

Extraction, purification and anti-TMV effects of α (β)-2,7,11-cembratriene-4,6-diol from tobacco leaves

Yongxin Feng^{1†}, Haijiang Jia^{2†}, Hui Guan¹, Weidong Zhang¹, Yanbin Zhou¹, Kaiyi Liu¹,
Yuejing Wang¹, Qiuli Li¹, Weihua Chen¹, Muhammad Aamir Sohail⁴, Jie Wang⁵, Shen
Huang^{3*}, Jianyu Wei^{2*}

¹ China Tobacco Hebei Industrial Co., Ltd.

² China Tobacco Guangxi Industrial Co., Ltd.

³ College of Food and Biological Engineering, Zhengzhou University of Light Industry,
Henan, 450002, China

⁴ Key Lab of Plant Pathology of Hubei Province, Huazhong Agricultural University, Wuhan
430070, Hubei, China

⁵ Key Laboratory of Tobacco Pest Monitoring Controlling & Integrated Management,
Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, 266101,
China.

[†] Both authors made equal contributions to this work.

* Correspondence: wangjie@caas.cn (S.-H); jtx_wjy@163.com (J.-Y.W.)

Abstract

Background: Acetone ethanol extracts from tobacco leaves have antiviral activity against TMV, but the antiviral effects of their specialized metabolites have not been systematically studied yet, especially the underlying mechanism is still unexplored.

Results: The tobacco cembranoids $\alpha(\beta)$ -2,7,11-cembratriene-4,6-diol ($\alpha(\beta)$ -CBD) were extracted and purified with an effective and green protocol including innovatively added 5% phosphoric acid for elution, one time silica gel chromatographic column separation and impurity removal and further HPLC purification. The results of antiviral activities against tobacco mosaic virus (TMV) with the local lesion counting method showed that $\alpha(\beta)$ -CBD have *in vivo* higher protective effects of 73.2% and 71.6%, at 75.0 μ M, respectively, than control agent Ningnanmycin (53.1%). Notably, The results of ELISA and and TMV-GFP fluorescent optical imaging assay indicated a obviously reduced viral protein and weaker GFP fluorescence signal and smaller infection area, which confirmed their anti-TMV activities at protein level. Furthermore, the enhanced production of SA and JA and the significantly increased transcription of of JA signaling pathway (*COII* and *PDF1.2*) and SA signaling pathway genes (*PR1*, *NPRI* and *EDSI*) in treated plants further conformed that exogenously applied $\alpha(\beta)$ -CBD can effectively elicit the tobacco plant immunity against TMV.

- 41 *Conclusions:* The $\alpha(\beta)$ -CBD mainly stimulates disease resistance of tobacco plants to resist
- 42 TMV and it can be used as bioagents to control TMV in the future.
- 43 **Keywords:** α -2,7,11-cembratriene-4,6-diol, β -2,7,11-cembratriene-4,6-diol, TMV, antiviral
- 44 activity

1. Introduction

Nicotiana tabacum L. (the flue-cured tobacco), an herbaceous plant belonging to the *Nicotiana* genus (Solanaceae family), is now cultivated worldwide and has long been used medicinally and recreationally as the most economically important industrial crops[1,2]. Moreover, *Nicotiana* species are commonly investigated for the biological activity of their specialized metabolites in responses to abiotic stress or biotic stress factors such as pathogens[3,4]. Tobacco mosaic virus (TMV) is the most ancient virus that causes massive economic losses to tobacco, pepper, cucumber and ornamental crops globally. Several findings suggested that acetone ethanol extracts from *N. tabacum* leaves have antiviral activity against TMV[5-8], but the antiviral effects of their specialized metabolites have not been systematically studied yet, especially the underlying mechanism is still unexplored.

Cembranoids, a group of natural diterpenoid products comprising four isoprene units of a 14-carbon macrocyclic skeleton, are mainly distributed in *Nicotiana tabacum*, *Pinus* genera, and some marine species (e.g., soft coral)[9,10]. In *Nicotiana* spp., cembranoids are most prevalent in the surface secretion of leaves and flowers[11]. A wide variety of bioactivities, including neuro-protective, anti-cancer, anti-invasive, antifungal, antiviral and anti-Inflammatory activities against animal viruses, have been carried out with two main cembranoids, α -cembratrien-diol (α -CBD) and C4 epimer (β -cembratrien-diol, β -CBD) (Figure 1)[12-14]. In addition to exhibiting suppression in spore germination of many fungal diseases (*Peronospora tabacina*, *Colletotrichum lagenarium*, *Candida albicans*, *Fusarium chlamydosporum*, *Aspergillus niger* and *Alternaria alternata*), exogenously applied $\alpha(\beta)$ -CBD also inhibited the growth of bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and

Proteus vulgaris)[15,16]. Interestingly, in response to tobacco mosaic virus infection, a substantial increase in cembranoids, in particular α/β -CBD, has been achieved with systemic acquired resistance (SAR) leaves[17,18]. However, it is scarcely examined whether the exogenous application of $\alpha(\beta)$ -CBD has inhibitory effects on tobacco virus infection or not.

In continuing efforts to identify natural product-derived antiviral agents against plant virus, we herein report the simultaneous extraction and identification of α -CBD and β -CBD from Yunyan 100 (a variety of *N. tabacum*). TMV-GFP based systemic host approach was used to assess the two compounds' anti-TMV potential in tobacco. The isolation, structural identification and biological evaluation of these compounds are listed here.

2. Materials and methods

2.1. Plant material

Fresh mature leaves of tobacco (Yuyan 100) for purification of α -CBD and β -CBD were harvested in Xuchang District (33.53 north latitude, 113.49 east longitude), Henan Province, China, in July 2014. Tobacco plants were cultivated in a light chamber at the light intensity of 10,000 lux with a long day photoperiod (18 h : 6 h, light : dark). The following experiments were conducted when *Nicotiana tabacum* L. cv. Samsun NN was selected at the seventh leaf stage for the local lesion counts, and *N.benthamiana* had grown 6 leaves for systemic TMV infection determination. TMV-GFP (a recombinant TMV encoding green fluorescent protein) was a gift from Professor Yule Liu, (Tsinghua University, Beijing, P. R. China).

