Amino Acids (2015) 47:2495–2503
DOI 10.1007/s00726-015-2040-z

ORIGINAL ARTICLE

Synthesis and biological activity evaluation of novel amino acid derivatives as potential elicitors against *Tomato yellow leaf curl virus*

Yufang Deng[1] · Shun He[1] · Qianqian Geng[1] · Yongheng Duan[1] · Mingcheng Guo[1] · Jianqiang Li[1] · Yongsong Cao[1]

Received: 27 April 2015 / Accepted: 24 June 2015 / Published online: 11 July 2015

Abstract Disease caused by *Tomato yellow leaf curl virus* (TYLCV) brings serious production losses of cultivated tomato worldwide. In our previous study, two novel amino acid derivatives exerted satisfactory antiviral activities against TYLCV. In this study, the variation of TYLCV, the transcriptional expression level of *Ty-1* and the enzyme activities of POD and PPO in tomato were monitored after treatment with two amino acid derivatives to illustrate the antiviral mechanism. The results showed the symptom severity caused by TYLCV was reduced significantly by two compounds and was associated with the inhibition of viral DNA level at the early stage. Among three levels of concentration, the highest inhibition rate of CNBF-His was 40.66 % at 1000 mg/L, for CNBF-Asn, the highest inhibition rate was 36.26 % at 2000 mg/L 30 days post-inoculation. Two compounds could also enhance the activities of PPO and POD and the transcriptional expression level of *Ty-1* which correlates with plant resistance in tomato. In the field test, two compounds increased the yields of tomato and the maximum increase of yield was 37.66 %. This is the first report of novel amino acid derivatives inducing resistance in tomato plant against TYLCV. It is suggested that amino acid derivatives have the potential to be an effective approach against TYLCV in tomato plant.

Keywords Amino acid derivative · Systemic resistance · *Tomato yellow leaf curl virus* · Peroxidase · Polyphenol oxidase · Transcriptional expression

Abbreviations

TYLCV	*Tomato yellow leaf curl virus*
SAR	Systemic acquired resistance
POD	Peroxidase
PPO	Polyphenol oxidase
BTH	S-methyl 1,2,3-benzothiadiazole-7-carbothioate
CNBF-His	2-((2,6-dinitro-4-(trifluoromethyl)phenyl) amino)-3-(1H-imidazol-4-yl))propanoic acid
CNBF-Asn	4-amino-2-((2,6-dinitro-4-(trifluoromethyl) phenyl)amino)-4-oxobutanoic acid
dpi	Days post-inoculation
CK	The control

Introduction

Disease caused by *Tomato yellow leaf curl virus* (TYLCV) is one of the most devastating begomoviruses diseases affecting tomato production in tropical and temperate areas worldwide (Navas-Castillo et al. 2011). The TYLCV is a monopartite geminivirus and transmitted by the whitefly (*Bemisia tabaci*). It brings serious production losses, especially when plants are infected at an early age, the yield loss up to 100 %. TYLCV was first reported in the Jordan Valley (now Israel) as early as 1939, and identified in 1961 (Cohen and Antignus 1994). To date, the virus has been reported in many areas, including the Middle East, Africa, North America, Europe, the Mediterranean Basin, and Asia (Lefeuvre et al. 2010). In China, it was first reported in 2006 in Shanghai (Wu et al. 2006). Subsequently, tomato

Handling Editor: J. Leban.

✉ Yongsong Cao
caoysong@126.com; caoys@cau.edu.cn

Yufang Deng
dengyufang456@163.com

[1] China Agricultural University, NO.2 Yuanmingyuan West Road, 100193 Beijing, China

yellow leaf curl disease broke out in many regions of China and had caused severe damage to tomato production (Zhang et al. 2009).

The management approaches of TYLCV mainly include breeding TYLCV-resistant tomato cultivars and controlling the vector population. Breeding TYLCV-resistant tomato cultivars is considered to be the best approach (Polston and Lapidot 2007). Currently, only a limited number of genotypes of commercial resistant cultivars are available. It is a challenge to combine high level of resistance with high fruit quality (Lapidot and Friedmann 2002). Controlling the vector population is the most important measure used in practical production, which usually requires heavy pesticide use and physical barriers (Seal et al. 2006). Heavy pesticide usage produces side effects, notably environmental contamination and the development of resistance of whitefly to pesticide (Palumbo et al. 2001).

