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Long-term protection against tobacco mosaic virus induced by the marine alga oligo-sulphated-galactan Poly-Ga in tobacco plants

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SUMMARY

In order to study the antiviral effect of the oligo-sulphated galactan Poly-Ga, the leaves of tobacco plants Xhanti^{NN} were sprayed with water (control), with increasing concentrations of Poly-Ga, for increasing numbers of treatments or cultivated for increasing times after treatment. Control and treated plants were infected with tobacco mosaic virus (TMV) and the numbers of necrotic lesions were measured in infected leaves. The number of necrotic lesions decreased with increasing concentrations of Poly-Ga, with increasing numbers of treatments and with increasing time after treatment, indicating a long-term protection against TMV that mimicks vaccination. In addition, control Xhanti^{nn} plants and plants treated with Poly-Ga and cultivated for increasing times after treatment were infected with TMV in the middle part of the plant, and the levels of TMV-capsid protein (CP) transcripts were measured in apical leaves. TMV-CP transcripts decreased in distant leaves, indicating that Poly-Ga induces systemic protection against TMV. The activities of the defence enzymes phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) and the amounts of several phenylpropanoid compounds (PPCs) were measured in control and treated plants without infection. A progressive increase in PAL activity was observed with increasing time after treatment, together with the accumulation of free and conjugated PPCs. In contrast, LOX activity remained unchanged. Interestingly, the increase in PAL activity showed a linear correlation with the decrease in necrotic lesions and the decrease in TMV-CP transcript level. Thus, Poly-Ga induced systemic and long-term protection against TMV in tobacco plants that is determined, at least in part, by a sustained activation of PAL and the accumulation of PPCs with potential antiviral activity.

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INTRODUCTION

Plant–pathogen interaction induces the release of oligosaccharides derived from plant and/or pathogen cell walls which can act as elicitors of plant defence responses (Darvill *et al*., 1994; Silipo *et al*., 2010). The best characterized oligosaccharide elicitors are oligogalacturonides (OGs) produced from pectin, chitooligosaccharides (COSs) derived from fungal cell walls, hepta-β-glucosides (HGs) obtained from the cell walls of *Phytophtora sojae*, lipochitooligosaccharides (LCOs) derived from the cell walls of Gram-negative bacteria (rhizobia) and rhamnolipids obtained from the cell walls of *Pseudomonas aeruginosa* (Aziz *et al*., 2004; Bishop *et al*., 1981; Denoux *et al*., 2008; Norman *et al*., 1999; Sharp *et al*., 1984; Varnier *et al*., 2009). These oligosaccharides activate defence mechanisms which protect plants against pathogens, such as bacteria, fungi and viruses (Aziz *et al*., 2007; Denoux *et al*., 2008; Varnier *et al*., 2009; Wang F. *et al*., 2009). In addition, marine algae sulphated oligosaccharides also stimulate plant defence responses and induce protection against bacteria, fungi and viruses (Aziz *et al*., 2003; Klarzynski *et al*., 2000, 2003; Laporte *et al*., 2007; Ménard *et al*., 2004).

With regard to the perception of plant/pathogen oligosaccharides in plants, it has been shown that these elicitors bind to specific receptors located in the plasma membrane (Itoh *et al*., 1997). Indeed, receptors for LCOs, HGs and COSs have been cloned and characterized recently (Fliegmann *et al*., 2004; Kaku *et al*., 2006; Madsen *et al*., 2003; Petutschnig *et al*., 2010). These receptors share a common LysM domain resembling the peptidoglycan-binding domain present in Toll-like receptors (TLRs) of mammalian cells, which is coupled to a kinase domain involved in signal transduction. The interaction of an elicitor with its receptor triggers a transient oxidative burst produced by a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located in the plasma membrane, which requires Ca^{2+} and phosphorylation for its activation (Ogasawara *et al*., 2008).