2.2. Large scale purification and isolation

The purification and isolation of $\alpha(\beta)$ -CBD was conducted as described previously with some revision[19,20]. The leave extracts from fresh mature tobacco leaves (200 kg) were extracted with dichloromethane (CH_2Cl_2) three times (3-5 sec each time at room temperature). The combined extracts were filtered to remove insoluble ingredients and green transparent filtrates were obtained. A brown residue (0.8 kg) was obtained after the removal of the solvent under reduced pressure at 45 °C, and completely dissolved under ultrasound radiation conditions in CH_2Cl_2 . CH_2Cl_2 extract was applied to silica gel (100-200 mesh) column chromatography eluted with $\text{MeOH} : \text{C}_4\text{H}_8\text{O}_2 = 3 : 1$, and the solvent was distilled under reduced pressure, and resuspended with CH_2Cl_2 (0.4 kg). CH_2Cl_2 dissolved matter was passed through two silica gel column chromatography of 200-300 mesh and 300-400 mesh in order eluted with $\text{CH}_2\text{Cl}_2 : \text{C}_4\text{H}_8\text{O}_2 = 3 : 1$. α -CBD (0.07 kg) with 70% purity and β -CBD (0.07 kg) with 70% purity were obtained. Finally, $\alpha(\beta)$ -CBD (0.07 kg) with 70% purity were applied C18 column chromatography (YMC-Pack ODS-A 250×20 mm, D.S-5 μm) eluted with $\text{C}_2\text{H}_5\text{N} : \text{H}_2\text{O} = 3 : 1$. And $\alpha(\beta)$ -CBD with 98 % purity were obtained.

The UPLC conditions for detecting CBD after purification were: capillary column: ACQUITY UPLC BEH C18 column (Waters, Milford, USA), 1.7 μm (2.1×50 mm); injection volume was 1 μL ; the mobile phase was a mixture of acetonitrile and water in a ratio of (60:40) at a flow rate of 0.5 mL/min; column temperature was 40 °C, and the UV wavelength was set at 210 nm.

Purified $\alpha(\beta)$ -CBD was checked by GC-MS (Agilent 7895A-5975C, USA). Chromatographic conditions were: capillary column: DB-5 ($60 \text{ m} \times 250 \mu\text{m} \times 0.25 \mu\text{m}$);

carrier gas: He; injection volume: 1 μ L; injector temperature: 180 °C; split ratio: 6:1; column temperature 120 °C for 1 min, programmed at 5 °C/min to 180 °C, 2 °C/min to 240 °C, and 5 °C/min to 280 °C. Mass spectrometer conditions were: interface temperature: 280 °C; quadrupole temperature: 150 °C; ion source temperature: 250 °C; solvent delay 8.5 min; mass scan range: 35–500 AMU.

After purification of the C18 column, we obtained two peaks containing CBD. The colorless compound 1 from the first peak was condensed to dryness and dissolved at 55 °C in N-pentane. Compound 1 solution was held at 25 °C, and was slowly transformed into the crystals. Compound 2 from the second peak was white and concentrated to dryness and dissolved in N-pentane at 55 °C. Compound 2 solution was kept at –5 °C, and was slowly transformed into the crystals. High-resolution GC-MS for determining $\alpha(\beta)$ -CBD is described in 2.3. NMR for resolving the structure of $\alpha(\beta)$ -CBD was done[21]. ^1H and ^{13}C experiments were carried out at 600 MHz on a Bruker Advance 600 MHz spectrometer (Bruker, Rheinstetten, Germany)[22].

2.3. Antiviral activity assays

TMV was According to Gooding's method[23], TMV virions maintained in *N. tabacum* cv. K326 were extracted and purified from the harvested leaves of systemically inoculated seedlings by homogenization in 0.01 M PBS (phosphate-buffered saline) buffer. The supernatants obtained by centrifugation at 1800 g for 3 min were stored at - 20 °C for use.

Purified $\alpha(\beta)$ -CBD was dissolved in DMSO and diluted with distilled H₂O containing

0.1% Tween 80 to the required concentrations (12.5, 25.0, 50, 75 µg/mL) for the following treatments. Ningnanmycin (200 µg/mL) was used as a positive control.

For the protective assay *in vivo*[24,25], The right half leaves of *N. tabacum* L. cv. Samsun NN were gently rubbed with α and β-CBD solution, respectively. After 6 h, the same area received the inoculation of 100 µL TMV (10.19 µg/mL), The positive control was the left half leaves smeared with TMV after applying DMSO solvent. The numbers of local lesions were counted 5 days after inoculation. Five replicates were implemented for each sample.

For the inactive assay *in vivo*[24,25], the TMV particles (10.19 µg/mL final concentration) were firstly mixed with the tested agents solutions at room temperature for 15 min, and then the mixed solution was spread on the left half leaves of *N. tabacum* L. cv. Samsun NN, whereas the right side was smeared with TMV mixed with DMSO solution. The numbers of local lesions were counted five days after inoculation. Five replicates were conducted for each sample.

For the curative assay *in vivo*[24,25], the whole leaves of *N. tabacum* L. cv. Samsun NN were inoculated with TMV particles (10.19 µg/mL final concentration). α and β-CBD were smeared onto the left half leaves 24 h after TMV inoculation, while the DMSO solution was smeared onto the right side as a negative control. The numbers of local lesions were counted five days after inoculation. Three replicates were conducted for each sample.

The inhibition rate of viral infection was calculated according to the following formula:

$$\text{Inhibition rate (\%)} = \frac{C_0 - C}{C_0} \times 100$$

$C0$: the mean value of local lesions for the control treatment; C : the average number of local lesions for the treatments.

2.4 TMV-GFP infection foci measurement

The third leaf from the top of *N. benthamiana* plant was mechanically inoculated with TMV by rubbing the extracts of TMV-GFP infected leaves at 24 h after applying $\alpha(\beta)$ -CBD (75.0 μ M) as foliar spray. Each treatment experiment was performed with five plants (replicates).

Fluorescent optical imaging of TMV-GFP inoculated *N. benthamiana* leaves was conducted under identical illumination and exposure conditions with an imaging fluorimeter Fluorcam FC 800-O (Photon System Instruments, Drasov, Czech Republic) at five and seven days post-inoculation (dpi), respectively. ImageJ v1.80 was used for the quantitative analysis of the GFP expression level and the areas of fluorescent infection sites [26].