Systemic acquired resistance (SAR) is an inducible defense mechanism that plays a significant role in plant defense (Hammerschmidt 2009). The resistance lasts a long time, sometimes for the lifetime of the plant, and is effective against a broad spectrum of pathogens (Durrant and Dong 2004). SAR can be activated by avirulent pathogen or certain chemicals such as s-methyl 1,2,3-benzothiadiazole-7-carbothioate (BTH) (Faoro et al. 2008), β-aminobutyric acid (Barilli et al. 2010), 2,6-dichloroisonicotinic acid (INA) (Dann et al. 1998). These plant activators can protect many species of plants against diseases caused by bacterial, fungal and even viral pathogens. Thus, inducing systemic resistance in tomato is also considered to be an effective approach against TYLCV. The development of SAR is accompanied with a series of physiological and biochemical responses. The increases of some enzyme activities were taken as a criterion to evaluate the disease resistance. These plant enzymes include peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia-lyase and so on. POD is an active oxygen-scavenging enzyme, which can regulate the production of reactive oxygen species. PPO involves in the oxidation of polyphenols into quinones (antimicrobial compounds) and lignification of plant cells during the microbial invasion. POD and PPO were found to be the typical enzyme correlating with disease resistance in plants (Gozzo 2003; Mohammadi and Kazemi 2002). Molecularly, the resistance is characterized by the increased expression of a large number of genes. There are five TYLCV resistance genes that have been exploited for resistance breeding, including *Ty-1*, *Ty-2*, *Ty-3*, *Ty-4*, *Ty-5*. *Ty-1* is the major gene in a gene work contributing to the resistance against TYLCV in tomato, which is associated with the virus accumulation in host and the long-distance transport of virus (Michelson et al. 1994). Tomato carrying *Ty-1* and challenged by the virus shows low level of viral replication and systemic spread with moderate or no visual symptom (Verlaan et al. 2013). It is meaningful to analyze the transcriptional expression level of *Ty-1* in tomato with induced resistance.

In our previous work, thirteen amino acid derivatives were synthesized from 4-chloro-3,5-dinitrobenzotrifluoride and amino acids, including glycine, alanine, valine, isoleucine, proline, aspartic acid, asparagine(Asn), glutamic acid, glutamine, serine, threonine, phenylalanine and histidine(His), respectively. The appropriate reaction conditions were ascertained and the activities against TYLCV in tomato plants were tested. The results showed that 2-((2,6-dinitro-4-(trifluoromethyl)phenyl)amino)-3-(1H-imidazol-4-yl))propanoic acid (CNBF-His) and 4-amino-2-((2,6-dinitro-4-(trifluoromethyl)phenyl)amino)-4-oxobutanoic acid (CNBF-Asn) exerted satisfactory antiviral activities against TYLCV. The aim of this study is to illustrate the mechanisms of two compounds against TYLCV by monitoring the amount of viral DNA, the transcriptional expression level of *Ty-1* and the enzyme activities of POD and PPO in tomato after inoculating TYLCV.

Materials and methods

Melting points were recorded by an electrothermal digital apparatus which are uncorrected. The ^{1}H NMR spectra were recorded with a Bruker Avance DPX 300 MHz NMR spectrometer. ^{13}C NMR spectra were obtained with the same instrument at 75 MHz. The MS spectra were measured with Agilent LC-MSD-Trap-VL Electrospray ion (ESI) mass spectrometer. The elemental analysis data were measured by an LECO-183 CHNS analyzer. BTH (Actigard 50WG) was purchased from Syngenta Co. Ltd (Shanghai, China). All other solvents and chemicals are reagent grade and used without further purification.

Synthesis of CNBF-His and CNBF-Asn

4-chloro-3,5-dinitrobenzotrifluoride (1.4610 g, 5.4 mmol), sodium bicarbonate (0.5040 g, 6 mmol), histidine (0.9310 g, 6 mmol) and 10 mL H_2O was added into 50 mL round-bottom flask equipped with a condenser. The reaction mixture was heated to 70 °C and sodium bicarbonate solution was dropwise-added to keep the pH at 8.5–9.0. After stirring for 2 h, the solution was cooled and the pH was adjusted with concentrated hydrochloric acid. The yellow solid was obtained after filtration.

Plant material

Tomato plants (*Lycopersicon esculentu* Mill. cv. Moneymaker) were grown in a mixture of peat:sand (1:1) in an insect-proof greenhouse at 20–24 °C, under a 16:8 h

light:dark photoperiod. Experiment was performed with plants each having two–three expanded leaves.

Inhibition rate was calculated from the disease severity index by the following formula:

$$\text{Inhibition rate} = \frac{\text{Severity index of the control} - \text{severity index of each treatment}}{\text{Severity index of the control}} \times 100\,\%$$

Pot experiment

To induce resistance, CNBF-His and CNBF-Asn with three concentrations, 500, 1000, 2000 mg/L, were sprayed on leaves. The control plants were treated with distilled water. In addition, BTH at a final concentration of 100 mg/L was used as positive control. Plants were treated with test compound for two times separately at 7, 3 days before inoculation. After inoculation, all plants were maintained in an insect-free greenhouse.

Agro-inoculation of plants

Agrobacterium tumefaciens containing the infectious clone of TYLCV-[CN:SH2] friendly provided by Zhejiang University (Hangzhou, China) was used in all experiments (Zhang et al. 2009). Agro-inoculation of tomato plants was performed with the method previously reported (Xie et al. 2006). *Agrobacterium tumefaciens* cultures were grown at 28 °C for 48 h ($OD_{550} = 1$). A 21-gauge needle was used to inject 0.2 mL of bacterial culture into stems or petioles of plants.

