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Tomato aspermy virus elimination improves medicinal quality of chrysanthemum

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Abstract: *Chrysanthemum morifolium* cv. 'Huaihuang', a medicinal chrysanthemum of China, undergoes long-term asexual reproduction and virus infection that change its quality characteristics. Our previous studies have shown that tomato aspermy virus (TAV) is the main virus infecting 'Huaihuang'. Many studies indicate that plant virus elimination can improve plant growth, but only a few studies have focused on the effects of detoxification on the medicinal components of medicinal plants. In this paper, the content of medicinal components, including chlorogenic acid, luteoloside and 3,5-O-dicaffeoylquinic acid, was compared between the TAV-free and TAV-infected (control) chrysanthemum. In addition, the reason why TAV elimination improves the medicinal components of chrysanthemum is explored. Our results suggest that TAV elimination significantly improves plant growth, enhances the enzyme activities of phenylalanine ammonia-lyase, cinnamate-4-hydroxylase and 4-coumarate:CoA ligase, and increases the levels of *CmHCT* and *CmCHS* expression, thereby greatly improving the medicinal quality of chrysanthemum.

Key words: chrysanthemum; tomato aspermy virus (TAV) elimination; chlorogenic acid; luteoloside; 3, 5-O-dicaffeoylquinic acid

INTRODUCTION

Chrysanthemum morifolium cv. 'Huaihuang' is a well-known medicinal plant produced mainly in the ancient Huaiqing area of Henan in China, and it has been used for medicinal and ornamental purposes, as well as food and tea. 'Huaihuang' contains abundant chlorogenic acid (CA), luteoloside (LO) and 3,5-O-dicaffeoylquinic acid (DCQA, an isomer of CA). Thus, CA, LO and DCQA have been chosen as standard compounds for evaluating the medicinal quality of chrysanthemum [1]. Asexual reproduction, such as cuttings and plant division propagation, is the main cultivation method for chrysanthemum, but virus contaminations greatly reduce chrysanthemum yield and quality [2-4]. Our previous studies showed that the main virus infecting 'Huaihuang' is tomato aspermy virus (TAV) [5], which causes severe mottling, dwarfing and twisting [6]. Long-term accumulation of TAV leads to slow growth, fewer flowers and substantial reduction in the amount of CA, LO and DCQA. These negative effects limit the large-scale cultivation and human consumption of 'Huaihuang', rendering it urgent to remove the virus and improve the quality of 'Huaihuang'.

Many studies have shown that the agronomic traits of virus-free plants, such as plant height, crown width, leaf number and yield, are significantly higher than those in infected plants [7,8]. For medicinal plants, virus elimination improves not only the agronomic traits, but also the medicinal quality. However, the effect of detoxification on medicinal quality has received little scientific attention.

In this study, we focused on the effect of TAV elimination on the improvement of the medicinal quality of chrysanthemum. Additionally, the relationship between the improvement of medicinal quality and TAV elimination was examined by studying the changes in enzyme activities of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL), and gene expression

levels of *CmHCT* and *CmCHS* in the biosynthesis of medicinal components in chrysanthemum.

MATERIALS AND METHODS

Plant material and growth conditions

Naturally TAV-infected plants of 'Huaihuang' growing in the ancient Huaiqing area of Henan in China were used in the present study. Stems with a young axillary bud (about 1 cm in length) were excised and used as explants for the establishment of stock cultures *in vitro*. After surface disinfection with 75% alcohol for 5 min and 0.1% $HgCl_2$ for 40 s, and then washing three times in sterile distilled water, the explants were grown on solid Murashige and Skoog (MS) medium supplemented with 2 mg/L kinetin and 0.5 mg/L α-naphthylacetic. Stock cultures were maintained at a temperature of 25±2°C under a 14 h light and a 10 h dark photoperiod with a light intensity of 36.0 μmol/(m^2 s) at the Engineering Technology Research Center of Nursing and Utilization of Genuine Chinese Crude Drugs in Henan Province (Henan Normal University, China). Subculturing was performed every four weeks.