2.5 Quantitative RT-PCR and TAS-ELISA

Total RNA was extracted with the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher Scientific, Shanghai, China) following the user guide. cDNA was prepared by using the PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan). Quantitative RT-PCR (qRT-PCR) reactions were implemented with PlatinumTM SYBRTM Green qPCR SuperMix - UDG (Invitrogen, Thermo Fisher Scientific) using the ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA). The reference gene *EF-1a* was used for quantitative analysis. The relative expression levels of the tested genes were calculated with the $2^{-\Delta\Delta C_t}$ method [27]. The primer sequences used are shown in Supplementary Table 1.

According to the previous studies[28], the accumulation of TMV protein was quantified by Triple antibody sandwich enzyme-linked immunosorbent (TAS-ELISA) assay kit (Agdia, Elkhart, IN, USA) following the user guide. was conducted to quantify in TMV infected tobacco leaves using an assay following the manufacturer's instructions .

The inhibition level of viral proliferation was recorded and calculated according to the following formula:

$$\text{Inhibition rate (\%)} = \frac{C-T}{C} \times 100$$

C : the viral content in the positive control; T : the viral content in the treated leaves.

Only TMV infected leaves were adopted as positive control. TMV concentration was calculated by the standard curve with the A_{405} value of TMV at concentrations of 8, 4, 2, 1, 0.5, 0.25 and 0.125 $\mu\text{g/mL}$. Absorbance at 405 nm was monitored with an iMark microplate reader (iMark13083, Bio-Rad, USA). All experiments were repeated three times and consisted of at least five tobacco seedlings per replicate.

2.6 Determination of JA and SA

The quantification of SA and JA from plant crude extracts were performed by LC-MS/MS following the previous description[29]. Approximately 200 mg harvested leaves were ground to a fine powder in liquid nitrogen. D6-SA (2-hydroxybenzoic acid-[2H6]) obtained from Sigma-Aldrich and H2-JA (dihydrojasmonic acid) obtained from OlChemim were used as internal standards. The supernatants after the removal of residues were analyzed on HPLC-tandem mass spectrometry (1200 L LC-MS system, Varian, American). Each treatment was consist of five replicates per sample .

2.7 Statistical analysis

All the experiment data presented with mean $\pm$ SD were pooled across three independent repeated experiments. Analysis of variance (ANOVA) was performed using SPSS v 19.0 (SPSS Inc., Chicago, IL, USA). The statistical differences between α -CBD and β -CBD treatment were considered significant with a Student's *t*-test at $P < 0.05$.

3. Results

3.1 structural identification of CBD

To obtain $\alpha(\beta)$ -CBD with the high purity, an efficient and green extraction and purification method was established. In contrast to previous research, the extracts were first washed in 5% phosphate, rather than the mixed n-hexane/(methanol-water) solution, which has a remarkable effect on eliminating significant impurities including nicotine and water-soluble sugar. The prepared samples were then applied to the silicon column, and eluted by different ratios (1:1, 3:1, 5:1 and 7:1) of petroleum ether/ethyl acetate to determine which ratio was best for purifying CBDs and removing other components. The fractions eluted by a 1:1 ratio of petroleum ether/ethyl acetate had some CBTs, with a higher abundance around 50 min. However, there were still many other components (Fig. 2A). When the ratio was changed to 3:1, many CBTs were found, and the other components were decreased in quantity and abundance (Fig. 2B). The fractions eluted by a 3:1 ratio of petroleum ether/ethyl acetate had the largest number of CBDs than the other ratios (5:1 and 7:1), while the slightest number of other components was obtained in quantity and abundance

(Fig. 2 C-D).

We performed semi-preparative HPLC using the UPLC condition to obtain CBD with a 60:40 ratio of acetonitrile: water. Two distinct peaks emerged clearly within 90 sec. After the samples were eluted using the silicon column and applied to semi-preparative HPLC, two targeted peaks were entirely separated from other components and even the CBDs themselves within 35 min (Fig. 3-4). The purity of the two compounds done by semi-preparative HPLC was >98%, confirmed by UPLC (data not shown). The yield of compound 1 and compound 2 were 0.005% and 0.007%, respectively. The structures of the compounds 1 and 2 were shown in Fig. 1, and the ^1H and ^{13}C NMR data of 1 and 2 were listed in Figures S1–S4, respectively.

The crystals of Peak 1, which melted at 65–66°C, were colorless. The crystals of Peak 2 were white, and the melting point was 125–126°C. The chemical structures of the two compounds were confirmed by high-resolution LC-MS and NMR, respectively. This experimental process is simple, easy to operate, and the obtained compounds have high purity, both above 98%.

The molecular formula of compound 1, $\text{C}_{20}\text{H}_{34}\text{O}_2$, was deduced from MS, and its molecular weight was 306. Some useful information about compound 1 was established, including: m/z 329.24493 was $[\text{M}+\text{Na}]^+$, m/z 289.25262 was $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$ and m/z 635.50128 was $[2\text{M}+\text{Na}]^+$ (Fig. 5A). In secondary MS of compound 1 (Fig. 5B), m/z 329.24 became 311.17 after losing one H_2O . In triple MS, after losing both H_2O and $-\text{C}_3\text{H}_6$, m/z 293.00 became 251.33 (Fig. 5C). Compound 1 was preliminarily determined as 2,7,11-cembratriene-4,6-diol. Both the fine structure and the absolute configuration of the

chiral carbon atom at C₄ of α-2,7,11-cembratriene-4,6-diol was confirmed by ¹³C-NMR and ¹H-NMR (Figure S1-S2).

The molecular formula of compound 2, C₂₀H₃₄O₂, was deduced from MS, and the molecular weight was 306. Some useful information about Compound 2 was established, including: m/z 329.24316 was [M+Na]⁺, m/z 289.25107 was [M-H₂O+H]⁺ and m/z 635.49762 was [2M+Na]⁺ (Fig. 6A). In secondary MS of Compound 2 (Fig. 6B), m/z 329.24 became m/z 311.17 after losing one H₂O. In triple MS, after losing both H₂O and -C₃H₆, m/z 293.00 became 251.33 (Fig. 6C), which was similar to that of α-2,7,11-cembratriene-4,6-diol. Compound 2 was preliminarily determined as β-2,7,11-cembratriene-4,6-diol, and confirmed by ¹³C-NMR and ¹H-NMR (Figure S3-S4).

