ISOLATION, PURIFICATION AND CHARACTERIZATION OF VIRUS INHIBITORY AGENT'S (VIA'S), SYSTEMICALLY INDUCED IN *CYAMOPSIS TETRAGONOLOBA*

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Key message: The virus inhibitory proteins called as Virus Inhibitory Agent (VIA) were purified from Cyamopsis tetragonoloba, induced by using CIP-29, a systemic resistance inducing protein from Clerodendrum inerme to resist virus infections, and characterized. Experimental observations found it tested positive for a glycoprotein

ABSTRACT

In the process of biological control measures in the present work the CIP-29, a known 29 kDa systemic antiviral resistance inducing protein isolated from Clerodendrum inerme, has been used to induce systemic resistance in Cyamopsis tetragonoloba against Sunn-hemp rosette virus (SRV). Paper reports the detection of virus inhibitory activity in induced-resistant leaf sap of C. tetragonoloba, and the purification of two virus inhibitory agents (VIAs) thereof has been done to establish the efficacy of biological control. VIA activity was recorded as a reduction in lesion number of SRV and Tobacco mosaic virus, when they were incubated separately with resistant sap and inoculated onto susceptible test hosts C. tetragonoloba and Nicotiana tabacum cv. Xanthi-nc respectively. The two VIAs were isolated from resistant C. tetragonoloba plant leaves using combinations of column chromatography. Both were basic proteins, and since their Mr was 32 and 62 kDa, these VIAs were called CT-VIA-32 and CT-VIA-62, respectively, on the basis of their molecular mass and the host. CT-VIA-62 displayed better activity, and was thus studied further for raising antibodies in albino rabbits. It tested positive for a glycoprotein, and was serologically detected only in leaf tissue post-induction

Keywords: *Induced systemic resistance, Tobacco mosaic virus, Antiviral, Virus inhibitory agent, CIP-29*

ABBREVIATIONS

AVF	Antiviral factor
CAP-34	34 kDa Systemic antiviral resistance inducing protein from Clerodendrum aculeatum
CIP-29	29 kDa Systemic antiviral resistance inducing protein from Clerodendrum inerme

ISR	Induced systemic resistance
IVR	Inhibitor of virus replication
PGPR	Plant growth promoting rhizobacteria
PR-protein	Pathogenesis-related protein
PRSV	Papaya ring spot virus
Mr	Molecular weight
RIP	Ribosome inactivating protein
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRV	Sunn-hemp rosette virus
TMV	Tobacco mosaic virus
VIA	Virus inhibitory agent

INTRODUCTION

Plant virus infections lead to huge losses in crop productivity. Agricultural resources face grave challenges from pathogen invasion that outrun conventional methods of control, and need eco-friendly approaches to overcome the loss. Agrochemicals (chemotherapeutics) detrimental to crop plants, paint a bleak picture in the long run upon living system as a whole, and its environment. Breeding for resistant varieties, though a promising control measure, suffers from the intrinsic problem of narrow range of resistance and its reversal due to strain specific properties of viruses. Tissue culture (virus free apical meristem culture) and micro-propagation techniques proven quite successful but provide no assurance to re-infection by viruses. Biological control agents against broad spectrum of pathogens (viruses), have emerged effective in ensuring good crop productivity and ecological safety. Resistance induction mechanisms, developing host resistance from within the plant by using biological agents, have gained recognition these days. The natural defence response stimulation in plants is carried out by various agencies including chemotherapeutic agents, viral, bacterial and fungal infections (cross protection) and plant extracts from a few higher plants. Among these agencies plant protein based method of control of viruses, emerges par excellence in terms of viability and feasibility.

Induced resistance triggers repressed pre-existing genes upon exposure to exogenous substances. It may be either induced systemic resistance, that is visible at sights away from that of treatment, or induced local resistance that offers protection only at site of application. The resistance inducers, of biological or chemical origin, are categorized as Localized Resistance Inducers (LRIs) and Systemic Resistance Inducers (SRIs). Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR) differ on the basis of the nature of elicitor and the regulatory pathway involved, and presence or absence of PR-proteins etc. Virus Inhibitory Agents (VIAs) are implicated in the development of induced antiviral resistance in plants, with possible functional similarities with the Antiviral Factor (AVF), the Inhibitor of Virus Replication (IVR) and PR-proteins. The VIA is also an inducible gene product. In the proposed work, VIAs has to be isolated from resistant *Cyamopsis tetragonoloba,* induced by partially purified CIP-29, a basic protein, obtained from *Clerodendrum inerme* Gaertn and used as an exogenous source of systemic resistance induction.

