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Purification and Characterization of a New Ribosome Inactivating Protein from Cinchonaglycoside C-treated Tobacco Leaves

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Abstract

A new ribosome-inactivating protein (RIP) with a molecular weight of 31 kDa induced by Cinchonaglycoside C (1) designated CIP31, was isolated from tobacco leaves. Analysis of this protein sequence indicated that it belongs to the RIP family and it was distinct from the other plant RIPs reported previously at its N-terminal amino acid sequence. CIP31 can directly impair synthesis of coat protein (CP) of tobacco mosaic virus (TMV), which resulted in inhibition of TMV long distance movement and multiplication in tobacco plants at concentrations of ng/mL. Furthermore, no toxicity was shown to the growth and fertility of the plants. CIP31 was synthesized only in the presence of Cinchonaglycoside C (1) and was independent of the salicylic acid (SA) signal pathway. We provided evidence for the SA-independent biological induction of resistance.

Key words: antiviral protein; Cinchonaglycoside C (**1**); coat protein; medical plant; ribosome inactivating protein; salicylic acid; tobacco mosaic virus.

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Plants react to virus infection by stimulating the production or activation of antiviral proteins (Sela 1981). Ribosome-inactivating proteins (RIPs) have been proposed as antiviral agents in plants (Taylor et al. 1994). For instance, many different kinds of RIP such as PAP (pokeweed antiviral protein), dianthin 32, tritin, barley RIP and ricin A-chain with antiviral activity against tobacco mosaic virus (TMV) infection, have been purified from plants (Taylor et al. 1994). RIPs are widely distributed and possibly ubiquitous in the plant kingdom (Stirpe and Barbieri 1986). There are two classes for RIPs: in type I RIPs have a single polypeptide chain, and in type II RIPs have two polypeptide chains, including an active A chain and a B chain, which is a galactosebinding lectin (Stirpe et al. 1992). Apart from these two RIP types, a 60-kDa protein (JIP60) has been identified in methyl jasmonate-treated plants (Reinbothe et al. 1994). RIPs are attracting considerable interest for their possible use as immunotoxins and antiviral agents as well as for their effects on viruses, protozoa, insects, and fungi (Barbieri et al. 1982; Cenini et al. 1988; Leah et al. 1991; Brandhorst et al. 1996).

Recently, different RIPs have been reported from about 50 plant species covering 17 families. Some families include many RIP-producing species, particularly Cucurbitaceae, Euphorbiaceae, Poaceae, and families belonging to the superorder Caryophyllales (Grasso 1978; Stirpe and Barbieri 1986; Kwon et al. 2000). A number of reports indicate that many RIPs are inducible by biotic or abiotic factors, such as salicylic acid triggering the expression of two single-chain RIPs named beetins 27 and 29 (Girbes et al. 1996). A salicylic-independent systemic induction of type 1 RIPs has also recently been described (Stirpe et al. 1996; Song et al. 2000). In this paper,

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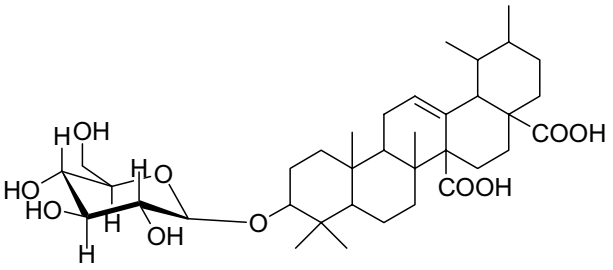

Figure 1. Chemical structure of compound (**1**) Cinchonaglycoside C.

Chemical formula: $C_{36}H_{56}O_{10}$. Exact mass: 648.387.

we describe the isolation and characterization of a new RIP induced by Cinchonaglycoside C (**1**) in tobacco plants. The protein damages TMV coat protein (CP) synthesis, resulting in the blocking of TMV long distance movement in plants.