3.2 Exogenous CBDs Enhance Resistance to TMV

The local lesion host *N. tabacum* L. cv. Samsun NN pretreated with α- and β-CBD had fewer lesions than DMSO pretreated plants. As shown in Table 1, obvious inhibitory effects of α-CBD and β-CBD against TMV in a dose-dependent manner was obtained by the half-leaf method in *N. tabacum* L. cv. Samsun NN. α- and β-CBD treatments showed higher protective effects (73.2% and 71.6%) at 75.0 μg/mL than Ningnanmycin treatment (53.1%) at 200 μg/mL. Compared with the protective activity, α- and β-CBD at 75.0 μg/mL possessed relatively lower curative (35.0% and 38.7%) and inactive activities (37.4% and 33.2%).

TMV-GFP was used to analyze which virus infection steps involved in the inhibitory effects of α-CBD and β-CBD (Fig. 7). Compared with DMSO and Ningnanmycin treatments along with a higher GFP intensity, a significantly weaker viral GFP fluorescence signal and

smaller infection area were obtained in α -CBD and β -CBD pretreated *N. benthamiana* (Fig. 7A). There are no clear differences in the foci number of TMV-GFP each plant and foci size per foci between α -CBD and β -CBD pretreatment (Fig. 7B and C). The meantime for TMV-GFP moving to apical leaves in α -CBD and β -CBD pretreatment was 2 days later than the Ningnanmycin treatment, although this difference was not statistically significant ($P=0.08$) (Fig. 7D).

The results of qRT-PCR indicated that any distinct differences in the relative expression of the TMV-CP gene were not detected among the control and both CBDs treatments, while an approximately 55% decrease of the TMV-CP gene in Ningnanmycin treatment was obtained (Fig. 8A). However, TAS-ELISA results showed that the viral protein synthesis was prominently inhibited in CBDs treated leaves (Fig. 8B). At 75 μ g/mL, α -CBD and β -CBD treatments caused 50% and 48% decreases in the contents of TMV-CP protein compared to the control, respectively, while 22% reduce in the viral protein content was gained in Ningnanmycin treatment (Fig. 8B and Table 2). These results suggest that CBDs may mainly obstruct the virus protein biosynthesis processes.

3.3 Effects of CBDs on phytohormone levels

The concentrations of JA in the CBDs-treated leaves under TMV infection were almost 1.8 times at 24 hpi and 1.5 times at 36 hpi higher than that of the control treatments, respectively, while the contents of SA in the treated leaves were almost 2.0 times at 24 hpi and 1.4 times at 36 hpi higher (Fig. 9). Meanwhile, no significant differences were obtained

between α -CBD and β -CBD treatments. These results implied that CBDs could evidently enhance the generation of JA and SA in the treatment leaves.

The transcription of JA signaling pathway (*COII* and *PDF1.2*) and SA signaling pathway genes (*PR1*, *NPRI* and *EDSI*) has made for obvious differences in performance. The larger elevation and longer duration of all these marker genes expression in CBDs pretreated plants occurred than that in the water pretreatment under TMV stress. The highest expression levels of JA and SA signaling pathway genes were observed at 24 hpi and 36 hpi. However, there were no significant differences in the expression of the tested genes between α -CBD+ TMV and β -CBD+TMV treatments. These transcriptions have similar trends in the diversification of JA and SA (Fig. 3G and H).

4. Discussion

Cembranoids are carbocyclic diterpenes and have gained widespread attention for the past few years and become one of the main focuses on natural product chemistry[11]. In this study, we successfully obtained $\alpha(\beta)$ -CBD with high purity (98%) using an efficient and green extraction and purification method. Compared with previous reports, the present method eliminates liquid-liquid extraction process of the n-hexane/(methanol-water) mixed solution, and innovatively added 5% phosphoric acid to wash for 1 to 2 times, then washed with deionized water for 2 to 3 times to neutral in impurities process, which has a significant effect on removing major impurities such as nicotine and water-soluble sugars. Furthermore, the present method is simple and easy to operate. Large amounts of high-purity compounds

can be obtained after one time silica gel chromatographic column separation and impurity removal and further purification by HPLC method.

Several natural products extracted from *N. tabacum* exhibited anti-TMV activity, such as flavonoids from the roots and stems of Yunyan-202[6], biphenyls from the leaves of *Honghuadajinyuan*[7], sesquiterpenes and benzolactones from the leaves of Yunyan-201[8]. Here, we found that $\alpha(\beta)$ -CBD from Yunyan 100 leaves exhibited higher protection effects than the curative and inactive activities in *N. tabacum* L. cv. Samsun *NN* by the half-leaf process in compared with the previously mentioned anti-TMV components from different varieties of tobacco. For initial screening, TMV and its local lesion host *N. tabacum* L. cv. Samsun *NN* was used. However, sometimes only the counts of local lesion for the screen of antiviral agents may not be enough in susceptible plants. Therefore, *N. benthamiana* for tracking assay by green fluorescent-protein tagged TMV. The tendency of their antiviral activities was confirmed by ID-ELISA in K326 and tracking images of TMV-GFP in *N. benthamiana*.

CP is essential for viral systemic infection and replication, which is involved in cell to cell and long-distance transport of TMV and the host response[30]. Our qRT-PCR and TAS-ELISA results indicated that $\alpha(\beta)$ -CBD significantly interfere with either TMV CP protein biosynthesis or post-translational modification processes, which is consistent with the observed TMV-GFP images of inoculated *N. benthamiana* leaves. However, full exploration are still needed whether CBD directly affect the structure of virus particle and stereoscopic assembly of the virus.

Besides, $\alpha(\beta)$ -CBD can elicit plant defense against virus infection without cost to the plant's fitness. Much of previous researches have reported that tobacco diterpenes play diverse roles, functioning as signaling molecules, or activating agents in plant defense. Seo et al. (2003) documented that Z-abienol-related diterpene (11E,13E)-labda-11,13-diene-8 α ,15-diol could stimulate tobacco plants defense response against TMV infection[5]. Fujimoto et al. (2015) showed that sclareol as an elicitor-related substance boosts the resistance of *Arabidopsis* and tomato roots against nematode penetration[31]. The signaling molecules SA and JA play critical roles in the defense response of tobacco plants to TMV. The enhanced production of SA and JA and the significantly increased transcription of the tested genes *COII*, *PDF1.2*, *PRI*, *NPRI* and *EDSI* further conformed that exogenously applied $\alpha(\beta)$ -CBD can effectively elicit the tobacco plant immunity against TMV.

