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转 *MhGlu* 基因烟草灰霉病抗性及光合特性研究

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摘　要:以湖北海棠盆栽及组培苗叶片为材料,经 NaCl、PEG-6000 及 4 ℃下 ABA 处理后,通过 RT-PCR 技术克隆了湖北海棠 β-1,3-葡聚糖酶基因 *MhGlu*;构建 *MhGlu* 基因的植物表达载体,通过农杆菌介导法将 *MhGlu* 基因转入烟草中,并通过 PCR 和 RT-PCR 检测,成功获得了 4 个转基因株系 T6、T8、T11 和 T18;以转基因烟草株系 T6 及 T8 和非转基因对照植株为材料,对 *MhGlu* 基因的功能进行了进一步分析。结果显示:(1)半定量 qRT-PCR 显示,NaCl、PEG-6000 及 4 ℃下 ABA 处理均可以诱导湖北海棠盆栽及组培苗叶片 *MhGlu* 基因的表达;NaCl 和 PEG-6000 处理 48 h 内 *MhGlu* 基因的表达随处理时间延长逐渐增强,4 ℃下 ABA 处理的 *MhGlu* 基因表达量在 4 h 时开始上调,12 h 时略降低,48 h 时又达到最大。(2)半定量 RT-PCR 检测转基因烟草植株几个病程相关基因 *PRs* 的表达量,表明过表达的 *MhGlu* 基因诱导并增强了烟草病程相关基因 *NtPR*1、*NtPR*3 和 *NtPR*5 的表达。(3)用灰霉病侵染烟草叶片,转基因烟草株系 T6、T8 均表现出较强的抗灰霉病特性。(4)测定烟草植株光合特性参数,转 *MhGlu* 基因烟草株系的净光合速率(P_n)、蒸腾速率(T_r)和气孔导度(G_s)较对照组均显著提高,且 T8 的净光合速率和蒸腾速率均显著高于 T6,而 T8 与 T6 的气孔导度差异不显著。*MhGlu* 基因在烟草中的过量表达能诱导病程相关基因 *PRs* 的表达,激活了烟草的光合特性保护机制,提高了转 *MhGlu* 基因烟草植株的灰霉病抗性。

关键词:*MhGlu*;灰霉病;抗性;光合特性;烟草

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Overexpression of *MhGlu* Enhances *Botrytis cinerea* Resistance in Tobacco

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Abstract: In our study, *MhGlu*, a β-1,3-glucanases gene of *Malus hupehensis* was cloned from leaves of *M. hupenhensis* treated with NaCl, PEG-6000 and ABA at 4 ℃ by RT-PCR. A plant binary expression vector of *MhGlu* was constructed. The vector was transformed into tobacco through *Agrobacterium*-mediated method. PCR and RT-PCR results showed that four transgenic tobacco lines T6, T8, T11 and T18 were obtained. The function of *MhGlu* was further analyzed with the transgenic tobacco lines T6, T8 and wild type tobacco plants (WT). Results showed that: (1) The expression of the gene in *M. hupehensis* was monitored in a 48 h course after treated with NaCl, PEG-6000 and ABA at 4 ℃, respectively. Semi-quantitative RT-PCR revealed that expression of *MhGlu* was induced by NaCl, PEG-6000 and ABA at 4 ℃ treatment in both potted seedlings and seedlings cultured *in vitro*. Treated by NaCl and PEG-6000, the expression of *MhGlu* gene increased gradually over time; while ABA treatment at 4 ℃ up-regulated *MhGlu* 4 h post treatment, the expression decline at 12 h, then reached to maximum at 48 h. (2) Compared with the WT plants, several pathogenesis-related genes (*PRs*) in tobacco (*NtPR*1, *NtPR*2, and *NtPR*3) were up-regulated in tobacco plants with overexpression of *MhGlu*, detected by semi-quantitative RT-PCR. (3) Being infected with *Botrytis cinerea*, T6 and T8 lines of transgenic tobacco plants showed stronger tolerance to *B.*