Results

Identification of Cinchonaglycoside C(1)

During the screening for antiviral compounds from the medical plants, a compound Cinchonaglycoside C (**1**) with antiviral activity against TMV was isolated from S*trobilanthes cusia*. Its chemical structure was identified as 3-O-β-D-glucopyranosyl-urs-12-ene-27,28-dioic acid (**1**) (Figure 1) by analysis of MS (mass spectrometry) ^{1}H NMR (nuclear magnetic resonance) and ^{13}C NMR spectral data and compared with those of Cinchonaglycoside C (Bashir 1996).

Cinchonaglycoside C (1) enhanced resistance to TMV infection in tobacco leaves

Exogenous application of Cinchonaglycoside C (**1**) to the surface of Xanthi-nc NN plant leaves revealed that the number and size of lesions developed by TMV were greatly reduced compared with the control (Figure 2B). The rate of inhibition was enhanced linearly with an increase in the concentration of Cinchonaglycoside C (**1**). The minimum inhibitory concentration was 100 nmol/L. At a concentration of 500 nmol/L the rate of inhibition was 92% (Figure 2A). To assess whether Cinchonaglycoside C (**1**) inhibits TMV multiplication in plants, Western blot analysis of TMV CP accumulation in the presence of different concentration of the Cinchonaglycoside C (**1**) was carried out. TMV CP accumulation was greatly reduced with the increase of Cinchonaglycoside C (**1**) concentration (Figure 2C). The inhibition of TMV multiplication under different administration methods implied that Cinchonaglycoside C (**1**) did not directly inhibit TMV replication, but induced a plant defense response (data not shown).

Effect of Cinchonaglycoside C (1) on accumulation of salicylic acid in tobacco plants

In order to study whether Cinchonaglycoside C (**1**) induced a plant defense response to TMV infection by the salicylic acid (SA) signal pathway, total SA levels were determined in Xanthi-nc NN plants that had been treated with different concentrations of Cinchonaglycoside C (**1**). The increase of SA levels was not observed even in plants treated with 500 nmol/L of Cinchonaglycoside C (**1**), compared with control (DMSO (dimethyl sulfoxide) -treated plants) (Table 1).

Purification and characterization of antiviral protein from Cinchonaglycoside C (1)-treated tobacco leaves

Infection of tobacco plants by TMV was prevented by leaf extracts from Cinchonaglycoside C-treated tobacco plants. The antiviral activity of plant extracts has been a clue to the identification of antiviral protein. A new antiviral protein (CIP31) possessing a molecular weight of 31 kDa and pH 5.37 was isolated from Cinchonaglycoside C (**1**)-treated Xanthi-nc NN plant leaves. The isolation procedure is described in Materials and Methods. Analysis of the CIP31 sequence showed high similarity to the lectin from mistletoe, a type II RIP. However, the N-terminal amino acid sequence identity of the two proteins was only 10%. Although the CIP31 was definitely RIP family (data not shown), it differed from all of the other plant RIPs at its N-terminal amino acid sequence (Figure 3). Analyzed the N-terminal amino acid sequence of the protein, we did not find any sequence homology with other known proteins. Thus, we determined that this protein is a new protein with RIP function.

Cinchonaglycoside C (1) induced CIP31 in tobacco plants

To examine the expression of CIP31 in response to Cinchonaglycoside C (**1**), Xanthi-nc NN plants were sprayed with different concentrations of solutions of Cinchonaglycoside C (**1**), DMSO or SA (300 μmol/L), and assayed for the accumulation of this protein by western blots. After being sprayed for 6 h, the protein was not detected in the cases of DMSO- or SA-treated tobacco plants, but expression of the protein closely paralleled the rise in concentration of the Cinchonaglycoside C (**1**) solution. The minimum concentration of Cinchonaglycoside C (**1**) required to induce the protein was 100 nmol/L. Furthermore, application of exogenous SA did not induce this protein expression (Figure 4). These results suggest that the protein express only in the presence of Cinchonaglycoside C (**1**) and independence of the SA signal pathway.