In conclusion, α -CBD and β -CBD with high purity (98%) were separated and purified from tobacco leaves using an efficient and green extraction and purification method. Exogenously applied $\alpha(\beta)$ -CBD showed excellent inhibitory activity against TMV infection in a dose-dependent manner. In particular, α - and β -CBD at 75.0 μ g/mL exhibited higher protective effects with the values of 73.2% and 71.6% than that of Ningnanmycin (53.1%) at 200 μ g/mL. The significant antiviral activity is due to its ability to disturb either TMV CP protein biosynthesis or post-translational modification processes and active natural immunity of tobacco plants by inducing the increased activity of defense-related enzymes and the improved transcription of SA signaling pathway genes. Collectively, $\alpha(\beta)$ -CBD have potential as new disease resistance elicitors to control TMV in the future.

Author Contributions: Y.-X.F., H.-J.J. and H.G. isolated and purified of active compounds and analyzed the structure. W.-D. Z., Y.-B. Z., M. A. S. tested anti-TMV activities of α -CBD and β -CBD. D.-S.G. tested Transmission Electron Microscope (TEM). W.D. Z., Y.B. Z., K.Y. L., Y.J. W., Q.L. L., W.H. C. determined the defense-related enzymes activity and the relative expression of SA and JA signaling pathway genes. S.-H. and J.-Y.W. designed the experiments and supervised the research. J. W., J.-Y.W. and Y.-X.F. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Table 1. Inhibition effects of α and β -CBD on TMV in *N. tabacum* L. cv. Samsun NN by half leaf method

Treatment	Concentration ($\mu\text{g/mL}$)	Protection effect (%)	Curative effect (%)	Inactivation effect (%)
α -CBD	12.5	30.5 $\pm$ 7.6a	21.4 $\pm$ 3a	21.5 $\pm$ 4a
	25.0	43.4 $\pm$ 4.9b	28.1 $\pm$ 9b	32.8 $\pm$ 6b
	50.0	58.0 $\pm$ 5.3c	33.0 $\pm$ 8c	33.7 $\pm$ 5b
	75.0	73.2 $\pm$ 4.2d	35.0 $\pm$ 8c	37.4 $\pm$ 4b
β -CBD	12.5	38.0 $\pm$ 6.3b	27.2 $\pm$ 6b	25.3 $\pm$ 6a
	25.0	45.9 $\pm$ 5.4b	33.4 $\pm$ 3c	27.0 $\pm$ 9ab
	50.0	60.6 $\pm$ 7.1c	36.5 $\pm$ 2cd	30.5 $\pm$ 7b
	75.0	71.6 $\pm$ 8.4d	38.7 $\pm$ 8cd	33.2 $\pm$ 4b
Ningnanmycin	200	53.2 $\pm$ 5.5c	51.4 $\pm$ 4d	56.0 $\pm$ 6d

All results are expressed as means $\pm$ SD; Numbers with different letters are statistically different at $p < 0.05$, $n = 3$ for all groups.

Table 2. Inhibition effects of α and β -CBD in *N. benthamiana* on TMV by TAS-ELISA

Sample	Concentration ($\mu\text{g/mL}$)	Protective effect (%)	Inactive effect (%)	Curative effect (%)
α -CBD	75	68.7 $\pm$ 3a	36.4 $\pm$ 5a	34.1 $\pm$ 4a
β -CBD	75	65.2 $\pm$ 5a	39.2 $\pm$ 3a	32.2 $\pm$ 3a
Ningnanmycin	200	51.3 $\pm$ 2b	56.7 $\pm$ 5b	47.5 $\pm$ 3a

473 All results are expressed as means $\pm$ SD; Numbers with different letters are statistically different at $p <$
 474 0.05, $n = 3$ for all groups.

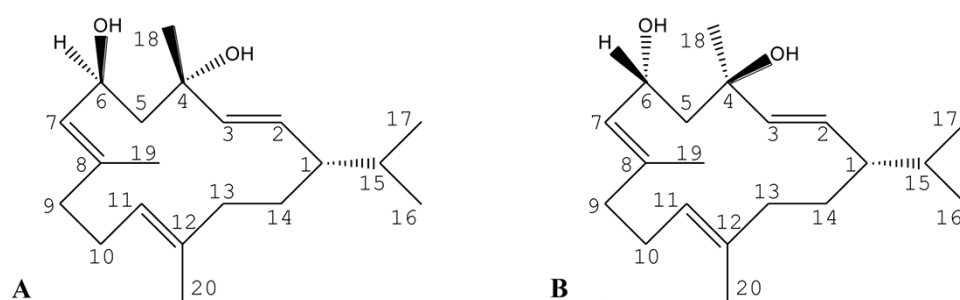
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481 **Figure 1. Molecular structure of α -CBD (A) and β -CBD (B).**