Systemic acquired resistance(SAR) and plant growth promoting rhizobacteria (PGPR) as well as phytoprotein-induced systemic resistance (ISR) are the two worthwhile strategies that could be developed to curb virus infection and spread (reviewed by Van Loon et al. 1998; Lugtenberg and Kamilova 2009; Prasad et al. 2012; Fu and Dong 2013). SAR, induced by localized pathogen infection (Ross 1961) and chemicals, in particular salicylic acid and its functional analogues like Benzothiadiazole (BTH) and 2,6 dichloro-isonicotinic acid (INA) (Antoniw and White 1980; Friedrich et al. 1996; Dann et al. 1998) provide resistance to subsequent pathogen invasion, while ISR does the same but is triggered by colonization of roots by non-pathogenic rhizosphere bacteria.

ISR also differs from SAR in not being associated with pathogenesis-related (PR-) proteins, and in its onset being more rapid (Prasad et al. 2012). Protection offered by antiviral proteins called ribosome-inactivating proteins (RIPs) is also under study (Girbe´s et al. 2004; Stirpe 2013). The mode of action of RIPs was first studied using rat rRNA where ricin, an RIP from Ricinus communis, could inactivate ribosomes by removing a specific adenine residue from the large rRNA (Endo et al. 1987), and was shown to possess RNA-N glycosidase activity. Depurination studies were later expanded to include non-mammalian ribosomes from plants, fungi and bacteria and a variety of substrates (Olivieri et al. 1996; Barbieri et al. 1997).

Proteins from Clerodendrum inerme (CIP-29) and C. aculeatum (CAP-34) exhibit RIP function and additionally induce systemic antiviral resistance in host plants (Prasad et al. 1995; Olivieri et al. 1996; Kumar et al. 1997). Furthermore, resistance could be abolished by the concomitant application of actinomycin-D in the host plants (Prasad et al. 1995). The systemic resistance induced by CAP-34 was accompanied with the simultaneous production of a virus inhibitory agent (VIA) in the treated plants (Verma et al. 1996). A partially purified preparation of VIA induced in Cyamopsis tetragonoloba plants following its induction by leaf extract from Bougainvillea spectabilis showed characteristics of a protein and inhibited viruses in vitro (Verma and Dwivedi 1984). Inducible antiviral proteins are known from tobacco plants reacting hypersensitively to virus infection. An antiviral factor (AVF) was purified from Nicotiana glutinosa plants infected with TMV (Sela and Applebaum 1962) that was found to be a phosphorylated glycoprotein of Mr 22 kDa (Mozes et al. 1978).

Drawing analogies of AVF with the antiviral interferon system in mammals, two antiviral proteins termed gp22 and gp35 were isolated from TMV infected tobacco through their affinity binding to monoclonal antibodies against interferon (Edelbaum et al. 1990). Interestingly, both were closely related to two pathogenesis-related (PR) protein families; gp22 related to PR 5, and gp35 to the PR 2 family, but neither of these PR-protein families are antiviral in function (Edelbaum et al. 1991). An inhibitor of virus replication (IVR) was reportedly released in the medium from TMV-infected cells of tobacco Samsun NN (Loebenstein and Gera 1981; Gera and Loebenstein 1983). IVR fraction inhibited virus replication when used as a spray and consisted of a 23 kDa protein and its dimer (Gera et al. 1990). Subsequently, NC3301, a cDNA clone isolated from induced-resistant leaf tissue of tobacco Samsun NN, was found to encode a 21.6 kDa polypeptide which reacted with IVR antibodies (Akad et al. 1999). Samsun nn tobacco transformed with the IVR gene was resistant to TMV and Botrytis cinerea (Akad et al. 2005), while transformed tomato showed partial resistance to B. cinerea, Alternaria solani, and Oidium neolycopersici (Loebenstein et al. 2010; Elad et al. 2012). In principle hence, alternative strategies are trying to utilize latent resistant mechanisms of susceptible plants to combat virus infections. The VIA, a suspected induced proteinaceous agent with antiviral functionalities, lends itself well to the line of biological control with efficient outcome of the inhibitory principles of investigations.

