under 50°C heat-shock treatment without pathogens inoculation; V, W, X: Micrographs of yam leaves under 50°C heat-shock treatment with pathogens inoculation. Bars= 500 μm).

The resistance of yam plants to disease was assessed using the cell death rate, with trypan blue staining employed to visualize dead cells. Following trypan blue staining, leaves from different yam groups were examined to evaluate changes post-heat treatment and fungal inoculation. The analysis revealed that, in the absence of fungal inoculation, leaves subjected to 50℃ heat shock exhibited a high cell death rate (Fig. 4D), whereas leaves under 40℃ and 45℃ heat shock conditions showed lower cell death rates (Fig. 4B and 4C). After inoculation with fungi on yam leaves, the highest cell death rate was observed in leaves without heat treatment (Fig. 4E), while leaves treated at 45℃ demonstrated the lowest cell death rate in the inoculated group (Fig. 4G).

In the inoculated group, leaves subjected to 40℃ and 50℃ heat shock exhibited a relatively high cell death rate (Fig. 4F and 4H). Microscopic examination of the cell death rate indicated that 45℃ heat shock treatment effectively mitigated cell death in yam leaves following fungal invasion (Fig. 5P, Q, R). Conversely, the highest number of dead cells was observed in yam leaves without heat shock treatment in the inoculated group (Fig. 5D, E, F). Leaves treated at 40℃ in the inoculated group showed a lower number of dead cells (Fig. 5J, K, L), whereas those under 50℃ heat shock had a higher number of dead cells compared to the 45℃ treatments in the inoculated group (Fig. 5V, W, X). The increased cell death at 50℃ may be attributed to excessive temperatures compromising cell structure and stomatal integrity. The trypan blue staining results confirmed that 45℃ heat shock treatment effectively counters *Curvularia lunata* invasion and reduces cell death in yam leaves.