Disease evaluation

The level of the resistance induced in tomato plants against TYLCV was evaluated by disease severity index and inhibition efficiency. Disease severity was investigated 30 days post-inoculation (dpi). Disease severity of all individual plants was graded using the rating scale as documented by Scott (Scott et al. 1996). The rating scale was from 0 to 4, 0 = no symptoms, 1 = slight symptoms visible only on close inspection, 2 = intermediate symptoms visible on part of the plant, 3 = severe symptoms over the entire plant, and 4 = severe symptoms and stunting. Disease severity index was calculated as reported earlier by Raupach (Raupach et al. 1996), from the disease rating by the following formula:

$$\text{Disease severity index} = \frac{\sum(\text{Disease grade} \times \text{number of plants in each grade}) \times 100}{\text{Total number of plants} \times \text{highest disease grade}}$$

Quantitative PCR

Total DNA was extracted using a modified CTAB protocol referenced method (Abarshi et al. 2010). A relative quantitation protocol to determine the amount of TYLCV-[CN:SH2] (GenBank NO.: AM282874) in tomato leaves was established. The primer was designed using the Primer Premier 5.0 software and is shown in Table 1. The nuclear-encoded large subunit ribosomal RNA gene (tomato 25S ribosomal RNA gene, GenBank Accession no. X13557) was selected as the endogenous tomato gene (Mason et al. 2008). Primers were purchased from Biomed Co. Ltd. (Beijing, China). Quantitative real-time PCR was carried out in a 7500 real-time PCR system (Applied Biosystems, America) using the SYBR® Premix DimerEraser™ kit (Takara, Dalian, China). Each 20 μL of final reaction volume contained 10 μL of SYBR Premix DimerEraser (2×), 0.4 μL of reference dye II, 0.6 μL of each primer, 7.4 μL of ddH_2O, and 1 μL DNA sample. Cycling parameters were as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 34 s (collecting fluorescent signal).

Viral DNA detection

For relative quantitation in plants, the second expanded leaf from the apex downwards taken from each treatment plants were collected every 3 dpi. Samples were extracted using the aforementioned CTAB method. Before preparation for reaction volume, each DNA sample was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific, America). DNA concentration of each sample was adjusted in the range from 1.0 to 10.0 ng/μL. The $2^{-\Delta\Delta C_T}$ method was used to calculate relative changes in gene expression in this real-time quantitative PCR experiment.

Table 1 Primers designed for the detection of TYLCV (CN:SH2) and the endogenous 25S rRNA tomato gene

Gene	Primer	Oligonucleotide sequence (5′-3′)	Product size (bp)
TYLCV(CN:SH2)	YG-3	GAGTTCCCCTGTGCGTGAA	139
	YG-5	CTGTTCGCAAGTATCAATCAAGGT	
25S rRNA tomato gene	UNIV(+)	ATAACCGCATCAGGTCTCCA	113
	UNIV(−)	CCGAAGTTACGGATCCATTT	

Quantitative RT-PCR

For gene expression analysis, the second expanded leaf from the apex downwards taken from each treatment plants were collected every 3 dpi. Total RNA was extracted using the SV Total RNA Isolation System (Promega, America) as described by the manufacturer. The cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China) following the protocol provided by the manufacturer. Quantitative Real-Time PCR was performed in 20 uL reactions in a 7500 real-time PCR system (Applied Biosystems, America) using the SYBR® Premix DimerEraser™ kit (Takara, Dalian, China) according to the protocol provided by the manufacturer.

The primer for quantitative RT-PCR of *Ty-1* was described by Maarten (Verlaan et al. 2013) with the forward primer: 180-F1 (5′-GGCAAAATATGCAGCCAGGCTTTCC-3′) and the reverse primer: 180-R1 (5′-TCAGTATGTATACGAGGTTCGCCGT-3′). As a reference, the *ACT* gene was used as described by Lillo (Løvdal and Lillo 2009) with primers: ACT-F (5′-GAAATAGCATAAGATGGCAGACG-3′) and ACT-R (5′-ATACCCACCATCACACCAGTAT-3′). Gene expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method.

Enzyme activity assay

Enzyme activity assay was performed on leaf extracts. Tomato leaves from each treatment plants were collected every 7 dpi. The POD and PPO activities were measured based on the method previous reported (Mohammadi and Kazemi 2002). 0.5 g leaf tissue was homogenized in 5 mL cold phosphate buffer (pH 6.0). After centrifugation at 12,000 rpm for 10 min, the supernatant was collected and used for enzyme assay (Rinder et al. 2008).

POD activity was carried out in Lambda 45 UV–Vis Spectrometer (Perkin Elmer, America). Before assay, the reaction solution was prepared as described in the following step. 28 μL guaiacol was added to 50 mL phosphate buffer (pH 6.0) and heated to dissolve it. Then 19 μL 30 % H_2O_2 was added, mixed well and stored at 4 °C as the reaction solution. The reaction mixture contained 0.2 mL enzyme extracts and 3.8 mL reaction solution. The absorbance at 470 nm was monitored for 2 min and POD activity was expressed as active unit per gram per minute. Each sample was assayed in triplicate.

PPO activity was assayed in triplicate using 3.5 mL phosphate buffer (pH 6.0), 1 mL 0.2 M catechol and 0.5 mL enzyme extracts. The absorbance at 525 nm was followed for 2 min and PPO activity was expressed as active unit per gram per minute.

