



Short Communication

Resistance of *Castanea mollissima* Shuhe-WYL strain to *Dryocosmus kuriphilus* and its molecular mechanism

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ABSTRACT. The resistance of *Castanea mollissima* Shuhe-WYL strain to *Dryocosmus kuriphilus* and its molecular mechanism were examined. The larvae of *D. kuriphilus* were inoculated on the Shuhe-WYL and Qingzha strains, and mortality was observed and compared; the relative mRNA content of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and the *bHLH* genes was detected using real-time polymerase chain reaction, and then compared between the Shuhe-WYL and Qingzha strains. Phenylalanine ammonia-lyase content was detected by western blotting and compared between the inoculated Shuhe-WYL, non-inoculated Shuhe-WYL, and inoculated Qingzha strains. The mortalities of larvae inoculated on the bud, bracteal leaf, and cardiac lobe were lower in Shuhe-WYL than Qingzha at 48 and 96 h after inoculation; the contents of *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, and *bHLH* in the cardiac lobe were higher in Shuhe-WYL than in Qingzha at 96 h after inoculation, but *KH domain protein* and *RNA-binding protein* were not significantly different. The content of phenylalanine ammonia-lyase in the cardiac lobe was higher in inoculated and non-inoculated Shuhe-WYL compared to inoculated Qingzha at 15, 30, 45, and 60 days, and higher in inoculated Shuhe-WYL than in non-inoculated Shuhe-WYL at 15, 30, 45,

and 60 days. The content of phenylalanine ammonia-lyase in the cardiac lobe of inoculated Shuhe-WYL had no significant difference between at 60 and at 45 days; and was higher at 60 and 45 days than at 30 and 15 days; and was higher at 30 days than at 15 days (60>45>30>15 days). The *C. mollissima* Shuhe-WYL strain was resistant to *D. kuriphilus*; high expression of *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, and *bHLH* and phenylalanine ammonia-lyase may explain the mechanism.

Key words: *Castanea mollissima*; *Dryocosmus kuriphilus*; Resistance; Phenylalanine ammonia-lyase

INTRODUCTION

Dryocosmus kuriphilus (chestnut gall wasp), belongs to Hymenoptera, Cynipidae and is an important pest in chestnuts. The larvae of *D. kuriphilus* infest the bud of the chestnut, which can wither the bud. The *Castanea mollissima* (Chinese chestnut) Shuhe-WYL strain is a hybrid of the wild CG-200 and Shuhe strains. During the course of breeding, the *C. mollissima* Shuhe-WYL strain showed resistance to *D. kuriphilus* (Zhang et al., 2000). Phenylalanine ammonia-lyase (PAL) is a key enzyme that catalyze sphenyl propanoid metabolism. The products of phenyl propanoid metabolism include the phenols phytoalexin and isoflavones phytoalexin, which positively influence the resistance to pests in plants (Kumar and Ellis, 2001). In the phenylpropanoid pathway, the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and *bHLH* genes are important in the production and activity of PAL (Zhao et al., 2001; MacDonald and D’Cunha, 2007; Pina and Errea, 2008). Additionally, they are critical for the production of phytoalexin.

In this study, we investigated the resistance of the *C. mollissima* Shuhe-WYL strain to *D. kuriphilus* and detected the relative content of mRNA of genes potentially related to the resistance and content of endogenous PAL. The aim of this study was to explain the mechanism of *C. mollissima* Shuhe-WYL strain resistance to *D. kuriphilus* and provide useful information for breeding chestnut strains with resistance to pests.

MATERIAL AND METHODS

Materials

Chestnut strains

The experimental strain used was the *C. mollissima* Shuhe-WYL strain, while the control strain was the *C. mollissima* Qingzha strain. The experimental field was in Jiangsu Province, China, which is the main habitat of the Chinese chestnut Shuhe-WYL strain. The experimental and control strains were grown in different gardens of the experimental field at the same time and infestation of *D. kuriphilus* was prevented. During the experiment, the experimental and control strains were given routine field management and insecticides were not used.

D. kuriphilus for experiments

The egg of *D. kuriphilus* was collected from the Qingzha strain in the field and was then incubated and a bred in the laboratory. The larvae of the F2 generation were used in the experiment.