Shoot tip culture and TAV detection

Apical and lateral shoots (about 0.3 mm) from virus-infected *in vitro* stock cultures were excised using a microscope under sterile conditions and were cultured in MS containing 0.03 mg/L kinetin; the medium was replaced every two weeks. After four weeks of culture, the leaves from the shoot-tip seedlings were sampled for total RNA extraction. Total RNA was used as a template for the synthesis of cDNA. The specific primers TAV-F (5' ATG GCC CAA AAC GGT ACG 3') and TAV-R (5' TCA CAC CGG GAG CGT TGA AG 3') were designed to amplify the TAV coat protein gene sequence (KF432415) that was obtained in a previous study [5]. The presence of TAV was determined using RT-PCR according to Zhao et al. [9].

Field growth

After RT-PCR, TAV-free plants of shoot-tip seedlings and TAV-infected plants (control) of stock cultures were transplanted into the field after subculturing and rapid propagation. In this experiment, a randomized block design including three blocks was adopted. Five hundred plants of the TAV-free group and 500 of the control group were transplanted in each block with a planting density of 60000 plants/hm^2. At the pre-harvest time (early November), the plant height and crown width of the TAV-free and control group plants were measured. Random sampling was performed according to the five-point sampling method [10], with 10 plants per point. At harvest time (mid-November), the plants were randomly harvested according to the five-point sampling method, with 20 plants per point. Flowers were harvested and dried at 55°C to a constant weight.

The content of main medicinal components

The concentrations of CA, LO and DCQA in dry flowers were determined using high-performance liquid chromatography (HPLC). The sample was ground to a powder and 0.25 g of the powder was suspended in 25 mL 70% methanol (v/v), ultrasonically processed for 40 min, and filtered through a 0.22-μm membrane prior to HPLC analysis.

An Agilent 1260 HPLC system (Agilent Technologies, Palo Alto, USA) was used to obtain HPLC chromatograms for each sample. Chromatographic separation was performed using solvents A (acetonitrile) and B (0.1% H_3PO_4) in the following separation program: 0-11 min (10-18% A), 11-30 min (18-20% A), and 30-40 min (20% A). The number of theoretical plates was ≥8000. The detection wavelength was set to 348 nm. The identification of CA, LO and DCQA was based on comparison between the retention times obtained for the standards and those in the samples run under the same experimental conditions. Each sample was injected three times with 5 μL each time. Preparation of the standard substances and calibration curves was as follows: three replicates of each standard CA, LO and DCQA (obtained from Wei Ke Qi Biological Technology in China, Sichuan) were injected (5 μL) and analyzed. The calibration curves were constructed by plotting the peak area and the total amount (μg) of each sample.

Enzyme activities and gene expression

Enzyme activities of PAL, C4H, 4CL, and gene expression of *CmHCT* and *CmCHS,* which are related to

the biosynthesis of the main medicinal components, were determined. All enzyme extraction procedures were conducted at 4°C. Fresh petals (0.1 g) obtained during the full-blossom period were homogenized in 1 mL 50 mM Tris-HCl (pH 8.9) containing 15 mM β-mercaptoethanol, 5 mM EDTA, 5 mM ascorbic acid, 10 μM leupeptin, and 0.15% (w/v) polyvinyl pyrrolidone. The homogenate was then centrifuged at 14650 *xg* for 20 min at 4°C. The supernatant was collected and used as a crude enzyme extract.

The activity of PAL was determined and expressed as the conversion rate of L-phenylalanine to trans-Cinnamic acid following Solecka and Kacperska [11] with slight modifications. The reaction mixture containing 0.5 mL crude enzyme extract and 2.5 mL 50 mM Tris-HCl (pH 8.9) buffer with 16 mM L-PAL, 3.6 mM NaCl was incubated for 1 h at 30°C. The reaction was stopped with 6 M HCl and the absorbance was measured at 290 nm. One unit of PAL was defined as the amount of enzyme that caused an increase in A_{290} of 0.001 units/min.