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cinerea than nontransgenic plants. (4)Overexpression *MhGlu* appeared to improve the photosynthetic characteristics of transgenic tobacco. The net photosynthetic rate (P_n), the transpiration rate (T_r) and stomatal conductance (G_s) of transgenic plants were increased compared with that of controls, and the difference was significant. The P_n and T_r of T8 were significantly higher than that of T6, while the difference of G_s between T8 and T6 was not significant. Overall, the overexpression of *MhGlu* can induce the expression of *PRs* genes, active the protection mechanism with photosynthetic characteristics and enhance the resistance to *B. cinerea* of tobacco.

Key words: *MhGlu*; *Botrytis cinerea*; resistance; photosynthetic characteristics; tobacco

β-1, 3-glucanases catalyze the hydrolysis of β-1, 3-glucans, which are found in the cell walls of many plant tissues and plant fungal pathogens[1] and are thought to play important roles in normal growth and development processes[2]. Two types of pathogen resistance with distinct signaling pathways have been recognized, induced systemic resistance (ISR) and systemic acquired resistance (SAR)[3]. In plants, SA and JA/ET are important regulators of SAR and ISR, respectively[4-6]. β-1, 3-glucanases are known as pathogenesis-related downstream components of the SAR pathway[7].

It is well known that β-1, 3-glucanases are considered pathogenesis-related (*PR*) proteins in plants[8]. *PR* genes, which are critical components induced by the SAR pathway, have been studied in many plants, especially model species, in efforts to describe plant defense reactions[9-10]. It is thought that *PR* genes likely play important roles in plant defense against fungal infections. β-1, 3-glucanase has been studied widely for its ability to elicit defense responses in plants[11-12]. β-1, 3-glucanase plays a direct role in plant defenses against fungi by hydrolyzing fungal cell walls and exhibiting other antifungal activity[13].

To date, pathogen-induced β-1, 3-glucanase genes have been widely studied in many plants. A number of glucanase genes have been isolated and characterized in bacteria, fungi, and higher plants, including *Bacillus amyloliquefaciens*[12], *B. licheniformis*[14-15], *B. subtilis*[16], *Clostridium thermocellum*[17], *Fibrobacter succinogenes*[18], *Paenibacillus* spp.[19], *Rhizopus arrhizus*[20], *Streptococcus bovis*[21], strawberry[22] and winter rye[23]. Among these species, *F. succinogenes* plays a key role in plant fiber degradation in the rumen[24] and thus the glucanase gene in this organism has received significant attention. Furthermore, β-1, 3-glucanase genes have been identified in a variety of herbal plants, as well as in rice[24], soybean[25], wheat[7,26], maize[27] and barley[28]. They have also been studied in woody plants, including the rubber tree[29] and banana[30]. Together, these studies showed that the activation of β-1, 3-glucanases might be a general response to pathogen infection in plants. In wheat, high expression of *TaGlu* was associated with resistance to stripe rust[7] and resistance to two economically important biotrophic fungal pathogens, *Erysiphe graminis* f. sp. *tritici* (powdery mildew) and *Puccinia recondita* f. sp. *tritici* (leaf rust)[31]. A peanut mustard defensin has been shown to confer resistance to the fungal pathogens *Phytophthora parasitica* pv. *nicotianae* and *Fusarium moniliforme* in transgenic tobacco, as well as against pathogens that cause leaf spot disease in peanut[32]. The overexpression of a tobacco glucanase in a transgenic groundnut produced resistance to *Cercospora arachidicola* and *Aspergillus flavus*[33]. This is the first report of the isolation of a β-1, 3-glucanase gene sequence from *M. hupehensis* and the study of its properties in a transgenic tobacco model.