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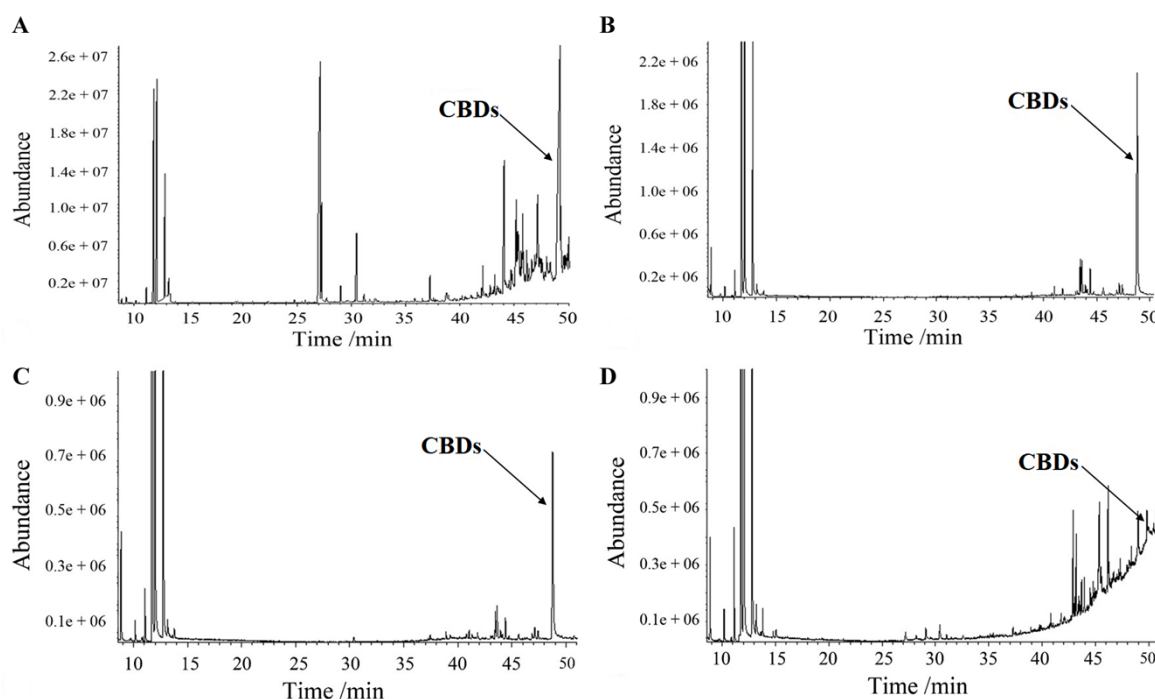


Figure 2. (A) Total ion current of fractions eluted by 1:1 ratio of petroleum ether/ethyl acetate. (B) Total ion current of fractions eluted by 3:1 ratio of petroleum ether/ethyl acetate. (C) Total ion current of fractions eluted by 5:1 ratio of petroleum ether/ethyl acetate. (D) Total ion current of fractions eluted by 7:1 ratio of petroleum ether/ethyl acetate.

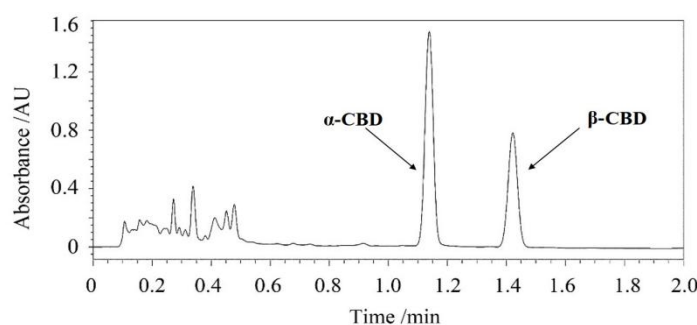


Figure 3. UPLC of mobile phase acetonitrile: water = 6:4 (v/v)

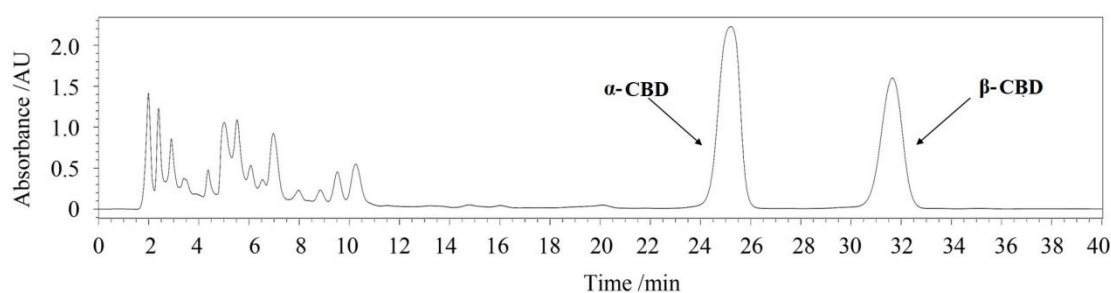


Figure 4. Semi-preparative HPLC of CBDs purified by C₁₈ column

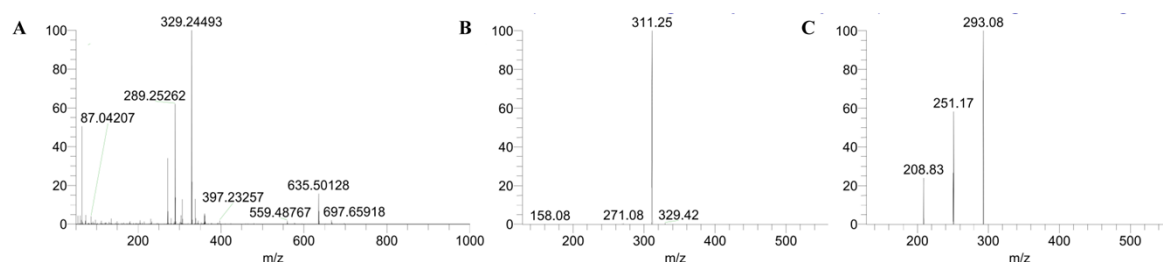


Figure 5. (A) High-resolution MS of Compound 1; (B) Secondary MS of Compound 1; (C) triple MS of Compound 1.

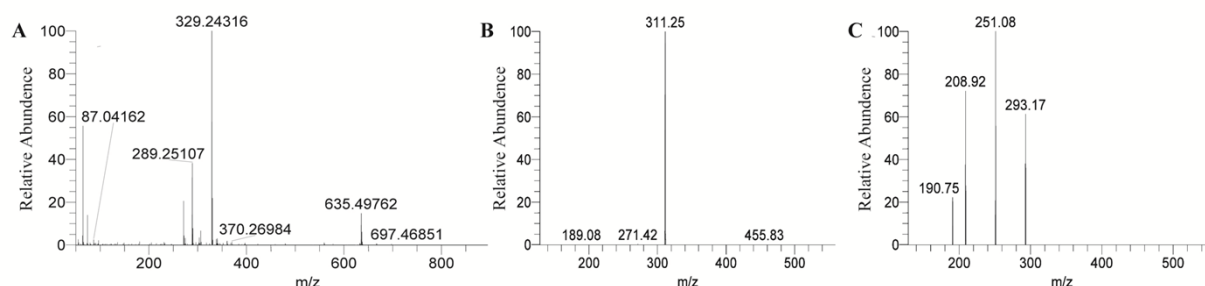


Figure 6. (A) High-resolution MS of Compound 2; (B) Secondary MS of Compound 2; (C) triple MS of Compound 1.