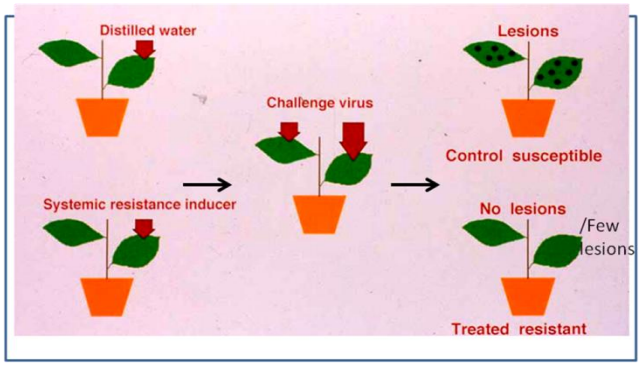
OBJECTIVES

Purification of inducer protein, CIP-29, from the leaves of *Clerodendrum inerme* Gaertn followed by biological assays in susceptible plants has to be done routinely. Induction of antiviral resistance which has been followed by bioassay's for production of VIAs in test plants. Isolation, purification and characterization of the CIP-29 induced virus inhibitory agent (VIA) in *Cyamopsis tetragonoloba* routinely undertaken simultaneously. A comparative study of VIAs obtained from *Cyamopsis tetragonoloba* in variable test hosts. The study of kinetics of production of VIAs is to be carried on. Further, raising of polyclonal antibodies in albino rabbits against purified VIA. Test of specificity and titre for polyclonal antibodies or antisera raised against VIA using immunoblot experiments (western blots).

METHODOLOGY

***Clerodendrum inerme* Gaertn., the source plant of CIP-29**

Fig.1



Assay procedure for systemic antiviral resistance induction in plants

Fig.2

Maintenance of test hosts and virus culture Test hosts Nicotiana tabacum cv. Xanthi-nc and C. tetragonoloba (L.) Taub. were grown in an insect-free glasshouse. Tobacco plants were used at a 2-month stage, with 5–6 fully expanded leaves, while C. tetragonoloba plants were used at 6-leaf stage, respectively. SRV and TMV strains were maintained in their systemic hosts *Crotalaria juncea* and *Nicotiana tabacum* L. var. NP-31 respectively and purification of TMV and SRV according to Gooding and Hebert (1967). The leaves of virus-infected plants, showing prominent mosaic symptoms, were harvested and homogenized in phosphate buffer (500 mM, pH 7.2, 1:1 w/v), were processed for use as virus inocula in routine bio-assays. *Cyamopsis tetragonoloba* and *Nicotiana tabacum* cv. xanthi nc were used as test hosts. These hosts show a hypersensitive response producing local lesions against Sunnhemp rosette virus (SRV) and Tobacco mosaic virus (TMV), respectively. The concentration of the purified viruses has been determined by spectrophotometry and djusted during experimentation to produce between 200 and 300 local lesions on the leaves of the control plants of N. tabacum cv. Xanthi-nc and C. tetragonoloba. For CIP-29 isolation, leaves of *Clerodendrum inerme* (Fig. 1) were first processed using standard homogenization, precipitation and centrifugation techniques in suitable buffer system. Induction of antiviral resistance, virus inhibitory agent and time course of virus inhibitory activity, the resistance inducing protein, CIP-29, purification has been done from the leaves of C. inerme Gaertn. (Prasad et al. 1995).

The biological assay followed as per the two lower leaves of C. tetragonoloba plants were sprayed with buffer (sodium acetate, 100 mM, pH 5.2) containing CIP-29 at a concentration of 25

µg/mL. Standard protein purification procedures using liquid column chromatography system, spectrophotometric observations to be carried out. Bio-assay procedures were undertaken to observe purified CIP-29 efficiency prior to its use as an inducer of virus inhibitory agents (VIAs) in the test hosts (Prasad et al. 1995) (Fig. 2).