Antiviral activity of exogenously applied CIP31 in tobacco plants

To test if CIP31 have antiviral activity, Xanthi-nc NN plants were inoculated with TMV in the presence of different concentrations

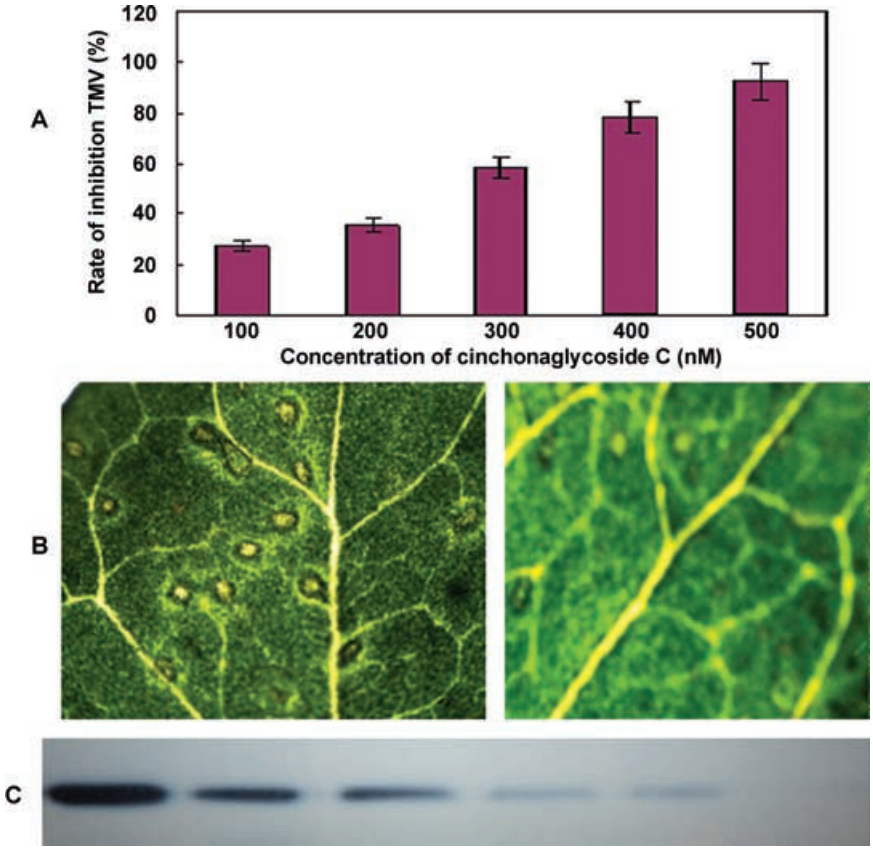

Figure 2. Tobacco plants were sprayed with DMSO or a solution containing the indicated concentrations of Cinchonaglycoside C (**1**) for 6 h, plants were inoculated with tobacco mosaic virus (TMV) (2 μg/mL), incubated at 25 °C for 5 d, and the number and size of lesion were measured.

(A) The rate of inhibition TMV by Cinchonaglycoside C (**1**) was measured with a stereoscopic microscope. **(B)** Necrotic lesions that had formed on the leaves treated with 300 nM Cinchonaglycoside C (**1**) (right) or DMSO (left). **(C)** Leaves that were treated with DMSO, 100, 200, 300, 400 or 500 nmol/L of Cinchonaglycoside C (**1**) were subjected to protein gel blot analysis with coat protein (CP) of anti-TMV antibody (Ab-TMV).

Table 1. Salicylic acid (SA) accumulation in Cinchonaglycoside C (**1**)-treated tobacco plants

Concentration (nmol/L)	SA level (ng/g fresh weight)
0	37.2 ± 6.1
1	36.8 ± 7.2
100	37.4 ± 6.4
300	36.9 ± 5.1
500	38.3 ± 5.8

SA levels in tobacco plants treated with different concentrations of Cinchonaglycoside C (**1**) were determined after administration for 6 h. Total SA content was determined as described as above. Four samples (0.5 g) were analyzed for each plant. The data presented are the means ± *SD*.

of CIP31. This resulted in a dramatic reduction in the numbers of TMV lesions on inoculated leaves compared with the control. Furthermore, the antiviral activity was dose-dependent (Table 2).