The activity of C4H was determined according to Lamb and Rubery [12]. The reaction mixture was composed of 0.8 mL crude enzyme extract and 2.2 mL 50 mM Tris-HCl (pH 8.9) buffer containing 2 μM trans-cinnamic acid2 μM $NADPNa_2$ and 5 μM glucose-6-PNa_2 and was incubated for 30 min at 25°C. The reaction was stopped with 6 M HCl and the absorbance was measured at 340 nm. One unit of C4H was defined as the amount of enzyme that caused an increase in A_{340} of 0.001 units/min.

The activity of 4CL was measured as the increase in A_{333} with *p*-coumarate as the substrate following Knobloch and Hahlbrock [13]. The reaction mixture was composed of 0.8 mL crude enzyme extract and 2.2 mL 50 mM Tris-HCl (pH 8.9) buffer containing 5 μM *p*-coumarate, 50 μM ATP, l μM CoA-SH, and 15 μM $MgSO_4 \cdot 7H_2O$. One unit of 4CL was defined as the amount of enzyme that caused a decrease in A_{333} of 0.01 units/min.

Quantitative RT-PCR (qRT-PCR) [14] was used to determine the expression levels of *CmHCT* and *CmCHS* at full-blossom period. Primers were designed based on the *CmHCT* and *CmCHS* sequences in the chrysanthemum transcriptome database (Primers F-5′ CAC CAG GTT ACT TTG GGA ATG 3′ and R-5′ CAG GCT GAA GTT CCA AGT AAT CGA 3′ for *CmHCT*; F-5′ GGC AGC CCA AGT CAA AGA 3′ and R-5′ CAG AGC AGA CGA CAA GAA CG 3′ for *CmCHS*). The housekeeping gene *UBI* of chrysanthemum was used as the internal reference (Primers F-5′ AGC TGA GCA GAC TCC CGA TG 3′ and R-5′ AGG CGA ATC ATC AGT ACC AAG T 3′ for *UBI*). The reaction program included initial denaturation at 95°C for 5 min, and 40 cycles at 95°C for 15 s and at 60°C for 45 s. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [14].

Statistical analysis

For all experiments, three biological replicates were used, and each was repeated three times. Student's *t*-test was carried out to determine significant differences in plant height, crown width, the main medicinal components, enzyme activities and gene expression in TAV-free and control plants. The differences between the two groups of data in all the experiments were evaluated as statistically significant ($*p<0.05$) or as extremely significant ($**p<0.01$).

RESULTS

Shoot-tip seedling acquisition and TAV detection

'Huaihuang' is mainly infected with TAV [5], so in this study we examined the effects of TAV elimination. After 6 days, the shoot tips began to swell and sprout. After 25 days, the shoot tips grew into robust plantlets. RT-PCR showed that for 35% of shoot-tip seedlings there was no specific amplification, and that a fragment of 657 bp was amplified in control plants (Fig. 1). Thus, TAV-free plantlets were obtained from the shoot-tip culture.

Effect of TAV elimination on 'Huaihuang' growth

Compared to the control, TAV-free plants grew well and did not present leaf yellowing, mottling and other illnesses (Fig. 2). The plant height and crown width of TAV-free plants were significantly higher than those of the control from July to October (Fig. 3). Comparative

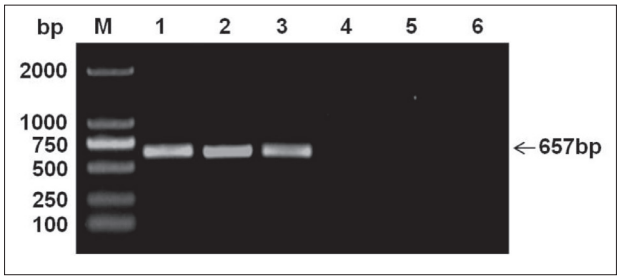

Fig.1. TAV detection in plantlets derived from control (lanes 1, 2, 3) and shoot-tip seedlings (lanes 4, 5, 6) of 'Huaihuang'.