The results of the antifungal and heat-shock gene expression

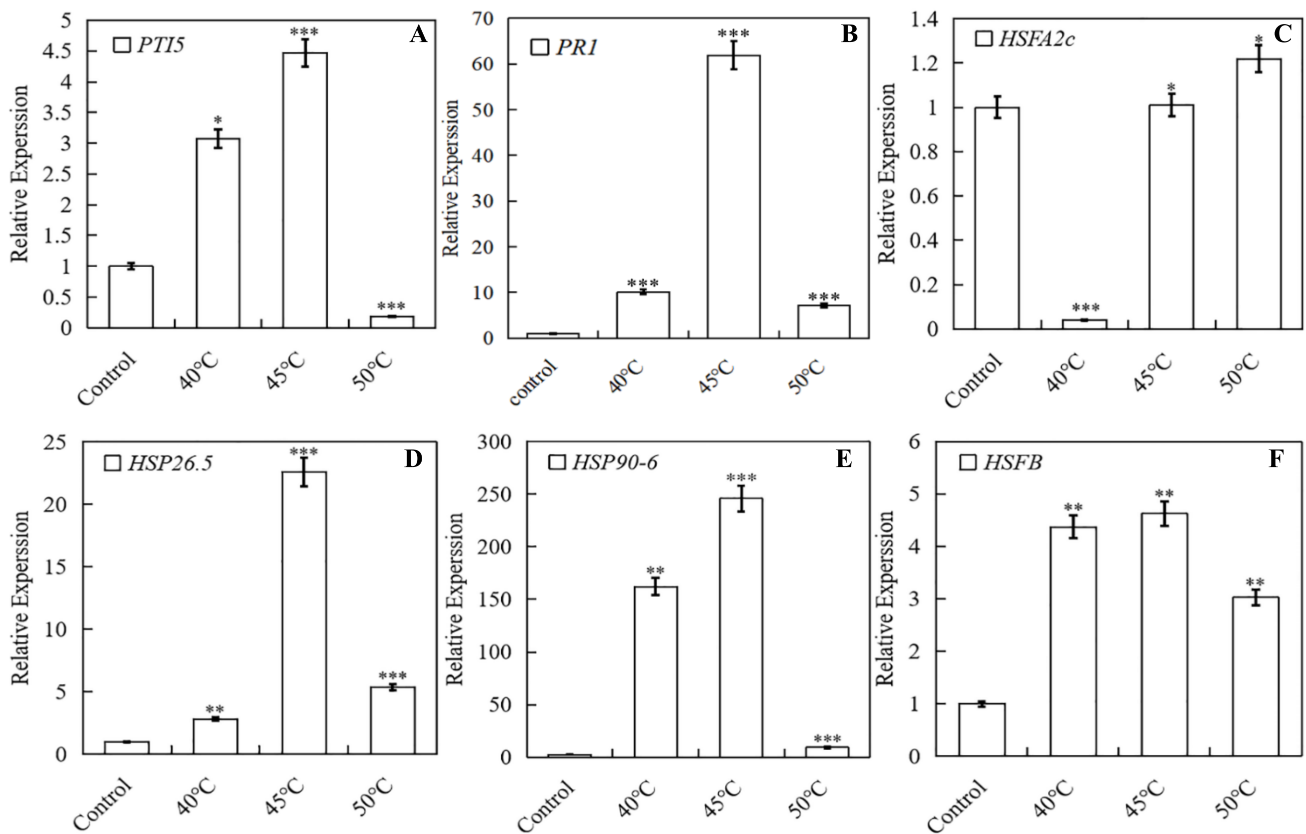

Fig. 6. qPCR results.
(Note: A and B show the expression of disease resistance genes *PTI5* and *PR1* in each group without pathogens inoculation; C, D, E and F show the expression of heat-shock genes *HSFA2c*, *HSP26.5*, *HSP90-6* and *HSFB* in each group without pathogens inoculation. * Indicates significant difference at p<0.05; ** indicates significant difference at p<0.01; *** indicates significant difference at p<0.001).

To elucidate the molecular basis of Yam's resistance to *Curvularia lunata* post-heat treatment, we examined the expression levels of resistance genes *PTI5* and *PR1*, alongside heat-shock genes, under varying heat treatment conditions. This investigation aimed to understand the effect of these genes on the fungus resistance of yam. The results showed that the relative expression of *HSFA2c* did not increase significantly under different heat-shock conditions and even declined under 40℃ heat-shock conditions Fig. 6C). The relative expression of *HSP26.5* increased significantly under different heat-shock conditions, particularly at 45℃ (Fig. 6D), whereas the expression levels were lower at 50℃ compared to that at 45℃. The relative expression of *HSP90-6* showed a substantial increase in expression at 40℃ and 45℃ and significant upregulation at 50℃, although it was notably lower than the former two conditions. The expression of *HSFB* exhibited a significant increase in expression levels across different heat-shock conditions, with the highest expression observed at 45℃. The relative expression of the resistance gene *PTI5* was significantly elevated at 40℃ and 45, with a higher expression at 45℃ than at 40℃ (Fig.

6A). The relative expression of the resistance gene *PR1* increased significantly under 45℃ heat-shock conditions. Analysis of the quantitative PCR results revealed that heat-shock treatment at 45℃ notably increased the expression of heat-shock genes and *PTI5*, consequently enhancing the resistance of yam to *Curvularia lunata*.

Determination of active ingredient content: Our research confirmed that heat stress could enhance yam's disease resistance, yet typically, increased resistance was associated with a decline in crop quality, as the disease resistance mechanism is energy-intensive and may negatively impact yield traits (Brown., 2002; Liu *et al*., 2019). To investigate the effect of heat treatment on the quality of yam, this experiment used HPLC technology to determine the content of Allantoin in the roots and stems of normal (Fig. 7B) and 45°C heat-treated yam (Fig. 7A). Additionally, we extracted polysaccharides using a water-alcohol precipitation method and quantified them using phenol-sulfuric acid. A standard curve was constructed with allantoin concentration (C) on the x-axis and peak area (A) on the y-axis, resulting in a linear regression equation of y=21,262,658.77x + 38,096.38 and R^2=0.9994 (Fig. 7C, D). Upon substituting values into the regression equation, the allantoin content was determined to be 0.035% in the normal group and 0.074% in the 45°C heat-treated group. Similarly, for polysaccharide concentration (C) as the independent variable and absorbance (A) as the dependent variable, a standard curve was plotted, yielding a linear regression equation of y=0.0071x + 0.1137 and R^2=0.9991 (Fig. 7 E). The polysaccharide content was calculated to be 1.061% in the normal group and 2.483% in the 45°C heat-treated group. Analysis of the allantoin and polysaccharide components revealed significantly higher levels in the most suitable heat-treated yam compared to the normal group (Fig. 7E, G). Heat treatment enhanced the synthesis of these beneficial components, leading to an overall improvement in the quality of yam.