Cinchonaglycoside C(1)-induced protein inhibit TMV systemic infection

Two approaches were used to address this matter. First, *Nicotiana tabacum* cv. K326 plants, a systemic host of TMV, were inoculated with TMV alone or TMV containing 50 ng/mL of CIP31, respectively. In the presence of CIP31, infection by TMV was limited to the inoculated leaves, which developed necrotic lesions at 5 dpi.(day post infection). No symptoms were observed in uninoculated leaves during the whole life span of these plants. By contrast, plants infected by TMV without CIP31, developed severer disease symptoms in inoculated and uninoculated leaves (data not shown). Second, a modified TMV expression vector (30B:green fluorescent protein) carrying a GFP gene placed downstream of the TMV CP subgenomic RNA promoter, which can systemically infect and express GFP in *N. benthamiana* plants (Deom et al. 1987), was used to facilitate tracking the movement of the virus vector in whole plants. *N. benthamiana* plants were infected with 30B:GFP through

CIP31 -ERDADG—EATGIEVIKIRELITLLRDEAD–SFSSRAAIPLE-
LectinA YERLRLR—VTHQTTGEEYFRFITLLRDYVS–SGSFSNEIPLL-
SNAIf -MRVVTK—LLYLVVLAICGLGIHGALTHTR-VTPPVYPSVSFN-
SNAV -MRVVAAAMLYFYIVVLAICSVGIQG-IDYPS-VSFNLDGAKS—
Ricin —IFPK—QYPIINFTTAGATVQSYTNFIRAVRGRLTTGADV–
BE27 -ADVTFD—LETASKTKYGTFLSNLRNIVK-DSKLVYEGIPMLP
Dianthin -MKIYLVA–AIAWILFQSSSWTTDAATAYTLNLANPSASQYS—
IRAb -MHKKVN–IKACLVSAIIWIWWAAIVGPAILVCSSSLSVTRG—

Homology(%)

CIP31 100 10 3 8 5 18 5 5
LectinA 10 100 11 5 3 13 3 8
RicinA 3 11 100 8 5 13 3 5
IRAb 8 5 8 100 8 0 15 15
Dianthin30 5 3 5 8 100 5 10 8
BE27 18 13 13 0 5 100 5 5
SNAIf 5 3 3 15 10 5 100 13
SNAV 5 8 5 15 8 5 13 100

Figure 3. N-Terminal amino acid sequences of the CIP31 from Cinchonaglycoside C (**1**)-treated tobacco plants in comparison with other members of the tobacco ribosome-inactivating protein (RIP), as well as antiviral RIP other plants.

Numbers within parentheses indicate the percentage of identity to the CIP31. Dots represent amino acid residues that match the CIP31. Lectin (GenBank accession no. AAL87006), ricin A-chain (X02388), IRAb (AAL55094), Dianthin30 (P24476), BE27 (AAS67266), SNAIf (AAC49989), SNAV (P33183). Sequence alignment was carried out by using the CLUSTALW program.

agroinfiltration. When an Agro35S-30B:GFP suspension containing 50 ng/mL of CIP31 was used to infect the plants, green fluorescent spots were clearly visible under long-wave UV light in inoculated leaves at 5 dpi. However, green fluorescence was only observed around the infiltrated areas and not in the upper uninoculated leaves (Figure 5, left). As a control, infection by Agro35S-30B:GFP without CIP31 generated green fluorescence not only in the inoculated leaves but also in upper systemic leaves (Figure 5, right). Together, theses results suggest that CIP31 can inhibit the replication of TMV in its host plants mainly through blocking the virus's long-distance transport.

Discussion

Pathogenic viruses can devastate populations of plants and animals. Yet, many plants are resistant to most plant viruses, despite lacking cell-based immune systems. For example, *Strobilanthes cusia* was not infected by TMV, compared to tobacco in our experimental results. During the screening for antiviral compounds from the medical plants, a compound

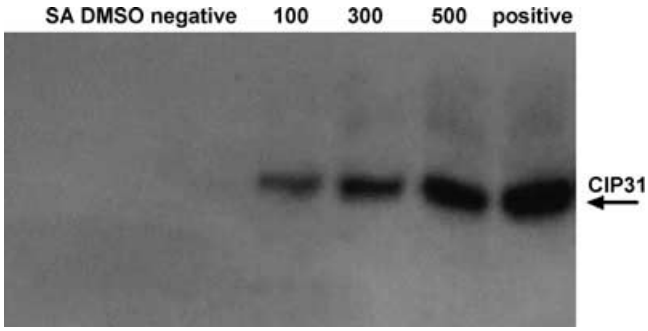

Figure 4. The effect of Cinchonaglycoside C (**1**) on accumulation of the CIP31.