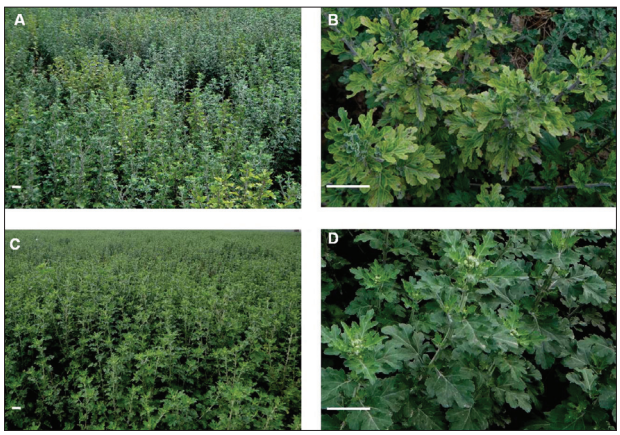

Fig. 2. The appearance of control (**A**, **B**) and TAV-free (**C**, **D**) 'Huaihuang' plants in the field. Disease symptoms were observed when the 'Huaihuang' seedlings were transplanted into the field in the fifth month. Compared with the control, TAV-free plants grow well and do not present leaf yellowing, mottling and other disease symptoms. A and C – long-range shoots; B and D – close-up shoots. Bar=5cm.

results of various agronomic traits showed that the growth of TAV-free plants was better than the control.

Effect of TAV elimination on the main medicinal components of 'Huaihuang'

We measured the content of CA, LO and DCQA in 'Huaihuang' dry flowers using HPLC with gradient elution (Fig. 4). The regression equations are as follows: $y=7.358x_1-5.529$ ($r^2=0.9999$), $y=13.39x_2+2.293$ ($r^2=0.9999$) and $y=6.8578x_3-1.3964$ ($r^2=0.9998$). Y represents the peak area, x_1, x_2, and x_3 are the concentrations of CA, LO and DCQA, respectively. Compared with control plants, the contents of CA, LO and DCQA in TAV-free plants increased by 3.92%, 33.33%, 10.27%, respectively (Table 1). Our results show that TAV elimination greatly improved the content of the main medicinal components of 'Huaihuang'.

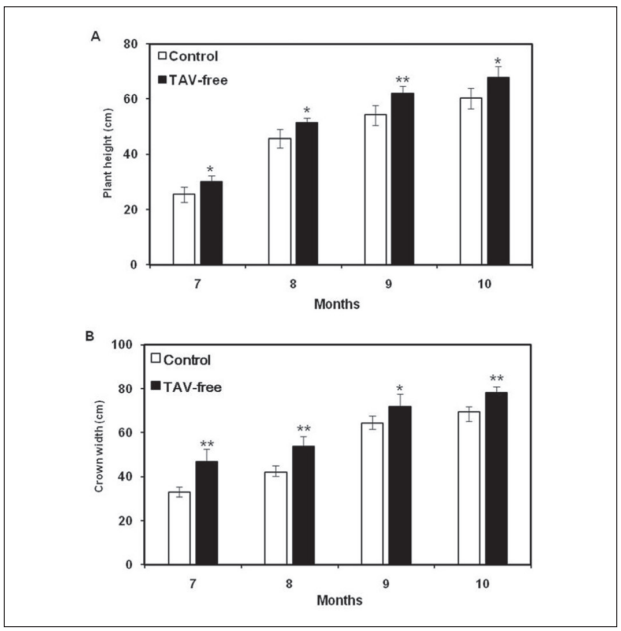

Fig. 3. Effect of TAV elimination on 'Huaihuang' plant height (**A**) and crown width (**B**). Plant height and crown width in TAV-free plants was significantly higher than in control plants (July to October). The data are the mean±SD of ten biological replicates. The asterisks indicate statistically significant differences between the plants from which TAV has been eliminated and control plants (*P<0.05; **P<0.01).