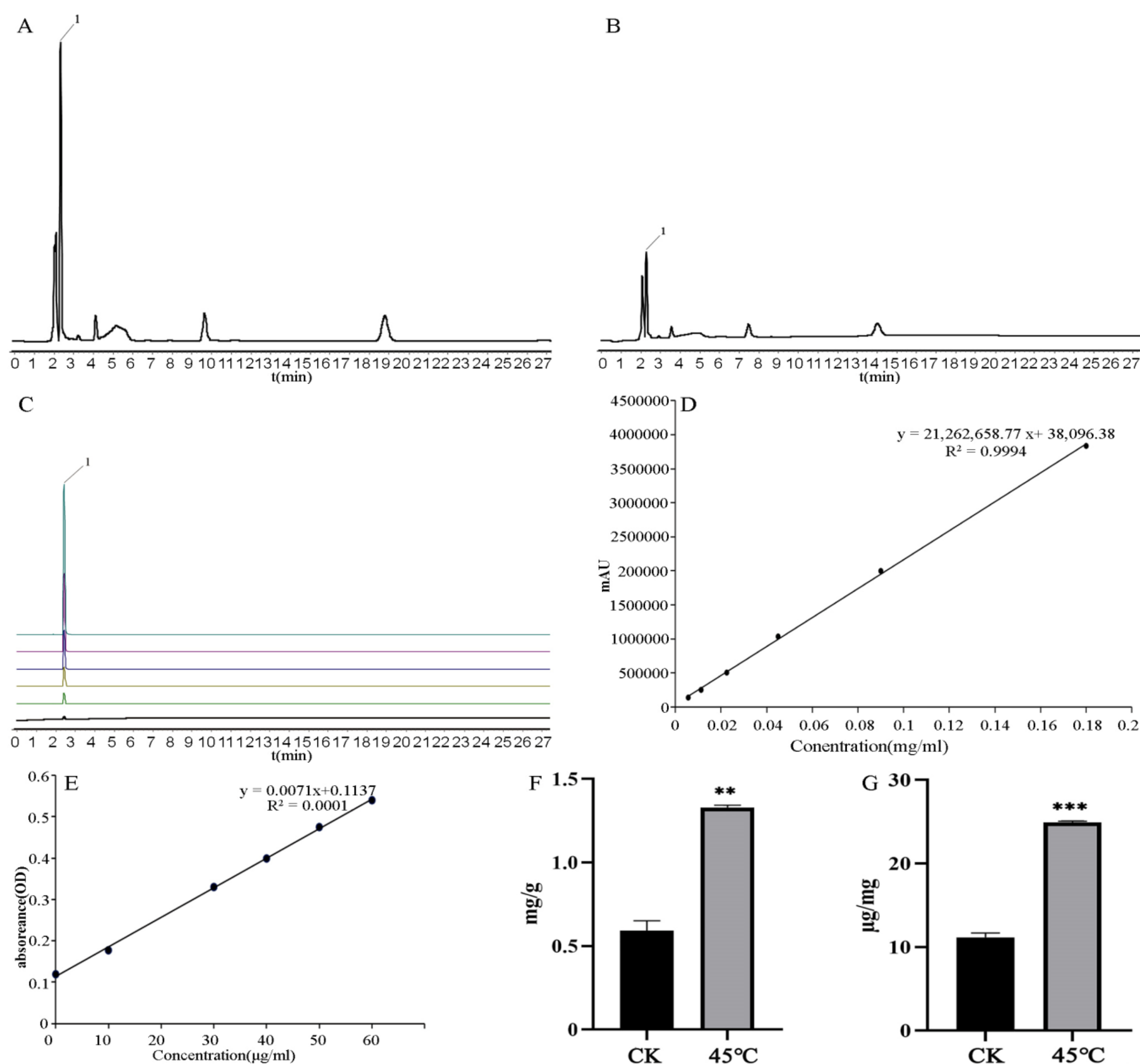

Fig. 7. Allantoin results of samples.

(Note: A: 45°C heat-shock group of allantoin; B: the normal group of allantoin; C, D: the standard curve of the allantoin; E: the polysaccharide standard curve; F: the content of the allantoin content in different groups, G: the polysaccharide content of different groups; ** indicates significant differences at $p<0.01$, 1 is the allantoin in Fig. *** indicates significant differences at $p<0.001$).