Methods

Infestation experiment

The underyearling bud, bracteal leaf, and cardiac lobe of the Shuhe-WYL strain and Qingzha strain each weighing 4 g were randomly collected in the experimental field and soaked for 12 h in 2% sodium hypochlorite, washed 5 times with sterile water, and placed into test tubes after air drying. For both the Shuhe-WYL strain and Qingzha strain, 45 test tubes were used (15 for the bud, 15 for the bracteal leaf, and 15 for the cardiac lobe). Each test tube was inoculated with 12 larvae of *D. kuriphilus*, and all larvae were the same age. The test tube was bound with carbasus and prostrated. The surviving larvae were evaluated at 48 and 96 h after inoculation.

Detection of the relative mRNA content of the *OsCDPK2*, receptor-like protein, *OsNAC6* protein, *KH* domain protein, *RNA binding protein*, and *bHLH* genes

Total RNA was extracted from the cardiac lobe of the Shuhe-WYL strain and Qingzha strain, which was inoculated for 96 h with the larvae of *D. kuriphilus*, and then reverse-transcribed into cDNA to be used as a template for real-time polymerase chain reaction. Both the Shuhe-WYL and Qingzha strains included 15 samples. The 20- μ L reaction system contained 10 μ L SYBR Premix ExTaqTM, 1 μ L upstream primer, 1 μ L down stream primer, 1 μ L cDNA, and 7 μ L deionized water. Primer sequences and polymerase chain reaction product sizes are shown in Table 1. The reaction conditions were pre-denaturation at 95°C for 3 min, denaturation for 95°C, for 30 s, and renaturation at 55°C, for 10 s for 40 cycles. Finally, the relative content of mRNA was analyzed using the $2^{-\Delta\Delta Ct}$ method (using β -actin as an internal reference).

Table 1. Sequence of the primers and the size of PCR products.

Gene	Sequence of primer (5'-3')	Size (bp)
<i>OsCDPK2</i>	F: ATCCAGAGGCAAACGGAGC R: CACATGGCAACATCTAACAG	107
<i>Receptor-like protein</i>	F: CGTCGCTCCAAATACACCAA R: CAGCGAAACATCCCCATCT	130
<i>OsNAC6 protein</i>	F: GCCAGCGGTGAGAAAGGA R: CAACCCTCCGACCATCAGTT	80
<i>KH domain protein</i>	F: GCGCTCATCTTCTCCGAAT R: CGCGGTCTGGTTATGTG	126
<i>RNA binding protein</i>	F: CTGGATCCAACAATGAGC R: ATCAAGATGGAGACCCA	84
<i>bHLH</i>	F: AGCGGGCGCAGATGAC R: CGTTCATGCACTCGCTGAA	115
β -actin	F: CACTCTTCAGCCTTCTCTC R: GGATGTCCACGTACACATTC	92

Detection of PAL content by western blotting

The cardiac lobe of the Shuhe-WYL strain and Qingzha strain was inoculated with 12 larvae on each leaf of *D. kuriphilus*. One hundred tagged leaves of the Shuhe-WYL and Qingzha strains were inoculated with the larvae of *D. kuriphilus*. At 15, 30, 45, and 60 days after inoculation, 15 tagged leaves of the Shuhe-WYL and Qingzha strains were randomly collected, while 15 leaves (cardiac lobes) of the Shuhe-WYL strain that had not been infested by the larvae were also

randomly collected. Total protein was extracted from the cardiac lobe. The BCA method was used for protein quantification. First, 30 µg protein was mixed with sample buffer. The sample was then boiled for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the percentage of the gel was 12%. The protein was transferred to nitrocellulose membranes and then blocked for 1 h with Tris-buffered saline-Tween 20 containing 50 g/L dried skim milk at room temperature. Rabbit anti-PAL and mouse anti-β-actin antibodies were added to the mixture, and the mixture was incubated at 4°C overnight. The mixture was washed 3 times with Tris-buffered saline-Tween 20, and goat anti-rabbit and goat anti-mouse secondary antibodies labeled with horseradish peroxidase were added. The mixture was incubated for 1 h at room temperature and then washed 3 times with Tris-buffered saline-Tween 20. Finally, the samples were visualized using electrochemiluminescence.

Statistical analysis

The mortality of the larvae of *D. kuriphilus*, the relative content of mRNA of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and *bHLH* genes, and the content of PAL were compared using Student *t*-tests. Statistical analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Significance was set at $P < 0.05$.