The *MhGlu* gene was a β-1, 3-glucanase gene in *M. hupehensis*, which was reported to be involved in *M. hupehensis* defending against apple ring spot pathogen and induced by salicylic acid (SA) motif, methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylic acid (ACC)[34]. In this study, the expression of the *MhGlu* gene inresponse to treatment with NaCl, PEG-6000, or ABA at 4 ℃ was studied in *M. hupehensis*. *MhGlu* gene was then transformed into tobacco to investigate whether *MhGlu* overexpression could induce resistance to the fungus *Botrytis cinerea*.

The results of our physiological experiments

also suggested that *MhGlu* transgenic tobacco plants exhibited stronger photosynthetic activity (P_n, T_r and G_s) than the nontransgenic plants did. This indicates that *MhGlu* plays an important role in normal plant growth and development processes.

This study provided us with information that is necessary and important for a better understanding of the molecular basis of *B. cinerea* infection and for further investigation of the role of *Malus hupehensis* β-1,3-glucanase in the defense against *B. cinerea*.

1 Material and methods

1.1 Plant materials and bacterial strains

The tobacco cultivar K326 was chosen for this study. Tobacco plants were subcultured *in vitro* in Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (6-BA, 2 mg/L Sigma) and naphthalene acetic acid (NAA, 0.1 mg/L Sigma). All cultures were maintained under a 16 h light (25 ℃)/8 h dark (25 ℃) cycle. *Escherichia coli* strain DH5a cells were used to clone β-1,3-glucan and its promoter and to propagate all recombinant plasmid vectors. The pCAMBIA-S1300＋and Pyk2478 vectors were used to construct the β-1,3-glucanase plant expression vectors. *Agrobacterium tumefaciens* EHA105 was used for transformation.

1.2 Induction of *MhGlu* expression in potted *M. hupehensis* seedlings and seedlings cultured *in vitro*

The leaves of three-week-old potted *M. hupehensis* seedlings were sprayed with solutions of 0.1 mmol/L NaCl (Sigma) and 0.02 mmol/L PEG-6000 (Sigma) for 4, 12 and 48 h, with water treatment as a control. Cultured *M. hupehensis* tissue was treated with 0.01 mmol/L ABA (Sigma) at 4 ℃, for 4, 12 and 48 h, with water treatment as a control. Each treatment group contained three plants. Leaves were collected and stored at −70 ℃ until use. Total RNA was isolated from young leaves as described by Cai *et al*[35].

Total RNA was treated with DNase I (TaKaRa, Code No: D2215) according to the manufacturer's instructions and reverse transcribed using the M-MLV RTase cDNA Synthesis kit (TaKaRa, D6130). Semi-quantitative RT-PCR was performed using r*Taq* DNA polymerase (TaKaRa, R10T1M) and the following primers: BGR2: 5′-GCAATAACTTCTGCTTGGGGT-GGTAAG -3′; BGR3: 5′-GGCATAGTTTCTGACAT-TGTTTTGGAC -3′. The RT-PCR reactions were performed under the following conditions: 94 ℃ for 4 min; 35 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s; and a final extension at 72 ℃ for 5 min. Semi-quantitative RT-PCR amplification was carried out in an Alpha Unit Block Assembly in a DNA Engine System (Bio-Rad). The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide.

1.3 *A. tumefaciens*-mediated transformation of tobacco

Leaf segments were excised from *in vitro*-grown shoots after 3 weeks of subculture. Transformation experiments were carried out as previously reported[36]. Briefly, leaves from 4-week-old micro-propagated tobacco shoots were inoculated with *Agrobacterium* grown overnight in yeast extract paste (YEP) media, centrifuged, resuspended in liquid MS media to an optical density (OD) of 0.8, and co-cultivated in MS regeneration media supplied with 2 mg/L 6-BA and 0.5 mg/L NAA in the dark for 3 days. Explants were then placed in regeneration media supplemented with 200 mg/L carbenicillin (Cb) to ensure resistance to *Agrobacterium* and 30 mg/L hygromycin B to select for transformants. The regeneration media was replaced every 2 weeks.