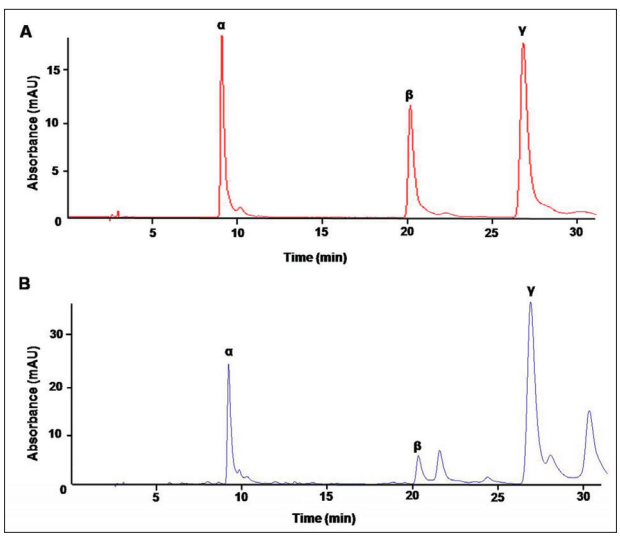

Fig. 4. Typical HPLC chromatograms of 'Huaihuang' flowers. **A** – CA, LO and DCQA standards. **B** – CA, LO and DCQA samples. The peaks represent CA (α), LO (β) and DCQA (γ), respectively. mAU: milli absorbance unit.

Table 1. The effect of TAV elimination on the content of the main medicinal components of 'Huaihuang' (%).

Plant	Chlorogenic acid	Luteoloside	3,5-O-dicaffeoylquinic acid
Control	0.485±0.027	0.087±0.005	1.714±0.066
TAV-free	0.504±0.006*	0.116±0.035*	1.890±0.234*

The data of all samples are presented as the mean±SD of three biological replicates. Student's t-test was carried out to determine significant differences in TAV-free and control plants. The asterisks indicate statistically significant differences (*P<0.05; **P<0.01).

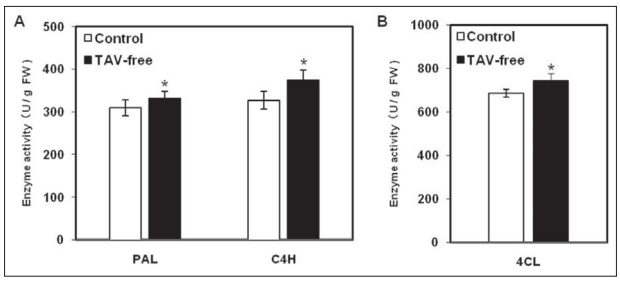

Fig. 5. Effect of TAV elimination on the enzyme activities of PAL and 4CL (**A**), C4H (**B**). PAL, 4CL and C4H are the key enzymes involved in the biosynthesis of the main medicinal components of 'Huaihuang'. TAV elimination significantly improved the activities of PAL, 4CL and C4H in TAV-free plants. FW – fresh weight. The data are the mean±SD of three biological replicates. The asterisks indicate statistically significant differences between TAV elimination and controls (*$P < 0.05$).