RESULTS

Mortality of *D. kuriphilus* larvae

The mortalities of the *D. kuriphilus* larvae are shown in Table 2. The mortalities of *D. kuriphilus* larvae inoculated on the bud, bracteal leaf, and cardiac lobe were lower in the Shuhe-WYL strain than in the Qingzha strain at 48 h after inoculation ($t = 20.042$, $P = 0.000$; $t = 14.548$, $P = 0.000$; $t = 24.547$, $P = 0.000$), and the mortalities of *D. kuriphilus* larvae inoculated on the bud, bracteal leaf, and cardiac lobe were also lower in the Shuhe-WYL strain than in the Qingzha strain at 96 h after inoculation ($t = 27.384$, $P = 0.000$; $t = 29.152$, $P = 0.000$; $t = 28.387$, $P = 0.000$).

Table 2. Mortalities of the larvae of *Dryocosmus kuriphilus* inoculated in the bud, bracteal leaf, and cardiac lobe of the Shuhe-WYL and Qingzha strains at 48 and 96 h after inoculation.

Strain	Inoculating position	No. of test tubes	Mortality at 48 h (%)	Mortality at 96 h (%)
Shuhe-WYL	Bud	15	33.1 ± 4.48	79.5 ± 5.25
	Bracteal leaf	15	23.5 ± 4.21	67.2 ± 5.08
	Cardiac lobe	15	31.5 ± 4.57	77.6 ± 6.02
Qingzha	Bud	15	4.2 ± 2.03	8.7 ± 3.15
	Bracteal leaf	15	3.7 ± 1.98	3.9 ± 2.12
	Cardiac lobe	15	2.3 ± 1.86	5.4 ± 3.04

Relative mRNA content of *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and *bHLH* genes

The relative mRNA content of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and *bHLH* genes is shown in Table 3. The relative content of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, and *bHLH* genes were higher in the Shuhe-WYL strain than in the Qingzha strain ($t = 11.028$, $P = 0.000$; $t = 12.257$, $P = 0.000$; $t = 3.056$, $P = 0.000$).

= 0.012; $t = 14.238$, $P = 0.000$). The relative contents of the *KH domain protein* and *RNA-binding protein* genes were not significantly different between the Shuhe-WYL and the Qingzha strains ($t = 0.034$, $P = 0.973$; $t = 0.158$, $P = 0.694$).

Table 3. Relative content of mRNA of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and *bHLH* genes in the cardiac lobe of the Chinese chestnut Shuhe-WYL strain and Chinese chestnut Qingzha strain at 96 h after inoculation.

Gene	No. of samples	Relative content of mRNA		<i>t</i>	P
		Shuhe-WYL	Qingzha		
<i>OsCDPK2</i>	15	0.82 ± 0.24	0.23 ± 0.17	11.028	0.000
<i>Receptor-like protein</i>	15	0.77 ± 0.18	0.15 ± 0.26	12.257	0.000
<i>OsNAC6 protein</i>	15	0.35 ± 0.22	0.22 ± 0.34	3.056	0.012
<i>KH domain protein</i>	15	0.76 ± 0.25	0.74 ± 0.27	0.034	0.973
<i>RNA binding protein</i>	15	0.34 ± 0.31	0.36 ± 0.29	0.158	0.694
<i>bHLH</i>	15	0.67 ± 0.16	0.13 ± 0.14	14.238	0.000

Content of PAL

PAL content in the cardiac lobe was higher in the inoculated Shuhe-WYL strain and non-inoculated Shuhe-WYL strain than in the inoculated Qingzha strain at the same time points ($t_{15, 30, 45, 60} = 15.254, 14.027, 10.157, 9.846$, and $P_{15, 30, 45, 60} = 0.000$ for inoculated Shuhe-WYL strain vs Qingzha strain; $t_{15, 30, 45, 60} = 7.236, 6.905, 5.269, 4.985$, and $P_{15, 30, 45, 60} = 0.000$ for the non-inoculated Shuhe-WYL strain vs Qingzha strain), and PAL content in the cardiac lobe was higher in the inoculated Shuhe-WYL strain than non-inoculated Shuhe-WYL strain at the same time points ($t_{15, 30, 45, 60} = 6.245, 5.458, 5.024, 5.921$ and $P_{15, 30, 45, 60} = 0.000$ for the inoculated Shuhe-WYL strain vs non-inoculated Shuhe-WYL strain). PAL content in the cardiac lobe of the inoculated Shuhe-WYL strain was 60 days \approx 45 days $>$ 30 days $>$ 15 days ($t_{60 vs 15} = 4.925$, $P_{60 vs 15} = 0.000$; $t_{60 vs 30} = 2.985$, $P_{60 vs 30} = 0.024$; $t_{60 vs 45} = 0.186$, $P_{60 vs 45} = 0.648$; $t_{45 vs 15} = 5.128$, $P_{45 vs 15} = 0.000$; $t_{45 vs 30} = 3.041$, $P_{45 vs 30} = 0.011$; $t_{30 vs 15} = 2.874$, $P_{30 vs 15} = 0.029$) (Table 4).