Discussion

A green and eco-friendly biological control technique has been investigated to mitigate the adverse effects of pesticides on yam quality and combat fungal infections such as *Curvularia lunata*. This approach involves employing a heat-shock treatment to enhance the expression of heat-shock genes (*HSP26.5*, *HSP90-6*, *HSFB*) and anti-fungal genes (*PTI5*, *PR1)* in yams, thereby inhibiting *Curvularia lunata* infection and bolstering disease resistance without compromising yam quality. This study aimed to foster the cultivation of yam seedlings capable of resisting fungal infections through pre-heat-shock treatment, offering an eco-friendly method to enhance yam disease resistance.

Yams are indigenous to tropical and subtropical regions and require high temperatures for optimal growth, exhibiting a notable tolerance to elevated temperatures. The primary yam cultivation regions in China are situated in the northern provinces, specifically Hebei, Shanxi, and Shandong. The growth period predominantly occurs during the summer months when temperatures and humidity levels are notably elevated, with an average summer temperature of 29°C, reaching a maximum of 41°C, and an average humidity of 70%. To align with the growth preferences of yams, heat-shock treatments were administered at 40, 45, and 50°C, surpassing their regular growth temperatures. Additionally, the yam samples were inoculated with *Curvularia lunata* within 70% humidity.

Experimental research indicated that suitable heat-shock treatments could enhance plant stress resistance, while excessive temperatures might damage plant tissues and trigger leaf senescence. *HSP* genes have been studied in rice, demonstrating that *HSPs* can influence disease resistance, bolstering defenses against maladies like white leaf blight and rice blast (Kuang, 2013). The expression of *HSFs* and stress-related genes in plants is correlated. *HSPs*, which are stress-inducible molecular chaperone proteins present in all organisms, play a crucial role in stress tolerance by aiding in the effective folding of newly synthesized proteins and maintaining the stability of existing proteins to prevent aggregation under pressure conditions (Haq *et al*., 2019; Muga & Moro, 2008; Hu *et al*., 2015; Lin *et al*., 2011). *HSPs* and *HSF* genes play a crucial role in plant stress response, enhancing cross-resistance to various stressors. The disease-resistance gene *PTI5* is mainly used to deal with diseases caused by fungal infections. The expression of *PTI5* gene in tomato can effectively reduce the damage caused by fungal infection and improve the disease resistance of tomato plants (Wang *et al*, 2021). Similarly, in wheat, the expression of the *PTI5* gene enhances resistance to powdery mildew from fungal infections. Moreover, in potatoes, the expression of the *PTI5* gene amplifies disease resistance and fortifies the plant's ability to combat pathogenic fungi stress (Xu, 2018). These findings underscore the significance of the *PTI5* gene in enhancing resistance to fungal infections. Heat-shock genes can modulate disease resistance genes, boosting their relative expression levels and thus improving the plant's capacity to ward off diseases. However, most existing yam studies have concentrated on single environmental stresses, with limited research on using heat-shock treatments to modulate yam's disease stress response (Shahzad *et al*., 2021; Kushawaha *et al*., 2021; Mohanty, 2021; Jiang *et al*., 2021).

This study employed DAB and Trypan Blue staining to assess phenotypic changes in yam leaves, utilized microscopy to observe cellular-level alterations and performed quantitative PCR to measure the relative expression levels of heat-shock protein genes and disease-resistant genes in various yam groups. Additionally, HPLC was used to determine the urea content in yams from both the optimal and normal heat treatment groups. The phenol-sulfuric acid method was applied to evaluate polysaccharide content changes in these groups, aiming to understand the impact of heat treatment on yam quality. Our study revealed that post 45°C heat treatment, yam leaves exhibited minimal damage and effectively countered *Curvularia lunata* infection. Quantitative PCR results indicated enhanced relative expression levels of heat-shock genes *HSFA2c*, *HSP26.5*, *HSP90-6*, and *HSFB* increased under 45°C heat-shock, and the relative expression level of *PTI5* was also increased. Conversely, under 50°C heat shock, the expression levels of *HSP26.5*, *HSP90-6*, and *HSFB* were lower than 45°C treatment, and the expression level of *PTI5* was also reduced compared to the non-heat-treated control, possibly due to excessive temperature harming the leaf structure and impacting gene transcriptional activity. Under 40°C heat shock, the relative expression level of *PTI5* was lower than 45°C, possibly due to the decrease in the relative expression level of *the HSFA2c* heat-shock gene. The analysis of yam leaf phenotypes and gene expression levels analysis demonstrated that 45°C heat shock effectively upregulated yam heat-shock genes. Heat-shock genes such as *HSP26.5*, *HSP90-6*, and *HSFB* induced the expression of the antifungal gene *PTI5*, PR1 and related stress genes. This induction facilitated the proper folding of newly synthesized proteins and the preservation of the structural integrity of existing proteins. Consequently, the harmful effects of fungal infections on yam seedling leaves were mitigated, preserving the functionality of critical enzymes involved in yam's primary and secondary metabolic pathways. This protective mechanism ultimately led to an enhancement in the levels of bioactive compounds, improving the overall quality of the yam product.