CIP-29 used to induce production of virus inhibitory agents (VIAs) in the test host *Cyamopsis tetragonoloba*. These plants constituted the treated set while the control set of plants were sprayed with buffer alone. The resistance induced to be determined by the local lesion assay. For this purpose, all the plants were challenge inoculated with SRV at a concentration adjusted to give 200–300 lesions, on both lower treated (site) and upper non-treated (remote site) leaves, 24 h after being sprayed with either CIP-29, or buffer alone. Local lesions that developed on the treated and control set of plants third day post-inoculation were counted and percent inhibition of virus infectivity (induced resistance) to be calculated as described earlier (Prasad et al. 1995). The presence of VIA in the induced set of plants was determined by bio-assay. The sap from the remote-site leaves of both CIP-29 treated (induced) or buffer-treated (non induced/control) plants will be incubated at room temperature for 4 h, with an equal volume of TMV and SRV. The incubated mixture was rubbed onto the leaves of the assay hosts, N. tabacum cv. Xanthi-nc and C. tetragonoloba, using carborundum powder (600 mesh) as an abrasive. Viruses incubated with buffer alone constituted additional controls. The local lesions that developed on these leaves 2 days (for C. tetragonoloba) and 5 days (for N. tabacum cv. Xanthi-nc) post-inoculation were counted and percent reduction in virus infectivity calculated. All experiments has been carried out in triplicate, using a minimum of five plants for each treatment. Three leaves per plant received the treatments. Numbers of local lesions were counted from 15 leaves, and standard error of mean calculated. The induction of VIA over a period of time has been conducted to determine the time period for maximum VIA induction.

The VIA has been purified from the tissue harvested at this point of time. Basal leaves of each plant in the treated set will be sprayed with CIP-29 (25 lg mL-1) while the control set of plants w being sprayed with buffer alone (sodium acetate, 100 mM, pH 5.2). The upper non-treated (remote-site) leaves harvested at time intervals of 4, 8, 12, 16, 20, 24, 48 and 72 h post-treatment and assayed for VIA activity as described above. Percentage reduction in virus infectivity was calculated and data subjected to regression analysis by the software Origin, Version 8.5.

The time period for maximum induction of VIA activity was taken into consideration while treating a larger set of plants for its purification. CIP-29, isolated from leaf extract of C. inerme, was purified as described earlier (Prasad et al. 1995) and used for induction of systemic antiviral resistance for purifying the associated virus inhibitory activity/agent in C. tetragonoloba plants. Purification of the Virus inhibitory agents has been undertaken from the remote-site leaves of plants whose basal leaves were treated with CIP- 29. 100 g leaves were harvested, washed and homogenized in two volumes of extraction buffer (200 mM sodium acetate, pH 5.2 with 0.1 % β-mercaptoethanol).

The sap sqeezed through two folds of muslin cloth and centrifuged at 10,000 rpm for 20 min. Ammonium sulfate (60 % w/v final concentration) to be gradually added to the supernatant, with continuous stirring. After chilling at 4C for 1 h, the precipitate to be recovered by centrifugation at 10,000 rpm for 10 min. The pellet suspended in 50 mL of a tenfold diluted extraction buffer and then subjected to another round of centrifugation prior to column chromatography. All chromatographic procedures has been carried out at room temperature.

RESULT AND DISCUSSION

Induction of antiviral resistance in C. tetragonoloba and detection of VIA in induced plants CIP-29 (25 µg/mL) induced resistance against SRV on C. tetragonoloba plants such that when SRV was

challenge inoculated 24 h after treatment, it failed to infect. There were no local lesions visible on both basal treated leaves (site) as well as the upper non-treated leaves (remote-site). Thus, the resistance induced was systemic in nature with a near 95 % reduction in lesion number on resistant leaves (Table 2). The tissue homogenate from the upper leaves of the control (buffer sprayed) and treated (CIP-29 sprayed) plants was mixed separately with an equal volume ofpurified SRV, TMV and PRSV and assayed for loss ofvirus infectivity in vitro due to the presence of VIA. Both of the viruses were inhibited on their respective hosts, C.tetragonoloba and N. tabacum cv. Xanthi-nc (Table 1). Inhibition of TMV on tobacco by VIA-containing sap from induced (CIP-29 treated) and in C. tetragonoloba plants is shown in Table-1 and kinetics shown in Fig. 3.

Induction of Virus inhibitory agent (VIA) in *Cyamopsis tetragonoloba* plants treated with CIP-29.