1.4 Confirmation of transgenic tobacco plants

Hygromycin-resistant tobacco plants were transplanted into pots and cultured in a greenhouse. To ensure that samples were free from *Agrobacterium* contamination, the top leaves were selected from each 3-month-old plant and grown in YEB agar medium for 3 days. Genomic DNA was extracted from the leaves and treated with RNase I, as described by Tong[37]. Total RNA was isolated from the young leaves as above. Total RNA was treated with Dnase I (TaKaRa, D2215) according to the manufacturer's instructions and reverse

transcribed using the M-MLV Rtase cDNA Synthesis kit (TaKaRa, D6130). The concentration of the isolated total RNA was estimated according to sample absorbance at 260 nm, as measured using a BioPhotometer (Eppendorf, Hamburg, Germany). RNA purity was verified by 260/280 nm absorbance ratio; values ranging from 1.80 to 2.00 were considered acceptably pure.

PCR and RT-PCR were performed using the primers BGF1: 5′-GCAATAACTTCTGCTTGGGGTGGTAAG -3′ and BGR1: 5′-GGCATAGTTTCTGACATTGTTTTGGAC -3′. PCR and RT-PCR were performed under the following conditions: 94 ℃ for 4 min; 35 cycles of 94 ℃ for 30 s, 59 ℃ for 45 s, and 72 ℃ for 2 min; and a final extension at 72 ℃ for 10 min. The PCR products (10 μL) were separated by electrophoresis on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide.

1.5 Analysis of *PR* gene expression in transgenic tobacco plants using semi-quantitative RT-PCR

Total RNA isolation, DNase I treatment and first strand cDNA synthesis were performed as described above. For semi-quantitative RT-PCR, reactions were performed using r*Taq* DNA polymerase (TaKaRa, R10T1M). Tobacco Tubulin, a housekeeping gene, was used as the control to normalize RT-PCR results. Semi-quantitative RT-PCR amplification was performed using an Alpha Unit Block Assembly in a DNA Engine System Thermal Cycler (Bio-Rad). The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide. PCR primers were designed as described by Zhang and colleagues[38], and their sequences are provided in Table 1. The resulting PCR products were cloned and sequenced to confirm that they represented the expected fragments of the tobacco *NtPR*1, *NtPR*3, *NtPR*5, and *NtTubulin* genes. Three biological replicates were run independently.

1.6 Pathogen challenge of transgenic tobacco plants

T6 and T8 were selected for this experiments. Hygromycin-resistant tobacco plants were transplanted into pots and cultured in a greenhouse. Pathogenic fungus inoculation was carried out by spraying a *B. cinerea* spore suspension (1×10^6 spores/mL) onto leaves at approximately the three-leaf stage. The inoculated plants were placed in a growth chamber under a 16 h light (25 ℃)/8 h dark (25 ℃) cycle with 80% relative humidity for 10 days. Conidia were observed 4 weeks later. The experiment was repeated three times independently.

Table 1 Sequences of primers used in this research

Gene	Primer name	Sequence 5′-3′	Size/bp
*NtPR*1	TUB-R TPR1-F	GCCGAAGAACTGACGAGAATC GCTGAGGGAAGTGGCGATTTC	165
*NtPR*5	TPR5-F TPR5-R	CGGAGACGAGGAAGATGAAGAATG GCCTAACAAGTGCTAAACCAAACC	153
*NtPR*3 (Chintase)	TCHI-F TCHI-R	GTGGTATGTTGAATGTTGCTCCTG TGATCTAACGAATCCTAGCCTTGG	85
NtTubulin	TTUB-F TTUB-R	AGATGTTCCGTCGTGTCAGTG TGCTTCCTCTTCATCCTCATATCC	200

1.7 Differences in photosynthetic characteristics between nontransgenic and transgenic tobacco

On July 23, 2011, from 11:00 — 12:00 pm (sunny), we tested the net photosynthetic rate (P_n), transpiration rate (T_r) and stomatal conductance (G_s) in nontransgenic and transgenic tobacco using an open gas line and a photosynthesis analyzer model produced in the US. Three strains were selected from each line, and leaf function was tested 3 times in each strain. All data was processed by SAS software for single-factor analysis of variance, followed by the average being weighted using Duncan's new multiple range test for multiple comparisons.