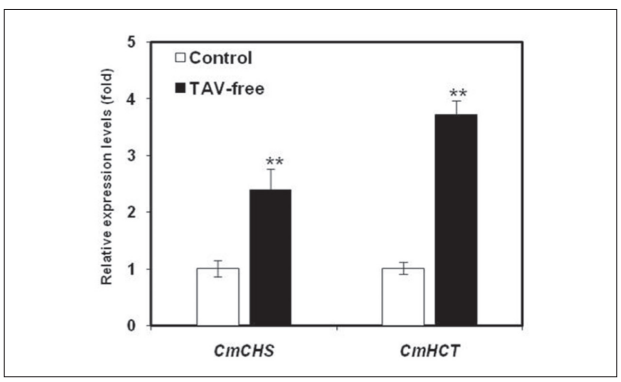

Fig. 6. Real-time PCR quantification of *CmHCT* and *CmCHS* in control and TAV-free 'Huaihuang' plants. *CmHCT* and *CmCHS* are the principal genes involved in the biosynthesis of the main medicinal components in 'Huaihuang'. TAV elimination significantly increased the expression of *CmHCT* and *CmCHS*. The data are the mean±SD of three biological replicates. The asterisks indicate statistically significant differences between the TAV-free and control plants (**P<0.01).

The effect of TAV elimination on enzyme activities and gene expression

As shown in Fig. 5, the activities of PAL, 4CL and C4H, which are the key enzymes involved in the biosynthesis of the principal medicinal components of 'Huaihuang', are significantly higher in TAV-free plants than in control plants. The relative expression levels of *CmHCT* and *CmCHS*, which are genes encoding for the major enzymes involved in the biosynthesis of the main medicinal components, were 2.39- and 3.71-fold higher, respectively, in TAV-free plants when compared to control plants (Fig. 6). These results show that the elimination of TAV increases the activities of the major enzymes and the relative expression levels of genes encoding the key enzymes related to the biosynthesis of the main medicinal components, thus improving the principal medicinal component content of 'Huaihuang'.

DISCUSSION

For most crops, research is focused on establishing a suitable detoxification method and obtaining virus-free plants [15-18] while little is known about the effect of detoxification on the contents of the plants' medicinal ingredients. 'Huaihuang' is a traditional Chinese herbal medicine, and the content of its main medicinal components (CA, LO and DCQA) is one of the main criteria for evaluating its value. Whether virus elimination can maintain or improve its medicinal value is the key factor determining whether virus elimination technology should be popularized and further applied. In this study, we increased the content of three medicinal components of 'Huaihuang' by TAV elimination, which indicated that detoxification can improve the medicinal value of 'Huaihuang'.

CA, LO and DCQA are all secondary metabolites generated by the phenylalanine metabolic pathway. In this pathway, coumaroyl-CoA is an important intermediate product that can be further transformed into CA and LO, and CA can be isomerized to DCQA. Phenylalanine can be converted to coumaroyl-CoA through sequential catalysis by PAL, C4H and 4CL. Hydroxycinnamoyl-CoA transferase (HCT) and chalcone synthase (CHS) are the key enzymes for CA and LO synthesis, respectively. Coumaroyl-CoA and shikimic acid are catalyzed by HCT to synthesize CA,

and coumaroyl-CoA and 3 × malonyl CoA are catalyzed by CHS to synthesize LO [19]. It is speculated that the levels of the three in 'Huaihuang' are closely related to the activities of PAL, C4H, 4CL, HCT and CHS. Therefore, in this study we measured the enzyme activities of PAL, C4H and 4CL and the expression levels of *CmHCT* and *CmCHS* as the reasons for the improvement of the main medicinal components of TAV-free chrysanthemum.

Our results showed that TAV elimination significantly improved plant growth and enhanced the activities and gene expression levels of the principal enzymes involved in the biosynthesis of the main medicinal components of the plant, thereby greatly improving its medicinal quality. Moreover, this study also proves that detoxification is an effective method to improve the medicinal quality of chrysanthemum.

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Author contributions: Xiting Zhao and Liwei Jiang conceived and designed the study; Yingyuan Zhou and Ying Tian performed the experiments; Ke Liu and Mengdan Ma performed the data analysis; Xiting Zhao and Liwei Jiang wrote the paper; Mingjun Li revised the paper. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors declare that they have no competing interests.

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