Treatment*	Average lesion number± SEM	% Reduction in lesion number
C. tetragonoloba/SRV		
DW+SRV in sap	200.83±2.52	-
VIA+SRV in sap	9.16±0.98	95.43
DW+purified SRV	205.00± 1.96	-
VIA+Purified SRV	10.16 ± 0.60	95.04
Nicotiana tabacum cv. Xanthi nc/TMV		
DW+TMV in sap	215.16±1.72	-
VIA+TMV in sap	18.00±0.96	91.63
DW+purified TMV	219.00 ± 2.38	-
VIA+Purified TMV	16.00 ± 1.15	92.69

*Basal leaves of *Cyamopsis tetragonoloba* were treated with purified CIP-29 (25 µg/mL) and the remote site (untreated) leaves were harvested after 24 hours of treatment. Leaf tissue homogenate was incubated for 4 hours with purified SRV/ TMV or viruses in centrifuged leaf saps. The mixture was assayed on *C. tetragonoloba*/*N.tabacum* cv. Xanthi nc, local lesion hosts for SRV/TMV.
SEM: Standard error mean

Table-1

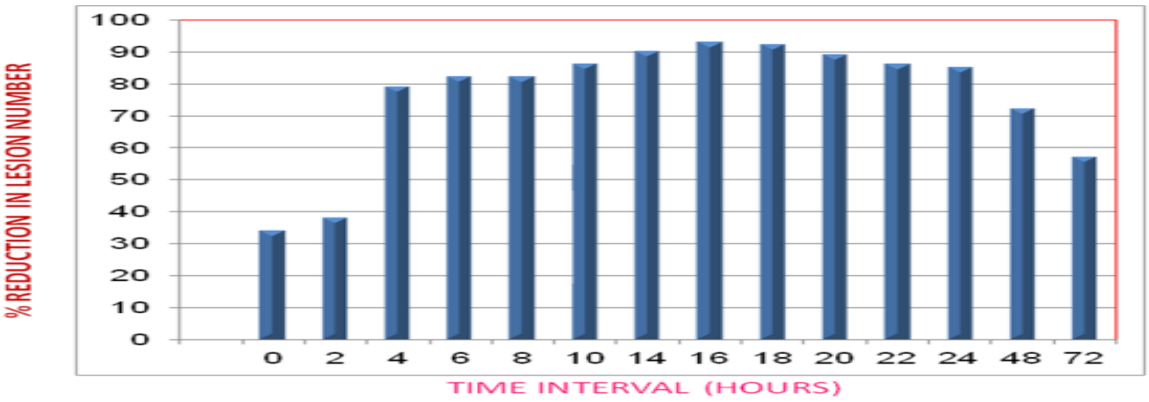
Purification of VIAs and determination of its molecular weights VIAs were purified from the leaves of CIP-29 induced C. tetragonoloba plants through sequential column chromatography as described in ‘‘Materials and methods’’

Kinetics of VIA induction in *C. tetragonoloba*

Time Interval (Hrs)*	Average lesion number± SEM	% Reduction in lesion number
Control (DW)	204.00 ± 2.54	-
0	134.00 ± 4.00	34.31
2	126.00 ± 5.00	38.23
4	43.00 ± 2.00	78.82
6	37.50 ± 2.50	81.61
8	36.00 ± 1.00	82.35
10	28.00 ± 1.00	86.25
14	20.00 ± 1.00	90.18
16	13.50 ± 1.50	93.38
18	17.00 ± 3.00	91.66
20	22.00 ± 1.00	89.00
22	28.00 ± 3.00	86.25
24	31.00 ± 1.00	84.80
48	56.00 ± 2.00	72.00
72	85.50 ± 3.50	57.25

*Basal leaves of *Cyamopsis tetragonoloba* were treated with purified CIP-29 (25 µg/mL) and the remote site (untreated) leaves were harvested at time intervals of 0, 2, 4, 6, 8, 10, 14, 16, 18, 20, 22, 24, 48, and 72 hours of treatment. Leaf tissue homogenate from each set was incubated for 4 hours with purified SRV. The incubated mixture was assayed on *C. tetragonoloba* plants. Virus incubated with DW served as a control. SEM: Standard error mean

Table-2



Kinetics of VIA induction. Leaves from CIP-29 treated *C. tetragonoloba* plants were harvested at various time intervals and assayed for induction of VIA through inhibition of SRV on *Cyamopsis tetragonoloba*.