Field experiment

The field experiment was conducted in an experimental field in vegetable sheds, which were divided into subareas of 14 m^2, and within each subarea, approximately 30 tomato plants were planted. Randomized block design was adopted in this field test. Four varieties were used in the field experiment, including Moneymaker, Beidouxin'ao, Oudun and Zhongnong 105. Moneymaker was an extremely susceptible variety to TYLCV and three other varieties were the mainly cultivated tomato varieties in China. Each variety had six treatments, including the control (CK), BTH as positive control, CNBF-His and CNBF-Asn with two concentrations, 1000, 2000 mg/L, respectively. Three repeated tests were performed for each test. Plants were treated with test compound for two times separately at 7, 3 days before inoculation. After inoculation, all plants were agro-inoculate.

Table 2 The structure and molecular formula of CNBF-His and CNBF-Asn

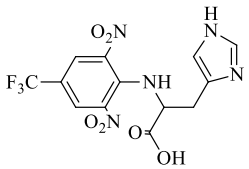
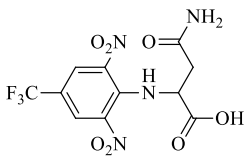
Compound	Molecular formula	Structural formula	Chemical name
CNBF-His	$C_{13}H_{10}F_3N_5O_6$		2-((2,6-dinitro-4-(trifluoromethyl)phenyl)amino)-3-(1H-imidazol-4-yl)propanoic acid
CNBF-Asn	$C_{11}H_9F_3N_4O_7$		4-amino-2-((2,6-dinitro-4-(trifluoromethyl)phenyl)amino)-4-oxobutanoic acid

Table 3 The disease severity index with treatment of CNBF-His and CNBF-Asn

Compounds	Concentration (mg/L)	Mean disease severity index	Inhibition rate (%)
CK	–	37.92 (a)	–
BTH	100	24.58 (bcd)	35.16
CNBF-His	500	28.75 (bc)	24.18
	1000	22.50 (d)	40.66
	2000	27.50 (bcd)	27.47
CNBF-Asn	500	30.42 (b)	19.78
	1000	27.92 (bcd)	26.37
	2000	24.17 (cd)	36.26

Data are presented as the mean of three trials each with 20 plants per treatment. Mean disease severity indexes within each column followed by different letters are significantly different at $P < 0.05$ by one-way analysis of variance

Table 4 The relative amount of viral DNA in plant with different disease grade

Disease Grade	The relative amount of viral DNA		
	10 dpi	20 dpi	30 dpi
1	1449.21 (a)	6677.94 (a)	12506.52 (a)
2	6402.41 (b)	29496.45 (b)	49619.35 (c)
3	11925.12 (c)	56458.65 (c)	24093.42 (b)
4	38652.12 (d)	24121.90 (b)	21405.61 (b)

Data are presented as the mean of 8 plants from each disease grade. Means within each column followed by different letters are significantly different at $P < 0.05$ by one-way analysis of variance

Results and discussion

Preparation of CNBF-His and CNBF-Asn

The structure and molecular formula of CNBF-His and CNBF-Asn are shown in Table 2. All compounds were separated and purified by recrystallization or silica gel chromatography. Their structures were identified by ^{1}H NMR, ^{13}C NMR, MS and elemental analyses.

Data for CNBF-His: yellow solid, yield 91 %, mp >250 °C. ^{1}H NMR (300.13 MHz; DMSO-D6; Me_4Si), δ 13.12 (br s, 2 H), 9.04 (d, 1 H, $J = 7.2$ Hz), 8.56 (s, 2 H), 8.04 (s, 1 H), 6.97 (s, 1 H), 4.01 (d, 1 H, $J = 5.4$ Hz), 3.12-2.91 (m, 2 H); ^{13}C NMR (75.48 MHz; DMSO-D6; Me_4Si), δ ppm = 29.18, 58.06, 115.11, 115.58, 116.18, 121.08, 124.68, 128.87, 131.65, 134.83, 138.91, 139.81, 171.41; MS-EI(*m/z*) calcd for $C_{13}H_{10}F_3N_5O_6$ $[M + H]^+$ 390.26, found 390.00; elemental analysis (%) found: C 40.25, H 2.42, N 18.03. Requires: C 40.11, H 2.59, N 17.99.

Data for CNBF-Asn: yellow solid, yield 92 %, mp 164–165 °C. ^{1}H NMR (300.13 MHz; DMSO-D6; Me_4Si), δ 13.11 (br s, 1 H), 8.86 (d, 1 H, $J = 9.6$ Hz), 8.60 (s, 2 H), 7.57 (s, 1 H), 7.10 (s, 1 H), 3.99-3.92 (m, 1 H), 2.87-2.69 (m, 2 H); ^{13}C NMR (75.48 MHz; DMSO-D6; Me_4Si), δ ppm = 36.61, 56.14, 116.22, 116.69, 121.10, 124.70, 129.10, 139.45, 141.12, 171.75, 172.05; MS-EI(*m/z*) calcd for $C_{11}H_9F_3N_4O_7[M - H]^+$ 365.22, found 364.80; elemental analysis (%) found: C 36.17, H 2.63, N 15.22. Requires: C 36.08, H 2.48, N 15.30.