2 Results

2.1 Induction of *MhGlu* expression in potted *M. hupehensis* seedlings and *M. hupehensis* tissue cultures

To induce the expression of *MhGlu*, potted seedlings of *M. hupehensis* were treated by NaCl or PEG-6000. The expressions of *MhGlu* after different treatments were evaluated using semi-quantitative RT-PCR, with *MhTubulin* as the internal control. Each treatment contained three replications, from which analogous results have been obtained. As shown in Fig. 1, A, *MhGlu* gene expression was

obviously enhanced at the 4 h，12 h，and 48 h time points，during NaCl treatment；the similar expression pattern was observed under PEG-6000 (Fig. 1，B). ABA treatment at 4 ℃ significantly increased the expression of the *MhGlu* gene at the 4 h and 48 h time points. Expression of *MhGlu* with ABA treatment at 4 ℃ for 12 h was also higher than control，but not much significant (Fig. 1，C).

2.2　Generation of transgenic tobacco overexpressing the *MhGlu* gene.

The *MhGlu* gene of *M. hupehensis* was transformed into tobacco (K326) selected in medium containing 300 mg/L hygromycin. Among the 40 initially transformed lines，only eight putative transgenic lines survived after several rounds of 30 mg/L hygromycin treatment over 2 months. After being moved to a greenhouse，four lines survived. All four of these lines showed a fragment with the expected size of the *MhGlu* gene after PCR (1 032 bp，Fig. 2，A)，and RT-PCR also amplified the expected 1 032 bp transcript of the transformed gene (Fig. 2，B). The results showed that these four tobacco lines had been successfully transformed with the *MhGlu* gene and that the *MhGlu* gene could be transcribed in transgenic tobacco plants.

2.3　*MhGlu* overexpression in tobacco induces the expression of *PR* genes

The *MhGlu* gene was transformed into tobacco plants to study whether the overexpression of the *MhGlu* transcript could induce *NtPR*1，*NtPR*3，or *NtPR*5 gene expression. The accumulation of *NtPR*1，*NtPR*3，and *NtPR*5 mRNAs was analyzed through semi-quantitative RT-PCR (Fig. 3). In the *MhGlu*-overexpressing tobacco plants，the expression of *NtPR*1 transcripts was higher in T8，T11，and T18 than in WT，but the expression in T6 was lower than that in WT. In our study，*NtPR*3 was detected in both transgenic plants and nontransgenic plants. Nevertheless，the expression levels of transgenic plants were greater than that of nontransgenic plants. *NtPR*5 transcripts were constitutively induced in the transgenic plants only.

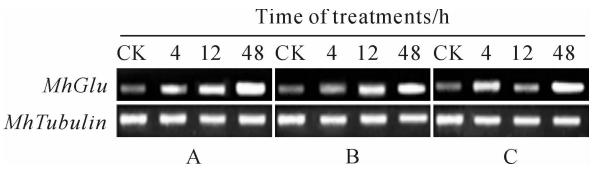

Fig. 1　Expression patterns of *MhGlu* in *M. hupehensis*

A. NaCl(Sigma)；B. PEG-6000 (Sigma)；C. ABA(Sigma) at 4 ℃

2.4　*MhGlu*-overexpressing tobacco plants exhibit enhanced resistance to *B. cinerea*

Two transgenic tobacco lines were chosen for an evaluation of disease resistance. Wild-type lines showed signs of damage by 3 days after infection with the fungus *B. cinerea*，and the lesion areas grew larger over time. In contrast，two transgenic tobacco lines infected with *B. cinerea* grew healthily，exhibiting no lesions and only a little wither.