Tobacco leaves were treated with different concentrations of Cinchonaglycoside C (**1**), salicylic acid (SA) (300 μmol/L) or an equal volume of DMSO solution as a control. The samples were taken after administration for 6 h. The accumulation of the protein was determined by Western blot analysis. Arrows indicate the positions of the protein. The experiment was repeated three times with similar results.

Table 2. Resistanc to tobacco mosaic virus (TMV) in the presence of exogenously applied CIP31

Protein applied (ng/mL)	Mean no. of lesions
0	58.3 ± 4.5
10	42.0 ± 3.2
30	26.3 ± 4.4
40	11.2 ± 5.1
50	2.6 ± 3.5

The number of lesions were counted at 5 d post inoculation. Mean values ± *SE* for the total number of lesions per plant are shown.

Cinchonaglycoside C (**1**) with antiviral activity against TMV was isolated from S*trobilanthes cusia*. Furthermore, we found that Cinchonaglycoside C (**1**) can not directly inhibit TMV replication but it can induce a new RIP. RIPs were stimulated in many plant species by a variety of biotic or abiotic factors (Reinbothe et al. 1994). In this study, we have purified a RIP (CIP31) from tobacco leaves treated with Cinchonaglycoside C (**1**) and shown that it possesses an antiviral capacity against TMV multiplication. Specifically, CIP31 was able to block TMV CP accumulation, resulting in the inhibition of TMV's long distance movement.

CIP31-treated plants with the highest protection levels showed a significant reduction in lesion numbers compared with control plants. The results show that CIP31 is capable of conferring local protection against TMV multiplication. CIP31 having no toxicity to the host suggests that the antiviral mechanism of CIP31 differs from that of the type-1 RIPs. The antiviral activity of type-1 RIPs is presumably due to its direct depurinating activity towards both viral nucleic acids and plant

Figure 5. Effect of the protein on tobacco mosaic virus (TMV) spread in *Nicotiana benthamiana* plants.

N. *benthamiana* plants inoculated on one of the lower leaves with Agro35S-30B:GFP containing 50 ng/mL of the protein (left). *N*. *benthamiana* plant inoculated with Agro35S-30B:GFP without the protein (right). The plants were photographed under long-wave UV light at 5 dpi.

ribosomes and hence, relies on a direct effect on both the invading viruses and the infected cells. As CIP31 does not act on plant ribosomes, the RIP can only act on the virus itself and not on the infected cells, which may explain why the protein retained the ability to inhibit virus accumulation without an obvious side effect on the host. Furthermore, we found that the expression of CIP31 depended on the concentration of Cinchonaglycoside C (**1**). It did not express without the presence of Cinchonaglycoside C (**1**). CIP31 differs from all other plant RIPs at its N-terminal amino acid sequence. Therefore, this protein is a novel RIP with remarkable antiviral capacity against TMV multiplication.

Ribosome-inactivating proteins have been implicated in plant defense roles (Stirpe et al. 1992) by virtue of their antiviral, antifungal, and antibacterial activities (Roberts and Selitrennikoff 1986; Na et al. 2002). Accordingly, we found that CIP 31 showed activity against TMV multiplication. Similarly, it is believed that RIPs are not found in all plants. In our studies, the protein was not detected in tobacco under normal conditions, whereas we detected a high expression of the protein in Cinchonaglycoside C (**1**)-treated plants. This report is in accordance with our observation that Cinchonaglycoside C (**1**) can induce plant defense responses to TMV multiplication. The fact that RIPs are general inhibitors of virus multiplication makes it an ideal candidate for developing antiviral agents. The results of the experiments described here provide the opportunity to produce a broad spectrum of antiviral agents of plant viruses. Validating plants as an effective resource for screening antiviral compounds becomes viable in light of our results. We do not yet understand the mechanism of protein inhibition of TMV multiplication. Further studies on protein will contribute to a better understanding of the mechanism of its antiviral action and facilitate the development of a broad spectrum of virus-resistant plants.