NADPH oxidase produces superoxide anions in the apoplast which are dismutated to hydrogen peroxide (Hammond-Kosack and Jones, 1996; Van Breusegem *et al*., 2008). In addition, the interaction of an elicitor with its receptor can activate calcium channels located in the plasma membrane (Hammond-Kosack and Jones, 1996; Jeworutzki *et al*., 2010), inducing a transient increase in intracellular calcium that leads to the activation of calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) that trigger defence gene expression (Boudsocq *et al*., 2010; Daxberger *et al*., 2007; Miya *et al*., 2007). The increase in intracellular calcium and reactive oxygen species (ROS) leads to the activation of defence enzymes and pathways and to the accumulation of compounds with antimicrobial activities (Hammond-Kosack and Jones, 1996; Jabs *et al*., 1997; Sels *et al*., 2008).

The expression of most defence genes is not constitutive, but is triggered during compatible or incompatible plant–pathogen interactions. One of the induced defence responses is the expression of pathogenesis-related (PR) protein families with antifungal and antibacterial properties (Sels *et al*., 2008). Plant–pathogen interaction also activates the defence phenylpropanoid and octadecanoid pathways leading to the synthesis of phenylpropanoid compounds (PPCs) and oxylipins with antimicrobial activities (Blée, 2004; Dixon, 2001). It is important to mention that the phenylpropanoid pathway is mainly regulated by the activity of phenylalanine ammonia lyase (PAL) and the octadecanoid pathway is regulated by lipoxygenase (LOX) (Howles *et al*., 1996; La Caméra *et al*., 2004). Interestingly, it has been shown that many PPCs have antiviral activities, such as benzoic acid, dihydroxybenzoic acid (DHBA), gallic acid (GA), caffeic acid (CA), chlorogenic acid (CHL), ferulic acid, scopoletin (SCO), esculetin (ESC), quercetin (QUE), kaempferol and rutin (RUT), among others (Schneider *et al*., 2010; Tian *et al*., 2009; Wang G. F. *et al*., 2009). These PPCs have antiviral activity mainly against animal viruses, and only SCO has been shown to inhibit plant virus replication (Chong *et al*., 2002). In addition, plants synthesize other compounds, such as terpenes, terpenoids and alkaloids, which have antiviral activities against animal and plant viruses (Wu *et al*., 2007; Yan *et al*., 2010; Zhang *et al*., 2007)

In a previous study, we prepared the oligo-sulphated galactan Poly-Ga with a molecular weight of around 8.5 kDa, which corresponds to 20 units of sulphated galactose (Laporte *et al*., 2007). In addition, we prepared a depolymerized fraction of mannuronic acid (Poly-Ma) obtained by acid hydrolysis of alginates extracted from a marine brown alga (Laporte *et al*., 2007). Poly-Ga and Poly-Ma sprayed on tobacco leaves at a concentration of 0.5 mg/mL once a week for 2 weeks, and cultivated for 15 additional days after treatment, induced protection against tobacco mosaic virus (TMV) of 74% and 22%, respectively (Laporte *et al*., 2007). Interestingly, Poly-Ga and Poly-Ma also induced an increase in growth and leaf biomass of 23% and 49%, respectively, after 7 days of treatment.

In this work, we analysed the antiviral effect of Poly-Ga against TMV in tobacco plants using increasing concentrations of Poly-Ga, increasing numbers of treatments and cultivating the treated plants for increasing times after treatment. In order to analyse the mechanisms involved in the antiviral effect, we determined the activities of PAL and LOX defence enzymes and the potential correlation between PAL and/or LOX activities and protection against TMV infection. In addition, we analysed the level of several PPCs with potential antiviral activity.

RESULTS

Poly-Ga induced a dose-dependent, treatment number-dependent and long-term protection against TMV

In order to study the antiviral properties of Poly-Ga, Xhanti^{NN} tobacco plants were treated with water (control), with increasing concentrations of Poly-Ga, with increasing numbers of treatments or cultivated for increasing time after treatment. Control and treated plants were infected with TMV and the number of necrotic lesions was determined in the infected leaves. Plants treated with 0.1, 0.3, 0.5 and 0.7 mg/mL of Poly-Ga showed decreases in the number of necrotic lesions of 47%, 53%, 59% and 73%, respectively, compared with control plants (Fig. 1A). In addition, plants treated with Poly-Ga for one, two, three and four times showed decreases in the number of necrotic lesions of 38%, 51%, 66% and 73%, respectively, compared with control plants (Fig. 1B). Furthermore, plants treated with Poly-Ga and cultivated for 15, 30, 45 and 60 days after treatment showed decreases in the number of necrotic lesions of 57%, 84%, 94% and 98%, respectively, compared with control plants (Fig. 1C,D). Thus, Poly-Ga induced a dose-dependent, treatment number-dependent and long-term protection against TMV infection, mimicking a vaccination effect.