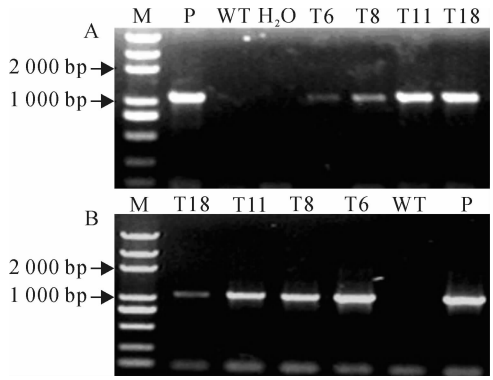

Fig. 2　Molecular conformation of *MhGlu* transgenic tobacco plants

A. PCR detection of *MhGlu*；B. Detection of *MhGlu* mRNA by RT-PCR；M. DL2000；H_2O. Blank control；P. Positive control (plasmid DNA)；WT. Non-transgenic tobacco plant；T6，T8，T11，T18. Transgenic tobacco plants

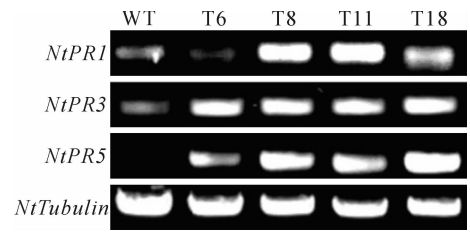

Fig. 3　Heterologous overexpression of *MhGlu* enhances the expression of *PRs* in transgenic tobacco plants by semi-quantitative RT-PCR

NtTubulin transcript levels were used to normalize the samples. Thirty-five cycles of PCR were performed for the *NtTubulin* and *NtPR*1 gene. Nt. *Nicotiana tabacum*. PR1. Pathogenesis-related protein 1；PR3. Pathogenesis-related protein 3；PR5. Pathogenesis-related protein 5

Images of representative leaves (one out of 10 for each genotype) were shown in Fig. 4. To control for the phenomena of leaf yellowing and decay over time, we also analyzed uninfected control plants. The uninfected control plants did not show any yellowing or lesion formation.

2.5 Differences of photosynthetic characteristics in nontransgenic and transgenic tobacco

The comparison of the photosynthetic parameters among transgenic and nontransgenic tested materials was shown in Table 2. Different letters within the same column indicate significant difference (P value) at 5% level. The net photosynthetic rates (P_n) of transgenic lines were higher than nontransgenic lines significantly. The similar tendency was exhibited by the stomatal conductance (G_s) and ranspiration rate (T_r). However, the difference of T_r between T6 and T8 was not significant, while P_n and G_s somewhat changed significantly in different transgenic lines.

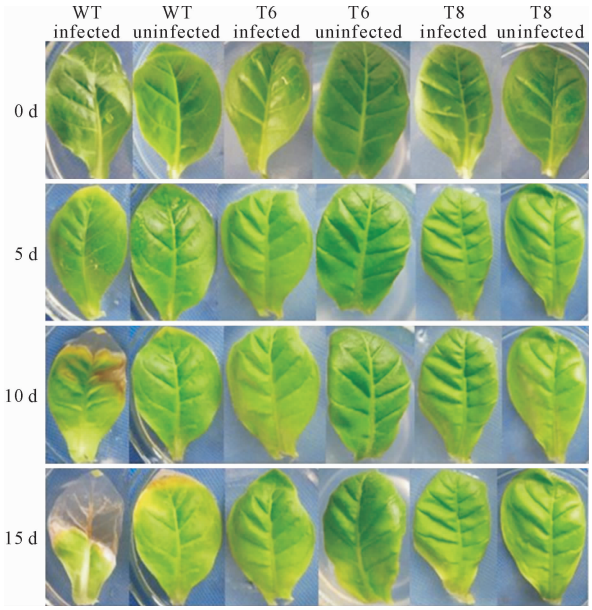

Fig. 4 Resistance to *B. cinerea* in transgenic tobacco plants expressing *MhGlu*

Table 2 Photosynthetic characteristics (P_n, T_r and G_s) in *MhGlu* transgenic plants