Efficacy against TYLCV

The disease severity indexes with treatment of CNBF-His and CNBF-Asn are shown in Table 3. Pre-treatments of tomato plants with CNBF-His and CNBF-Asn or BTH reduced significantly the disease severity caused by the virus. Under screening concentration, compounds show good activity against TYLCV with inhibition rates above 20 % at 30 dpi except for CNBF-Asn (500 mg/L). Among three levels of concentration, the highest inhibition rate of CNBF-His is 40.66 % at 1000 mg/L, for CNBF-Asn, the highest inhibition rate is 36.26 % at 2000 mg/L. BTH as the positive control, the inhibition rate is 35.16 % at the concentration of 100 mg/L. There is no significant difference ($P < 0.05$) in mean disease severity index among CNBF-His (1000 mg/L), CNBF-Asn (2000 mg/L) and BTH (100 mg/L). Therefore, this concentration for each compound was used for further analyses.

Effect on the viral DNA variation

To understand the disease development better, the viral DNA contents of inoculated plants were detected to show the level variation of symptom severity at 10, 20 and 30 dpi (Table 4). On the 10th day post-inoculation, only a few plants showed symptom and most of them showed slight sign of yellowing. There was a positive correlation between the viral DNA level and the grade of disease severity. More severe symptom the host had, the higher the viral DNA level was. While in the later period (20 dpi and 30 dpi), the viral DNA level was not positively correlated with symptom severity. On the 20th day post-inoculation, the virus level of grade 4 was not significantly different from that of grade 2. And on the 30th day post-inoculation, the grade 3 was also not significantly different from that of grade 4. Presumably, the competitive disease model may play a dominant role in the early stage of symptom and disease development. In this model, virus commandeers host transcriptional and translational machinery, host genes shutdown and the availability of virus synthesis increase. Thus, the viral DNA level has a positive correlation with symptom severity in the early stage. This is consistent with the previous study on the relationship between the resistance level of plants and the TYLCV accumulation level

(Rom et al. 1993). However, the competition for cellular resources is not a major contributor to the appearance of disease symptoms. Instead, when the symptom severity reaches a certain extent (grade 3 or 4), the interaction disease model begins to dominate. The virus level increases rapidly and then subsides (Culver and Padmanabhan 2007). Compared to competitive model, the interaction model seems to be more likely to explain the variations in disease severity when viral accumulation does not correlate with symptom severity. The virus–host interaction is clearly important but far from understood (Culver and Padmanabhan 2007; Dreher and Miller 2006).

In addition, the viral DNA variation of tomato leaf with time was monitored. As can be seen in Figs. 1 and 2, the virus level of each treatment increased slowly at the early stage (before 12 dpi) and then peaked twice in the later stage. Consistently, previous research reported that the viral DNA level peaked twice at 14 and 28 dpi (Lapidot et al. 1997). The control had higher virus level than any other treatments. The virus levels of treatments with CNBF-His (1000 mg/L) and CNBF-Asn (2000 mg/L) were lower than that of other treatments at the early stage and began to increase slowly until 18 dpi. There was a negative correlation between virus levels at the early stage and inhibition rates. Considering the possible relationship between the viral level and symptom severity, CNBF-His and CNBF-Asn may lower the viral DNA level in plants to reduce the symptom severity at the early stage.

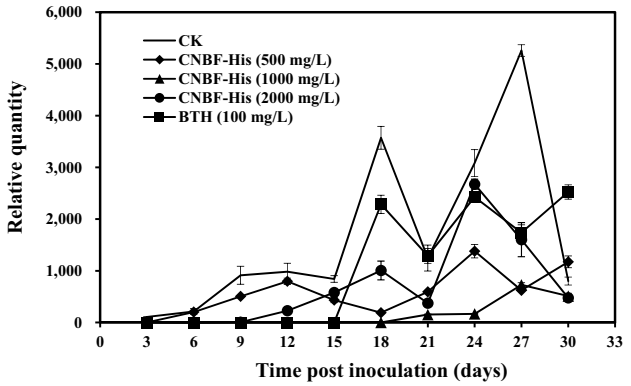


Fig. 1 The viral DNA variation of tomato treated by CNBF-His with time. *Vertical value* the relative amount of virus which presented in each sample and normalized to the amount of the endogenous 25S tomato gene, which is the mean of six replicates from two parallel experiments. *Bars* standard errors, $n = 6$

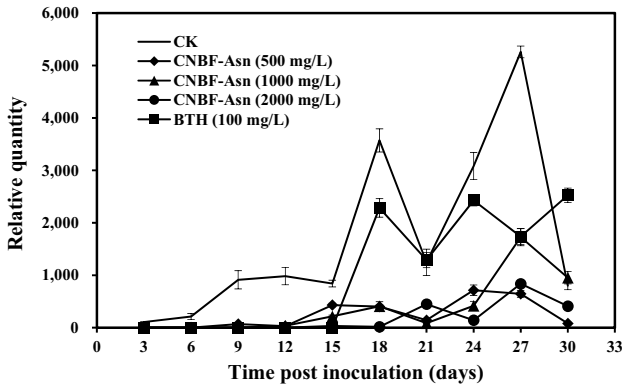


Fig. 2 The viral DNA variation of tomato treated by CNBF-Asn with time. *Vertical value* the relative amount of virus which presented in each sample and normalized to the amount of the endogenous 25S tomato gene, which is the mean of six replicates from two parallel experiments. *Bars* standard errors, $n = 6$