Poly-Ga induced a systemic protection against TMV

In order to detect whether Poly-Ga induced systemic protection against TMV, Xhanti^{nn} tobacco plants were treated with water (control) or with Poly-Ga and cultivated for increasing times after treatment. Control and treated plants were infected with TMV in the middle part of the plant and the relative level of TMV-capsid protein (CP) transcript was quantified in apical leaves using real-time reverse transcription-polymerase chain reaction (RT-PCR). Plants treated with Poly-Ga and cultivated for 15, 30, 45 or 60 days after treatment showed decreases in

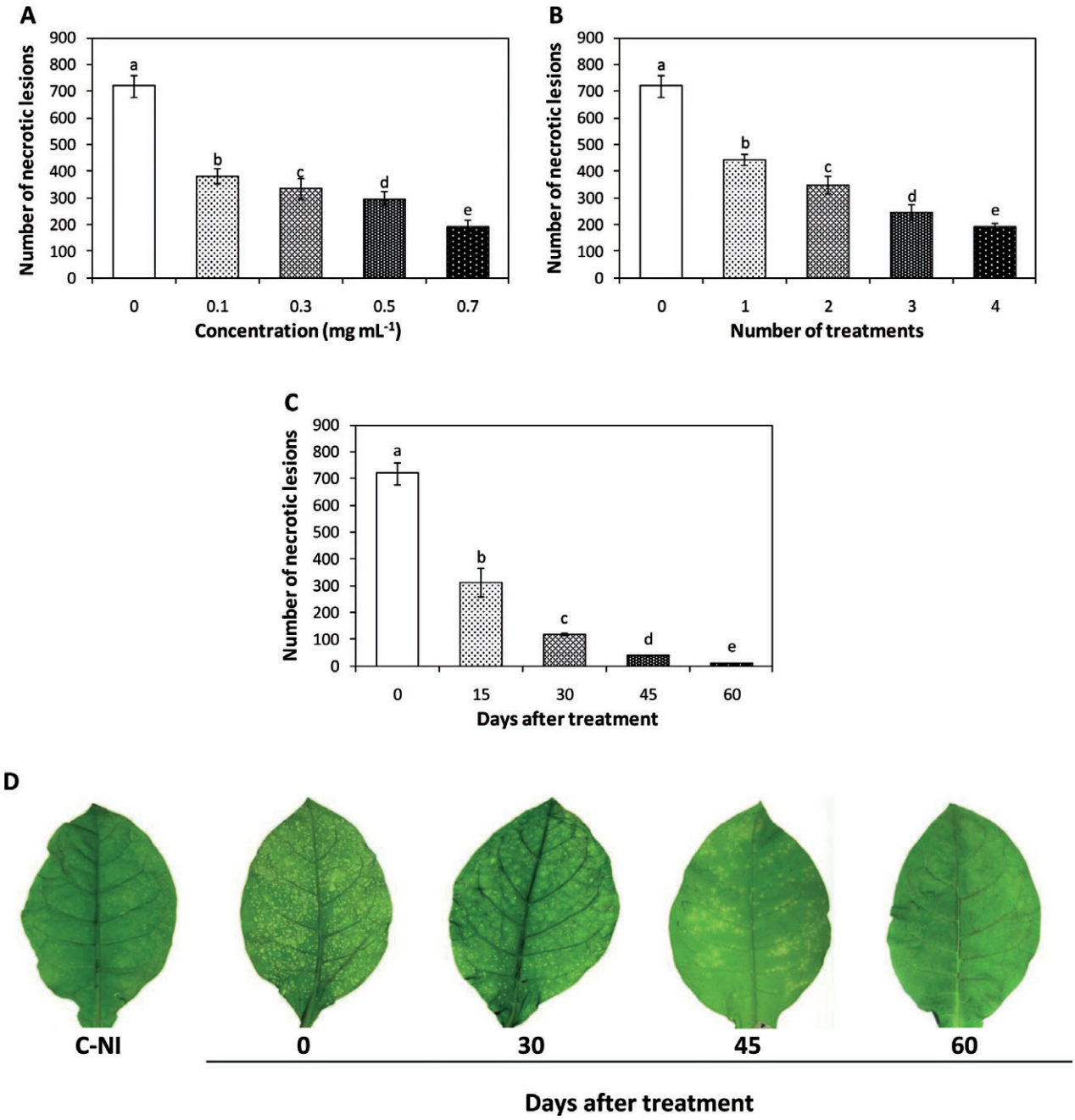

Fig. 1 Number of necrotic lesions in the infected leaves of control Xhanti^{NN} tobacco plants and in plants treated with increasing concentrations of Poly-Ga (A), with an increasing number of treatments (B) and cultivated for increasing times after treatment (C). Bars correspond to the mean values obtained from seven plants ± SD. Different letters indicate significant differences ($P < 0.05$). Leaf of a control Xhanti^{NN} tobacco plant not infected with tobacco mosaic virus (TMV) (C-NI); leaf of a plant treated with Poly-Ga and cultivated for 0, 30, 45 and 60 additional days after treatment and infected with TMV (D).

TMV-CP transcript level of 48%, 83%, 96% and 97%, respectively (Fig. 2A). This was consistent with the decrease in mosaic symptoms in the apical leaves of treated plants (Fig. 2B). Thus, Poly-Ga induced a systemic protection against TMV in tobacco plants.

Poly-Ga induced a progressive and sustained activation of PAL defence enzyme

In order to analyse the mechanisms involved in the antiviral effect of Poly-Ga, the activities of PAL and LOX defence