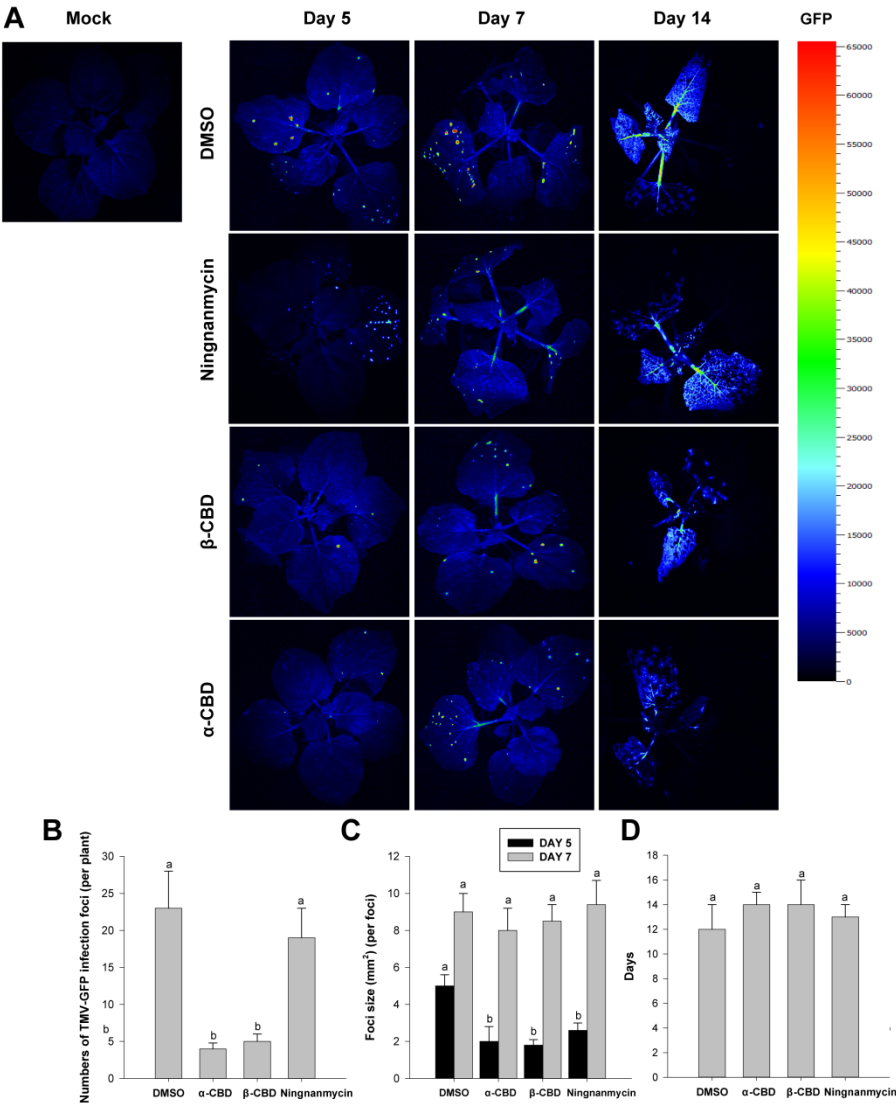


Figure 7. CBDs enhance *N. benthamiana*' resistance against TMV. DMSO,

Ningnanmycin, α -CBD and β -CBD pretreated plants were inoculated on the third leaf

from top with TMV-GFP; (A) Fluorescent optical imaging of systemic TMV-GFP

multiplication in different treatments was taken under UV light at 5, 7 and 14 days

post-inoculation (dpi); (B) Numbers of infection foci on inoculated leaves; (C)

TMV-GFP multiplication was quantified in the treated plants by calculating the foci

size per foci; (D) The mean time for TMV-GFP moving to apical leaves in each treatment. Statistical significance was tested by the Kruskal–Wallis test and significantly different groups are indicated by different letters for each time point ($P < 0.05$). The data were the mean $\pm$ SD of three replicates (at least 20 plants per replicate). Similar results were obtained in three independent repeats of the experiment.

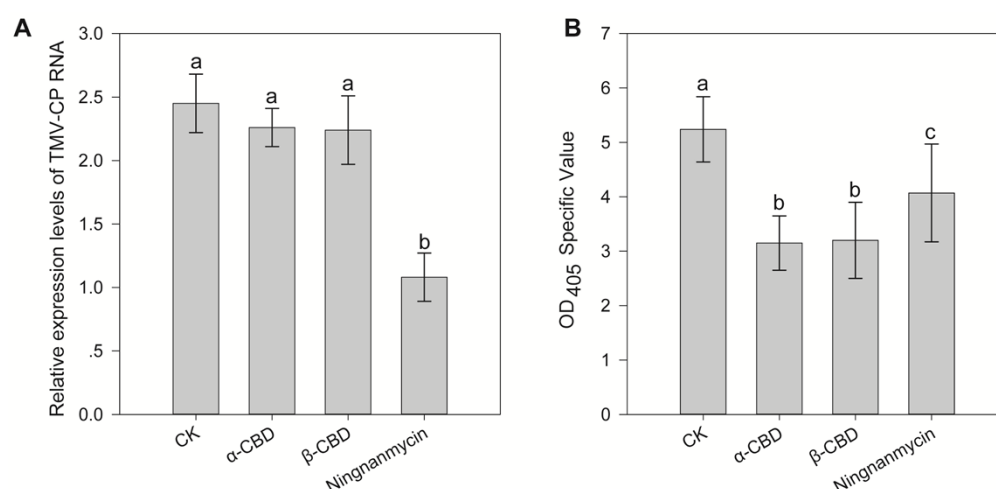


Figure 8. Protective effects of α and β -CBD in *N. benthamiana* on TMV. (A) Gene expression Levels of TMV CP in α and β -CBD-treated leaves; (B) Relative accumulation of TMV protein in tobacco leaves in α and β -CBD-treated leaves