Effect on the transcriptional expression level of *Ty-1*

The results showed that the transcriptional expression level of *Ty-1* of the control was significantly lower than

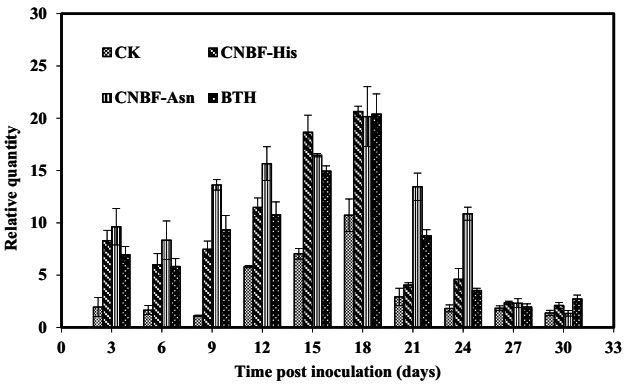


Fig. 3 The transcriptional expression level of *Ty-1* in tomato treated by CNBF-His or CNBF-Asn with time. *Vertical value* the transcriptional expression level of *Ty-1* which presented in each sample and normalized to the amount of the *ACT* gene, which is the mean of six replicates from two parallel experiments. *Bars* standard errors, $n = 6$

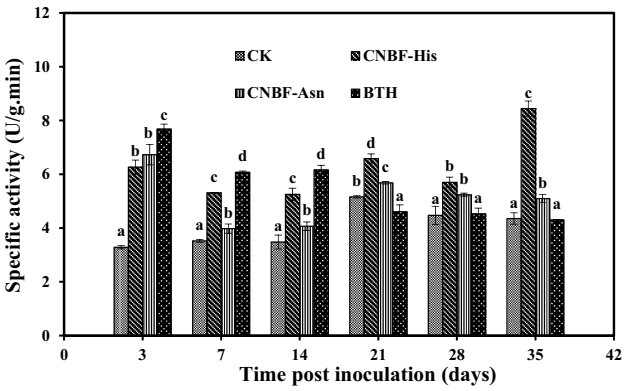


Fig. 4 PPO activity variation in tomato treated by CNBF-His or CNBF-Asn with time. Each value represents the mean of six replicates from two paralleled experiments. *Bars* standard errors, $n = 6$

any other treatments (Fig. 3). CNBF-His (1000 mg/L), CNBF-Asn (2000 mg/L) and BTH (100 mg/L) evidently induced the expression of *Ty-1*. The transcriptional expression level of *Ty-1* induced by CNBF-Asn increased the most by 12.21 in 9 dpi. Overall, the transcriptional expression level of *Ty-1* increased firstly and then decreased, peaked at 18 dpi. This is in line with the previous research that the expression of *Ty-1* was peaked at 19 dpi in the susceptible Moneymaker line (Verlaan et al. 2013). *Ty-1* is the major gene in a gene work contributing to the resistance against TYLCV in tomato, which is associated with the virus accumulation in host and the long-distance transport of virus (Michelson et al. 1994). Based on the combination of effect on the virus level and the transcriptional expression level of *Ty-1*, we could find that the transcriptional expression level of *Ty-1* declined while the virus level increased in 18 dpi. So, presumably two compounds activate the expression of *Ty-1* to inhibit the virus level. This conclusion brings us a new idea to screen efficient chemicals against TYLCV by monitoring the transcriptional expression level of *Ty-1*.

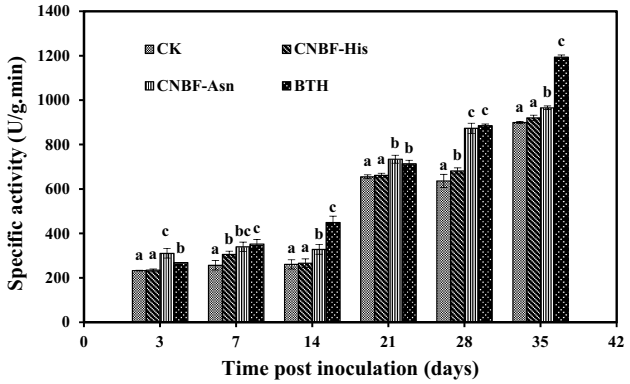


Fig. 5 POD activity variation in tomato treated by CNBF-His or CNBF-Asn with time. Each value represents the mean of six replicates from two paralleled experiments. *Bars* standard errors, $n = 6$