Test line	P_n /(μmol·m^{-2}·s^{-1})	G_s /(mmol·m^{-2}·s^{-1})	T_r /(g·m^{-2}·h^{-1})
T8	17.30±0.61a	0.852±0.02a	15.2±0.3a
T6	16.60±0.52b	0.664±0.02b	15.5±0.8a
WT	14.43±0.67c	0.530±0.01c	13.5±0.4b

3 Discussion

The overexpression of β-1, 3-glucanase in plants can enhance resistance to fungal pathogens, which have shown in wheat[7,26], barley[28], rice[24], banana[30], strawberry[22] and several other species. *MhGlu*, a β-1, 3-glucanase gene cloned from *M. hupehensis* (Pamp.) Rehd by in silico cloning, was reported to be induced by SA, MeJA and ACC and be involved in *M. hupehensis* resistance to *Botryosphaeria berengeriana*[34].

In this study, *MhGlu* was induced in *M. hupehensis* by the treatments of NaCl, PEG-6000 and ABA at 4 ℃, respectively. This result may suggest the multiple resistances to defense response of *MhGlu* in *M. hupehensis*. Cross adaptation, a phenomenon that plants espousing to a moderate stress can induce the resistance to other stresses, has been reported in different stresses combinations[39]. Under these abiotic stresses, we found that *MhGlu* gene was up-regulation, from which we can envisage that this gene may be associated with cross adaptation in plants.

MhGlu gene was transformed into the tobacco, and observed an increased resistance to *B. cinerea*. We creatively designed two different sets of control: infected WT plants and non infected ones. The lesion area in WT plants inoculated with *B. cinerea* grew larger over time, while nearly no symptoms of infection were observed in transgenic tobacco lines. Plants without infection showed no yellowing or lesion during the course of the experiments, which ensures that the leaf yellowing and decay was not caused by the passage of time. The spore suspension that was used in the experiment was not able to infect the transgenic tobacco lines, suggesting that *MhGlu* transgenic tobacco has an increased resistance to *B. cinerea*. In future studies, we will inoculate these transgenic plants with other tobacco fungal pathogens, and we speculate that the overexpression of *MhGlu* isolated from *Malus hupehensis* will enhance the resistance to pathogens other than *B. cinerea*. We will also study the mechanism of resistance in *MhGlu* transgenic apples in the future.

In this study, the SAR marker gene *NtPR1* was expressed at higher levels in the T8, T11, and T18 transgenic lines than in WT, but its expression was lower in T6 than in WT. *NtPR3* was expressed in both transgenic plants and nontransgenic plants, of which the expressions in transgenic plants were greater than nontransgenic ones. *NtPR5* transcripts were only detected from the transgenic plants. These results may reveal the interactions among different *PR* genes, in plants defence against disease stress.

To our knowledge, this is the first report of the effects of an exogenous β-1, 3-glucanase gene in the defense response of tobacco to *B. cinerea*. The pathogenesis-related induction of a 31 kD β-1, 3-glucanase was clearly established at the transcript and protein levels. Although the role of PR proteins in plant defense against *B. cinerea* is far from fully understood, our results support the involvement of β-1, 3-glucanase in resistance against *B. cinerea*, at least in tobacco. Whether β-1, 3-glucanase induction plays a direct causal role in tobacco resistance to *B. cinerea* should be studied further.

Photosynthesis is a critical function in all plants. The leaf photosynthetic rate directly affects tree growth and yield[38]. In our experiments, a comparison of the P_n of the tested materials showed that P_n was increased in T8 and T6 compared with nontransgenic lines, while T_r and G_s were all increased in the transgenic lines. These differences may be caused by gene insertion at different sites; this could produce different transgene expression patterns and levels, and thus different plant growth rates and behaviors.

Overexpression of the *MhGlu* gene in tobacco enhanced the resistance to *B. cinerea*, suggesting that SAR was induced in transgenic tobacco. Overexpression of the *MhGlu* gene also improved the plants' photosynthetic capacity.

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