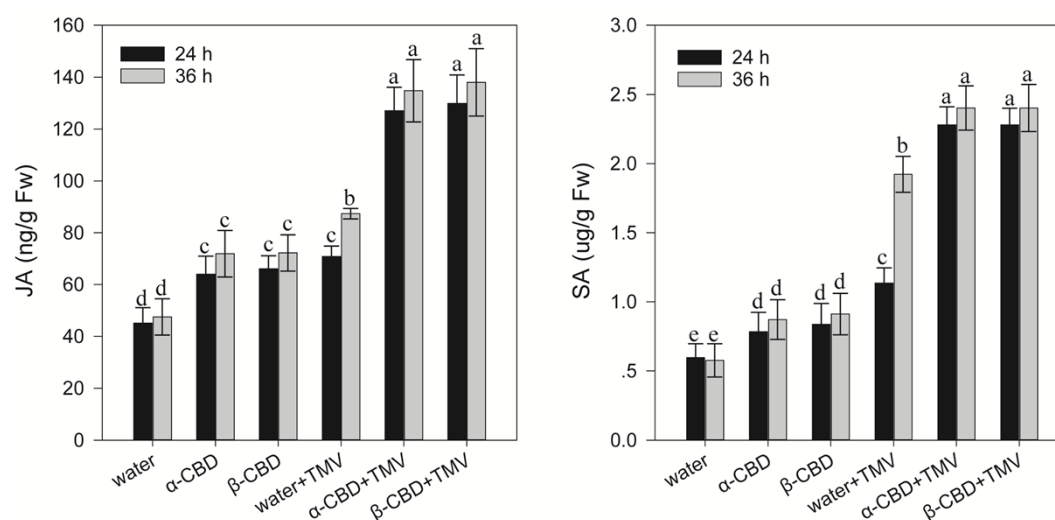


Figure 9. Effect of α -CBD and β -CBD on the contents of JA and SA at 24 h and 36 h after CBD treatment under TMV infection.

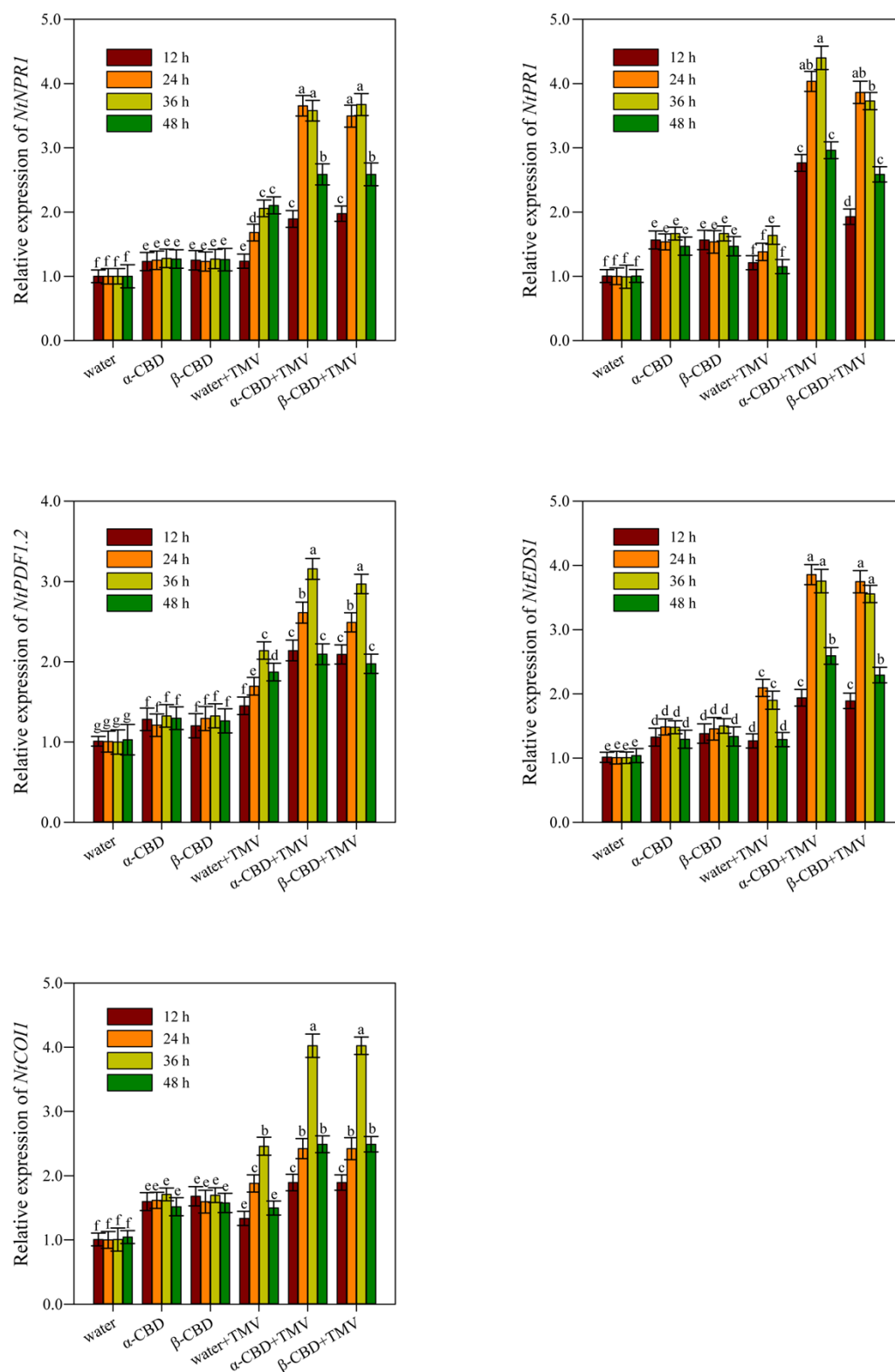


Figure 10. Relative expression of key genes of JA (COI1 and PDF1.2) and SA signaling pathway genes (PR1, NPR1 and EDS1) .

545 **Supporting information materials:**

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547 **Table S1. Groups of real-time quantitative PCR (RT-qPCR) primers used to amplify**

548 **genespecific regions.**

549

550 **Figure S1. ^{13}C - NMR spectra of α -CBD**

551 **Figure S2. ^1H -NMR spectra of α -CBD**

552 **Figure S3. ^{13}C -NMR spectra of β -CBD**

553 **Figure S4. ^1H -NMR spectra of β -CBD**