Table 5 Effect on the yield of different tomato varieties

Variety	Treatment (mg/L)	Yield (g/plant)	Yield increase (%)[a]
Moneymaker	CK	723 (a)	–
	BTH 100	975 (d)	34.85
	CNBF-His 1000	869 (b)	20.19
	CNBF-His 2000	867 (b)	19.92
	CNBF-Asn 1000	889 (c)	22.96
	CNBF-Asn 2000	969 (d)	34.02
Beidouxin'ao	CK	935 (a)	–
	BTH 100	1275 (d)	36.36
	CNBF-His 1000	1213 (bcd)	29.73
	CNBF-His 2000	1187 (bc)	26.95
	CNBF-Asn 1000	1168 (b)	24.92
	CNBF-Asn 2000	1250 (cd)	33.69
Oudun	CK	756 (a)	–
	BTH 100	1022 (e)	35.19
	CNBF-His 1000	944 (b)	24.87
	CNBF-His 2000	934 (b)	23.54
	CNBF-Asn 1000	961 (c)	27.11
	CNBF-Asn 2000	1012 (d)	33.86
Zhongnong 105	CK	762 (a)	–
	BTH 100	1037 (d)	36.09
	CNBF-His 1000	853 (b)	11.94
	CNBF-His 2000	845 (b)	10.89
	CNBF-Asn 1000	967 (c)	26.90
	CNBF-Asn 2000	1049 (e)	37.66

Data were present as the mean of three replicates. Yield was expressed as grams per plant. Mean yields within each column followed by different letters are significantly different at $P < 0.05$ by one-way analysis of variance

[a] Yield increase was calculated by the following formula: $\text{Yield increase} = \frac{\text{Yield of each treatment} - \text{Yield of the control}}{\text{Yield of the control}} \times 100\,\%$

Effect on the activity of PPO and POD

Many plant oxidative enzymes such as PPO and POD can catalyze the formation of lignin and other oxidative phenols that contribute to reinforcing the cell structure during the pathogens invasion. The results showed that PPO activities treated with CNBF-His or CNBF-Asn were significantly higher than that of the control and reached the most in 3 dpi, then maintained a high level and they were lower before 14 days and then significantly higher than that of BTH in later stage (Fig. 4).

POD activity treated with CNBF-His, CNBF-Asn or BTH increased greatly with time and POD activity treated with CNBF-Asn or BTH was significant higher than the control (Fig. 5). The enhanced activity ranged from 4 to 37 % compared to the control. Compared with BTH, CNBF-Asn had a slightly higher POD activity only in the 3 and 21 dpi. POD activity treated with CNBF-His was significantly higher than the control only in the 7 and 28 dpi.

Effect on the yield

The yield of tomato plants could be reduced by the infection of TYLCV due to the changes in the concentrations of phytochemical constituents (Tajul et al. 2011). CNBF-His and CNBF-Asn could reduce disease severity caused by TYLCV in pot experiment and a field experiment was designed to verify the effect on the yield of four different tomato varieties (Table 5). All tomato varieties treated with CNBF-His, CNBF-Asn or BTH had a higher yield compared to the control. For CNBF-His, the highest yield increase was 29.73 % at 1000 mg/L for Beidouxin'ao and the yields had no significant difference between the two concentrations. While for CNBF-Asn, the highest yield increase was 37.66 % at 2000 mg/L for Zhongnong 105 and the treatment concentrations were positively correlated with yield increases of tomato plants. CNBF-Asn was better than CNBF-His in the yield increase for all tomato varieties. CNBF-Asn at 2000 mg/L had a similar effect on yield increase with BTH at 100 mg/L. Considering that higher concentration may cause phytotoxicity and high cost, CNBF-Asn (2000 mg/L) may be more applicable in practical production.

Conclusions

In the present work, CNBF-His and CNBF-Asn significantly reduced the symptom severity caused by TYLCV, and the decreased symptoms severity was associated with the inhibition of viral DNA levels at the early stage. Among three levels of concentration, the highest inhibition rate treated with CNBF-His was 40.66 % at 1000 mg/L, and 36.26 % treated with CNBF-Asn at 2000 mg/L. The virus DNA levels of all treatment were lower than that of the control and increased slowly in the early stage and then peaked twice in the later stage, which implies that two compounds may lower the viral DNA level in plants to reduce the symptom severity. Two novel compounds could also enhance the activities of PPO and POD and the transcriptional expression level of *Ty-1* which correlate with plant resistance in tomato. In the field test, two compounds had higher yields of tomato than that of the control and the maximum increase of yield was 37.66 %. These promising results indicated that CNBF-His and CNBF-Asn were a potential plant activator against TYLCV and are being developed for future commercialization.

Acknowledgments This work was supported by National Department Public Benefit Research Foundation (201003065) and Research Fund for the Doctoral Program of Higher Education of China (20120008130006).

Conflict of interest The authors declare that they have no conflict of interest.