Fig. 3

Here, we report on the purification of two virus inhibitoryproteins, named CT-VIA-32 and CT-VIA-62 from C. tetragonoloba plants that were induced to resist virus infection through prior application of CIP-29, a systemic antiviral resistance inducing protein purified from the leaves of C. inerme. An earlier report described virus inhibitory activity in a number of plants treated with B. spectabilis leaf extract (Verma and Dwivedi 1984). VIAcontaining sap from all such plants inhibited TMVinfectivity when assayed on Datura stramonium. B. spectabilis leaf extract incited VIA activity in crude homogenate of C. tetragonoloba inhibited different viruses on their respective hypersensitive hosts viz. TMV on tobacco Samsun NN as well as D. stramonium, SRV on C. tetragonoloba, Tomato yellow mottle mosaic virus on tobacco Samsun NN and D. stramonium, and Physalis shoestring mosaic virus on D. stramonium. Thus, VIA produced in response to treatment with resistance inducing extract reduced virus infections in both heterologous and homologous hosts, and was neither virus nor host specific. The virus inhibitory property of the B. spectabilis VIAcontaining sap was destroyed when heated at 80 ˚C and was susceptible to protease digestion. The proteinaceous nature of VIA was thus suggested in an early effort towards its characterization (Verma and Dwivedi 1984).

Immunoblot to determine specificity of anti-VIA-II-serum. The antiserum recognized VIA-II Ab lot (lot used to generate antibodies) as well as VIA-II Non Ab lot (VIA-II from another batch of purification).

Fig. 4

Leaf tissue homogenate from induced C. tetragonoloba plants displayed antiviral activity in two pools of fractions from the cation-exchange matrix. After further purification by chromatography on Hydroxyapatite and Superose-6 matrices, a 32 kDa protein and a 62 kDa protein were obtained from pool A and pool B, respectively. The virus inhibitory activity associated with the fractions coincided with the elution profiles of the two proteins, and the elution maxima could reduce TMV infectivity by more than 80 and 90 percent, respectively, in the bio-assays conducted ontobacco Xanthi-nc. Based on the host plant (C. tetragonoloba) from which isolated, and the molecular weight (32 and 62 kDa), the respective VIAs were named CT-VIA-32 and CT-VIA-62. We tested the inhibitory effects of both purified proteins and found that for the same concentration used, CTVIA- 62 was twofold more active than CT-VIA-32. Hence, later investigations were restricted to CT-VIA-62. The inducer protein, CIP-29, is monomeric and inhibits protein synthesis ex vivo as well as in cells, and also deadenylates both RNA and DNA (Olivieri et al. 1996). In our case, the inducer and the induced protein CT-VIA-62 are clearly distinct from each other both in terms of molecular weight and in that peptides generated from CTVIA- 62 did not exhibit any homologies with the RIPs, even though several of them are also reported to inhibit virus infection in plants (Stevens et al. 1981; Wang and Tumer 2000).

The two VIAs were serologically unrelated and only CT-VIA-32 was recognized by anti-CIP-29-serum. Although premature, but this opens the possibility of CT-VIA-32 being an induced RIP. Purified CT-VIA-62 was determined to be a basic glycoprotein which was also resistant to proteinase K. The protein band of M_r 62 kDa, that was electrophoretically homogenous, was used to raise polyclonal antibodies against VIA-II in Albino rabbits. VIA-II was chosen as the antigen since it was higher purity, and also the yield was better than VIA-I. The immunoblot experiments were subsequently conducted successfully for its specificity and titre. The titre of the anti-62 kDa VIA-II antibody was 1:10000, indicating a high level of antigenicity for the VIA-II. The anti-VIA-II antiserum showed reaction against purified VIA-II from the same lot which was used for antiserum preparation (antigen lot) and also reacted against another batch of freshly purified VIA-II (non-antigen lot) Fig. 4. Antibodies raised against CIP-29 recognized VIA-I, but not VIA-II. On the contrary, antibodies to VIA-II did not recognize VIA-I. This was an interesting observation in that the VIA-I shared common antigenic epitopes with the inducer CIP-29.

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