Table 4. Content of PAL in the cardiac lobe of inoculated the Shuhe-WYL strain, non-inoculated Shuhe-WYL strain, and inoculated Qingzha strain on 15, 30, 45, and 60 days after inoculation.

Strain	15 days	30 days	45 days	60 days
Inoculated Shuhe-WYL	46.22 ± 4.035	65.24 ± 4.574	78.45 ± 5.147	76.55 ± 4.834
Non-inoculated Shuhe-WYL	21.46 ± 6.023	33.79 ± 3.846	40.12 ± 6.249	38.67 ± 5.748
Inoculated Qingzha	8.75 ± 5.146	13.67 ± 4.029	24.48 ± 4.249	23.59 ± 3.825

DISCUSSION

D. kuriphilus mainly harms the bud of chestnut. The newborn larva of *D. kuriphilus* can infest the bud, leading to branch death. In this study, we evaluated the resistance of the bud, bracteal leaf, and cardiac lobe of the Chinese chestnut Shuhe-WYL strain to the larvae of *D. kuriphilus*. The results showed that most of the larvae inoculated on the Shuhe-WYL strain had died by 96 h; the mortality rate was higher than that in the control strain. This indicates that the Shuhe-WYL strains are effectively resistant to damage caused by *D. kuriphilus*.

PAL is a key enzyme that catalyzes phenylpropanoid metabolism and is very important

for the resistance of plants to pests. When plants are infested by pests, the content and activity of endogenous PAL increases, accelerating phenylpropanoid metabolism. This results in increased phytoalexin production, including lignin and pyrocatechol. Pyrocatechol has been shown to be positively correlated with the resistance of chestnut to *D. kuriphilus*, and lignin can increase the lignification of the gallae endosporium of *D. kuriphilus*, limiting the feeding of *D. kuriphilus* larvae. Several studies have shown that the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, *KH domain protein*, *RNA-binding protein*, and *bHLH* genes are correlated with the production and activity of PAL. In this study, we found that the relative mRNA content of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, and *bHLH* genes was higher in the Shuhe-WYL strain than in the Qingzha strain inoculated for 96 h, and the content of PAL was higher in the inoculated Shuhe-WYL strain and non-inoculated Shuhe strain than in the Qingzha strain. PAL content increased with infestation time in the inoculated Shuhe-WYL strain. These results may explain the mechanism of the resistance of the Shuhe-WYL strain to *D. kuriphilus*.

In summary, the *C. mollissima* Shuhe-WYL strain was resistant to *D. kuriphilus*; the high expression of the *OsCDPK2*, *receptor-like protein*, *OsNAC6 protein*, and *bHLH* genes and PAL may explain the mechanism. However, other factors also lead to increased PAL expression, such as ashypothermia, perfunctory injuries, growth hormone, aethylenum, and infection (Hu et al., 2011). Therefore, further studies of the mechanism should be conducted.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

- Hu GS, Jia JM, Hur YJ, Chung YS, et al. (2011). Molecular characterization of phenylalanine ammonia lyase gene from *Cistanche deserticola*. *Mol. Biol. Rep.* 38: 3741-3750.
- Kumar A and Ellis BE (2001). The phenylalanine ammonia-lyase gene family in raspberry. Structure, expression, and evolution. *Plant Physiol.* 127: 230-239.
- MacDonald MJ and D'Cunha GB (2007). A modern view of phenylalanine ammonia lyase. *Biochem. Cell. Biol.* 85: 273-282.
- Pina A and Errea P (2008). Differential induction of phenylalanine ammonia-lyase gene expression in response to in vitro callus unions of *Prunus* spp. *J. Plant Physiol.* 165: 705-714.
- Zhang DL, Quantick PC and GrigorJM (2000). Changes in phenolic compounds in Litchi (*Litchi chinensis* Sonn.) fruit during postharvest storage. *Postharvest Biol. Tec.* 19: 165-172.
- Zhao J, Zhu WH and Hu Q (2001). Enhanced catharanthine production in catharanthusroseus cell cultures by combined elicitor treatment in shake flasks and bioreactors. *Enzyme Microb. Tech.* 28: 673-681.