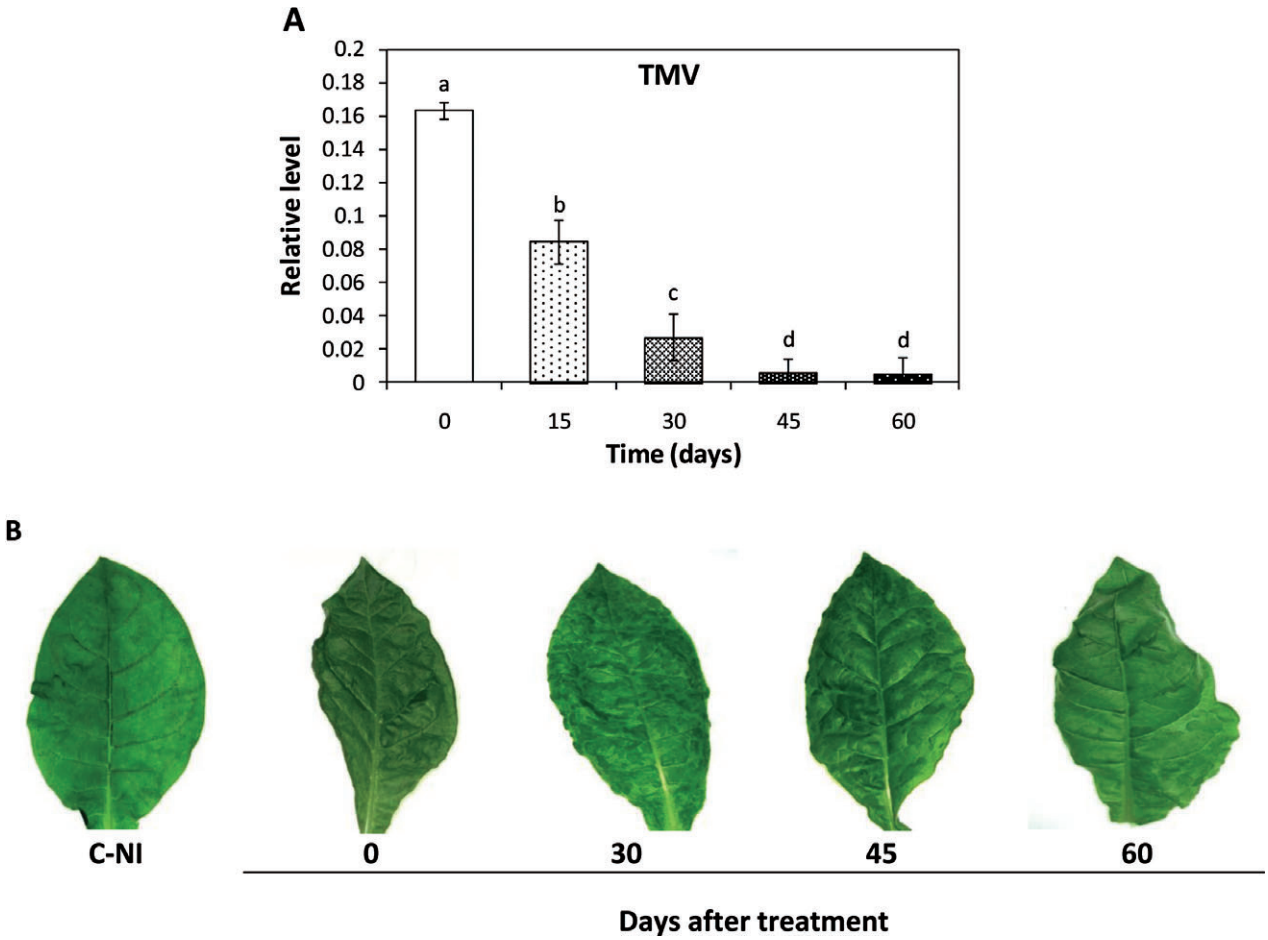

Fig. 2 Relative level of tobacco mosaic virus (TMV) transcripts in the apical leaves of control Xhanti^{nn} tobacco plants and plants treated with Poly-Ga and cultivated for increasing times after treatment and infected with TMV in the middle part of the plant (A). Bars correspond to the mean values of three independent experiments ± SD. Different letters indicate significant differences ($P < 0.05$). Leaf of a control Xhanti^{nn} tobacco plant not infected with TMV (C-NI); leaves of plants treated with Poly-Ga and cultivated for 0, 30, 45 and 60 additional days after treatment and infected with TMV (B). The relative level of TMV-capsid protein (CP) transcripts is expressed as $2^{-\Delta\Delta CT}$, which includes the mean value of the TMV-CP level in the control plant subtracted from the mean value detected in treated plants.

enzymes were determined in Xhanti^{NN} tobacco plants treated with increasing concentrations of Poly-Ga, for increasing numbers of treatment and cultivated for increasing times after treatment (and not infected with TMV). Plants treated with 0.1, 0.3, 0.5 and 0.7 mg/mL of Poly-Ga showed increases in PAL activity of 1.8, 2, 2.8 and 3.4 times, respectively, compared with the