This study has preliminarily explored the effects of heat shock in enhancing the resistance of yam to infection by *Curvularia lunata* and has identified vital disease disease-resistance genes. However, large-scale field experiments have yet to be conducted, and the detailed disease resistance signaling pathway remains unclear. Further research is necessary to investigate other unknown genes involved in the disease resistance pathway and the interactions among genes within the signaling pathway. Our heat shock experiments on yam showed that appropriately applying heat shock improved yam's resistance to *Curvularia lunata* while maintaining its quality. The ability of yam plants to resist *Curvularia lunata* is influenced by the expression of heat shock genes, which are crucial for cell growth and development, metabolic differentiation, tissue cell stability, and environmental adaptation. Analysis of the expression patterns of heat shock genes and disease resistance genes revealed a cross-reaction between the heat shock signaling

pathway and the disease resistance signaling pathway in yam under heat stress. These findings offer valuable insights and guidance for developing eco-friendly disease resistance methods in yam cultivation and analyzing and enhancing the molecular mechanisms involved in disease resistance.

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References

Brown, J.K. 2002. Yield penalties of disease resistance in crops. *Curr. Opin. Plant Biol*., 5(4): 339-344.

Ghorbel, M., I. Zribi, K. Missaoui, M. Drira-Fakhfekh, B. Azzouzi and F. Brini. 2021. Differential regulation of the durum wheat Pathogenesis-related protein (PR1) by Calmodulin TdCaM1.3 protein. *Mol. Biol. Rep*., 48(1): 347-362.

Haq, S.U., A. Khan, M. Ali, A.M. Khattak, W.X. Gai, H.X. Zhang, A.M. Wei and Z.H. Gong. 2019. Heat shock proteins: Dynamic biomolecules to counter plant biotic and abiotic stresses. *Int. J. Mol. Sci*., 20(21): 5321.

Hu, Y., Y.T. Han, W. Wei, Y.J. Li, K. Zhang, Y.R. Gao, F.L. Zhao and J.Y. Feng. 2015. Identification, isolation, and expression analysis of heat shock transcription factors in the diploid woodland strawberry *Fragaria vesca*. *Front. Plant Sci*., 6(10): 3313-3318.

Jiang, L.Y., W.J. Hu, Y.X. Qian, Q.Y. Ren and J. Zhang. 2021. Genome-wide identification, classification and expression analysis of the *Hsf* and *Hsp70* gene families in maize. *Gene*., 770: 145348.

Kuang, J. 2013. The function of small heat shock protein sHSP17.5 and heat shock factors in resistance to bacterial blight in rice. *Thejiang Normol University.*

Kushawaha, A.K., A. Khan, S.K. Sopory and N. Sanan-Mishra. 2021. Priming by high temperature stress induces MicroRNA regulated heat shock modules indicating their involvement in thermopriming response in rice. *Life (Basel)*., 11(4): 291.

Lin, Y.X., H.Y. Jiang, Z.X. Chu, X.L. Tang, S.W. Zhu and B.J. Cheng. 2011. Genome-wide identification, classification and analysis of heat shock transcription factor family in maize. *BMC Genom*., 12(1): 1-14.

Liu, G., Y.Y. Ji, N.H. Bhuiyan, G. Pilot, G. Selvaraj, J. Zou and Y. Wei. 2010. Amino acid homeostasis modulates salicylic acid-associated redox status and defense responses in Arabidopsis. *Plant Cell*, 22(11): 3845-63.

Liu, M.M., Z.Y. Shi , X.H. Zhang, M.X. Wang, L. Zhang, K.Z. Zheng, J.Y. Liu, X.M. Hu, C.R. Di, Q. Qian, Z.H. He and D.L. Yang. 2019. Inducible overexpression of Ideal Plant Architecture improves both yield and disease resistance in rice. *Nat. Plants*, 5(4): 389-400.