Materials and Methods

Plant materials and virus

Xanthi-nc NN plants, *Nicotiana tabacum* K326 and *N. benthamiana* were grown in an environmentally controlled growth chamber at 25 °C, with 75% relative humidity, and a 16 h photoperiod. Plants with expanded fifth leaves (5–6 weeks after seeding) were used for all studies. All of the TMV (strain U1) used in this work was purified from *N. tabacum* K326 leaves inoculated with TMV and harvested at 7 d post inoculation. The plants were inoculated mechanically in three rosette leaves with a solution of 5 μg/mL TMV in 20 mmol/L phosphate buffer, using a sterile cotton swab dusted with carborundum. TMV replication in infected plants was examined by enzyme-linked immunosorbent assay (ELISA). The number and size of lesions were measured under a microscope.

Activity-guiding isolation of Cinchonaglycoside C(1) from *Strobilanthesis cusia*

The stem and leaves of fresh *S. cusia* were collected in Yunnan Province, China. The plant material (25 kg) was extracted with methanol. The extract was divided into acidic, basic and neutral fractions. The neutral petrol-insoluble portion was partitioned into petroleum ether, $CHCl_3$, ethyl acetate, acetonitrile, and aqueous solution portions, respectively. Each fraction was evaporated to dryness, and the remaining residue was dissolved in DMSO to different concentrations (10 mg/mL, 100 mg/mL, 1 000 mg/mL). Aliquots of the solution were sprayed on Xanthi-nc NN plant leaves with DMSO as controls. After 6 h of spraying, plants were inoculated with TMV and maintained in a growth chamber at 25 °C with 14/10 h or 16/8 h light/dark photoperiods for 5 d. TMV replication was examined by ELISA as described

(Bendahmane et al. 1997). The active fractions were combined, evaporated, and separated on a C18 cartridge column (Waters, Milford, MA, USA) eluted with increasingly higher concentrations of methanol in water starting with 10% (v/v) methanol. The active fraction was subjected to a silica gel column eluted with $CHC1_3$ and $CHCI_3$-MeOH (in order of increasing polarity). The eluate from $CHCI_3$-MeOH (19:1) showed that two major spots on TLC were further separated on a reversed-phase HPLC (High-performance liquid chromatography) column (Li Chrospher 100 RP-18, 5 μm particle size, 4 mm × 5 cm; Hewlett-Packard, Palo Alto, CA, USA) and eluted with mobile phase A (water: methanol, 1:4 [v/v]) at a flow rate of 1 mL/min, monitored at 254 nm. Starting at 0.1 min after injection, 30 fractions, each containing 5 mL were collected. The active fractions were found with retention times of 10.2 to 18.5 min. These fractions were collected and separated further on a HPLC column (LiChrospher 100 RP-18, 5 μm particle size, 4 mm × 25 cm; Hewlett-Packard, America, Philadelphia) eluted with mobile phase B (water : acetonitrile, 4:6 [v/v]) at the same flow rate. A peak fraction with a retention time of 16.2 min showed activity and was collected to yield 21 mg of Cinchonaglycoside C (**1**) (Bashir 1996).

Quantification of TMV coat protein

The accumulation levels of TMV CP in different concentrations of Cinchonaglycoside C-treated tobacco plants were determined by Western blot analysis (Hull 2002).