References

Abarshi M, Mohammed I, Wasswa P, Hillocks R, Holt J, Legg J, Seal S, Maruthi M (2010) Optimization of diagnostic RT-PCR protocols and sampling procedures for the reliable and cost-effective detection of Cassava brown streak virus. J Virol Methods 163(2):353–359

Barilli E, Prats E, Rubiales D (2010) Benzothiadiazole and BABA improve resistance to Uromyces pisi (Pers.) Wint. in Pisum sativum L. with an enhancement of enzymatic activities and total phenolic content. Eur J Plant Pathol 128(4):483–493

Cohen S, Antignus Y (1994) Tomato yellow leaf curl virus, a whitefly-borne geminivirus of tomatoes. In: Advances in disease vector research. Springer, pp 259–288

Culver JN, Padmanabhan MS (2007) Virus-induced disease: altering host physiology one interaction at a time. Annu Rev Phytopathol 45:221–243

Dann E, Diers B, Byrum J, Hammerschmidt R (1998) Effect of treating soybean with 2, 6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by Sclerotinia sclerotiorum in field and greenhouse studies. Eur J Plant Pathol 104(3):271–278

Dreher TW, Miller WA (2006) Translational control in positive strand RNA plant viruses. Virology 344(1):185–197

Durrant W, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185–209

Faoro F, Maffi D, Cantu D, Iriti M (2008) Chemical-induced resistance against powdery mildew in barley: the effects of chitosan and benzothiadiazole. Biocontrol 53(2):387–401

Gozzo F (2003) Systemic acquired resistance in crop protection: from nature to a chemical approach. J Agric Food Chem 51(16):4487–4503

Hammerschmidt R (2009) Systemic acquired resistance. Adv Bot Res 51:173–222

Lapidot M, Friedmann M (2002) Breeding for resistance to whitefly-transmitted geminiviruses. Ann Appl Biol 140(2):109–127

Lapidot M, Friedmann M, Lachman O, Yehezkel A, Nahon S, Cohen S, Pilowsky M (1997) Comparison of resistance level to tomato yellow leaf curl virus among commercial cultivars and breeding lines. Plant Dis 81(12):1425–1428

Lefeuvre P, Martin DP, Harkins G, Lemey P, Gray AJ, Meredith S, Lakay F, Monjane A, Lett J-M, Varsani A (2010) The spread of Tomato yellow leaf curl virus from the Middle East to the world. PLoS Pathog 6(10):e1001164

Løvdal T, Lillo C (2009) Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Anal Biochem 387(2):238–242

Mason G, Caciagli P, Accotto GP, Noris E (2008) Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in Bemisia tabaci. J Virol Methods 147(2):282–289

Michelson I, Zamir D, Czosnek H (1994) Accumulation and translocation of tomato yellow leaf curl virus (TYLCV) in a Lycopersicon esculentum breeding line containing the L. chilense TYLCV tolerance gene Ty-1. Phytopathology 84(9):928–933

Mohammadi M, Kazemi H (2002) Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with Fusariumgraminearum and induced resistance. Plant Sci 162(4):491–498

Navas-Castillo J, Fiallo-Olivé E, Sánchez-Campos S (2011) Emerging virus diseases transmitted by white flies. Annu Rev Phytopathol 49:219–248

Palumbo J, Horowitz A, Prabhaker N (2001) Insecticidal control and resistance management for Bemisia tabaci. Crop protection 20(9):739–765

Polston JE, Lapidot M (2007) Management of tomato yellow leaf curl virus: US and Israel perspectives. In: Tomato Yellow Leaf Curl Virus Disease. Springer, pp 251–262

Raupach GS, Liu L, Murphy JF, Tuzun S, Kloepper JW (1996) Induced systemic resistance in cucumber and tomato against cucumber mosaic cucumovirus using plant growth-promoting rhizobacteria (PGPR). Plant Dis 80(8):891–894

Rinder J, Casazza A, Hoefgen R, Hesse H (2008) Regulation of aspartate-derived amino acid homeostasis in potato plants (Solanum tuberosum L.) by expression of E. coli homoserine kinase. Amino Acids 34(2):213–222

Rom M, Antignus Y, Gidoni D, Pilowsky M, Cohen S (1993) Accumulation of tomato yellow leaf curl virus DNA in tolerant and susceptible tomato lines. Plant Dis 77(3):253–257

Scott J, Stevens M, Barten J, Thome C, Polston J, Schuster D, Serra C (1996) Introgression of resistance to whitefly-transmitted geminiviruses from Lycopersicon chilense to tomato. Bemisia: 1995, taxonomy, biology, damage, control and management

Seal S, Jeger M, Van den Bosch F (2006) Begomovirus evolution and disease management. Adv Virus Res 67:297–316

Tajul M, Naher K, Hossain T, Siddiqui Y, Sariah M (2011) Tomato yellow leaf curl virus (TYLCV) alters the phytochemical constituents in tomato fruits. Aust J Crop Sci 5(5):575–581

Verlaan MG, Hutton SF, Ibrahem RM, Kormelink R, Visser RG, Scott JW, Edwards JD, Bai Y (2013) The tomato yellow leaf curl virus resistance genes Ty-1 and Ty-3 are allelic and code for DFDGD-class RNA–dependent RNA polymerases. PLoS Genet 9(3):e1003399

Wu J, Dai F, Zhou X (2006) First report of Tomato yellow leaf curl virus in China. Plant Disease 90(10):1359

Xie Y, Jiang T, Zhou X (2006) Agroinoculation shows Tobacco leaf curl Yunnan virus is a monopartite begomovirus. Eur J Plant Pathol 115(4):369–375

Zhang H, Gong H, Zhou X (2009) Molecular characterization and pathogenicity of Tomato yellow leaf curl virus in China. Virus Genes 39(2):249–255