Mignouna, D., A. Mank, N. Ellis, N.V.D. Bosch, R. Asiedu, M. Abang and J. Peleman. 2002. A genetic linkage map of water yam (*Dioscorea alata* L.) based on AFLP markers and QTL analysis for anthracnose resistance. *Theor. Appl. Genet*., 105(5): 726-735.

Mohanty, B. 2021. Promoter architecture and transcriptional regulation of genes upregulated in germination and coleoptile elongation of diverse rice genotypes tolerant to submergence. *Front Genet*., 12: 639654.

Muga, A. and F. Moro. 2008. Thermal adaptation of heat shock proteins. *Curr. Prot. Pept. Sci*., 9(6): 552-566.

Muthamilarasan, M. and M. Prasad. 2013. Plant innate immunity: an updated insight in-to defense mechanism. *J. Biosci*., 38(2): 433-449.

Shahzad, R., S. Jamil, S. Ahmad, A. Nisar, Z. Amina, S. Saleem, M. Zaffar Iqbal, R. Muhammad Atif and X. Wang. 2021. Harnessing the potential of plant transcription factors in developing climate resilient crops to improve global food security: Current and future perspectives. *Saudi J. Biol. Sci*., 28(4): 2323-2341.

Tamiru, M., S. Natsume, H. Takagi, B. White, H. Yaegashi, M. Shimizu, K. Yoshida, A. Uemura, K. Oikawa, A. Abe, N. Urasaki, H. Matsumura, P. Babil, S. Yamanaka, R. Matsumoto, S. Muranaka, G. Girma, A. Lopez-Montes, M. Gedil, R. Bhattacharjee, M. Abberton, P.L. Kumar, I. Rabbi, M. Tsujimura, T. Terachi, W. Haerty, M. Corpas, S. Kamoun, G. Kahl, H. Takagi, R. Asiedu and R. Terauchi. 2017. Genome sequencing of the staple food crop white Guinea yam enables the development of a molecular marker for sex determination. *BMC Biol*., 15(1): 86.

Tanabe, S., H. Onodera, N. Hara, N. Ishii-Minami, B. Day, Y. Fujisawa, T. Hagio, S. Toki, N. Shibuya, Y. Nishizawa and E. Minami. 2016. The elicitor-responsive gene for a GRAS family protein, CIGR2, suppresses cell death in rice inoculated with rice blast fungus via activation of a heat shock transcription factor, *OsHsf23*. *Biosci. Biotechnol. Biochem*., 80(01): 145-151.

Venkatesh, J., M. Jahn and B.C. Kang. 2016. Genome-Wide Analysis and Evolution of the Pto-Like Protein Kinase (PLPK) Gene Family in Pepper. *PloS One*., 11(8): e0161545.

Wang, Y., G.D. Feng, Z. Zhang, Y. Liu, Y.L. Ma, Y.Y. Wang, F. Ma, Y. Zhou, R. Gross, H.H. Xu, R.P. Wang, F.M. Xiao, Y.S. Liu and X.L. Niu. 2021. Overexpression of Pti4, Pti5, and Pti6 in tomato promote plant defense and fruit ripening. *Plant Sci*., 302: 110702.

Xu, J.N. 2018. Construction the plant expressing vector of transcription factors StPti5 from *Solanum tuberosum* and analysis its stress-induced expression characteristics. *Hunan Agricultural University*.

Yi, Q., R.A. Malvar, L. Álvarez-Iglesias, B. Ordás and P. Revilla. 2020. Dissecting the genetics of cold tolerance in a multi-parental maize population. *Theor. Appl. Genet*., 133(2): 503-516.

Zahid, M.A., M. Sandroni, R.R. Vetukuri and E. Andreasson. 2021. A fast, nondestructive method for the detection of disease-related lesions and wounded leaves. *Biotechniques*, 71(2): 425-430.

Zaid, R., R. Koren, E. Kligun, R. Gupta, M. Leibman-Markus, P.K. Mukherjee, C.M. Kenerley, M. Bar and B.A. Horwitz. 2022. Gliotoxin, an immunosuppressive fungal metabolite, primes plant immunity: Evidence from *Trichoderma* virens-tomato interaction. *mBiol*., 13(4): e0038922.

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