Purification of CIP31

Tobacco leaves were treated with either 500 nmol/L compound 1 or an equal volume of DMSO solution as a control. The samples (2.5 kg) were taken after compound 1 administration for 6 h and were ground in liquid N_2, homogenized in three volumes of extraction buffer (10 mmol/L-NaH_2PO/Na_2HPO_4/150 mmol/L-NaCl (pH 7.4)/0.02% (w/v) NaN_3), and then centrifuged for 30 min at 10 000*g*. The supernatant was brought to 20% (w/v) ammonium sulfate by continuous stirring. The mixture was chilled for 1 h and then centrifuged again for 30 min at 10 000*g*. The supernatant was fractionated by successive addition of solid $(NH_4)_2SO_4$ to 20–90% saturation, respectively. Each precipitate was collected after stirring for 1 h by centrifugation as described above. The final pellet was dissolved in a minimal volume (200 mL) of 20 mmol/L-Tris/HCl, pH 7.4, and dialyzed exhaustively against the same buffer over a period of 48 h. The resulting suspension was centrifuged at 25 000*g* for 1 h, and the conventional local lesion assay was used to examine each fraction containing virus-inhibitory proteins. The active fractions were pooled, concentrated, and desalted using cutoff ultrafiltration membranes (Millipore, Bedford, MA, USA). Further chromatographic steps were carried out by using a FPLC (fast protein liquid chromatography) system (AP). Fractions exhibiting antiviral activity were further purified on a Mono Q column after concentration. The target protein was eluted with a linear gradient of NaCl (50–1 000 mmol/L). Individual peaks were collected, concentrated, and desalted using ultrafiltration devices (Millipore).

Amino acid sequencing

Protein purified from Cinchonaglycoside C(**1**)-treated Xanthi-nc NN plant leaves was put into separate wells of a 96-well plate, and processed by a proteolysis workstation to yield tryptic peptide pools. A mass list of peptides from each protein was obtained by using a matrix-assisted laser desorption ionization (MALDI)-time-of-flight spectrometer (ELITE, PerSeptive Biosystems, Framingham, MA, USA). Fragmentation spectra from 1-Da mass windows (obtained from the MALDI mass list) were recorded by using a nanospray ionization source (Zspray, Micromass, Manchester, UK) on a Q-TOF, Micromass, Manchester, UK) instrument. The continuum fragmentation spectra were converted to centered spectra and used to search the SwissProt (version 36.0, October 1998) database with the SEQUEST computer program. Candidate sequences were confirmed when an ion series consistent with y-type fragmentation was observed for the complete peptide sequence (Biemann 1990).

Quantification of CIP31 in Cinchonaglycoside C-treated tobacco plants

Tobacco leaves were treated with different concentrations of Cinchonaglycoside C (**1**), SA (300 μmol/L) or an equal volume of DMSO solution as a control. The samples were taken after administration for 6 h. The accumulation of the protein was determined by Western blot. For Western blot analysis, rabbit anti-CIP31 antiserum was used as the primary antibody. Goat anti-rabbit IgG (Promega) were used as the secondary antibodies in the Western blot analysis. For color development, NBT/BCIP (Nitroblue Tetrazolium/5-Bromo-4-chloro-3-Indolyl Phosphate) (Promega) were used.

Determination of SA

SA levels in the leaves of Xanthi-nc NN plants were determined after Cinchonaglycoside C (500 nmol/L) was sprayed for 6 h. SA was extracted from leaf samples (0.5 g) and quantified by HPLC. Total SA was determined as described by Yalpani et al. (1993).

Assay of systemic movement of TMV

An engineered TMV-based expression vector 30B:GFP which can efficiently move into and express GFP in systemic leaves of *N. benthamiana* (Shivprasad et al. 1999) was used as a target to test the effect of this protein on inhibition of the

systemic movement of TMV. Delivery of 30B:GFP into *N. benthamiana* cells was achieved by pressure infiltration of an *Agrobacterium* (Agro35S-30B:GFP) harboring a plant expression plasmid p35S-30B:GFP in which the viral vector was placed under the control of the CaMV 35S promoter (Jia et al. 2003). The bacterial suspension with or without 50 ng/mL of this protein was inoculated onto a lower leaf of 4-week-old *N. benthamiana* plants. At 5 dpi, the plants were illuminated with a hand-held long-wave UV lamp (UV Products, model B100 AP). GFP fluorescence in plant tissues was directly visualized and photographed using a digital camera (Nikon Coolpix 995).

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