control (Fig. 3A), whereas LOX activity remained unchanged (data not shown). In addition, plants treated for 1, 2, 3 and 4 weeks with Poly-Ga showed increases in PAL activity of 1.5, 2, 2.6 and 3.3 times, respectively, compared with the control (Fig. 3B), whereas LOX enzyme activity remained unchanged (data not shown). Furthermore, plants treated with Poly-Ga and cultivated for 15, 30, 45 and 60 days after treatment showed increases in PAL activity of 2.6, 4.3, 5 and 5.5 times, respectively, compared with the control (Fig. 3C), whereas LOX activity remained unchanged (Fig. 3D). It is interesting to note that PAL activity did not change in control plants cultivated for 15, 30, 45 and 60 days after treatment (Fig. 3C). Thus, Poly-Ga induced a dose-dependent, treatment number-dependent and long-term activation of PAL enzyme. Interestingly, the increase in PAL activity correlated linearly with the decrease in necrotic lesions, with increasing concentrations of Poly-Ga (Fig. 4A), increasing numbers of treatment (Fig. 4B) and increasing times after treatment (Fig. 4C), as well as with the decrease in the TMV-CP transcript level in apical leaves (Fig. 4D). Thus, PAL activation may determine, at least in part, the antiviral effect induced by Poly-Ga.

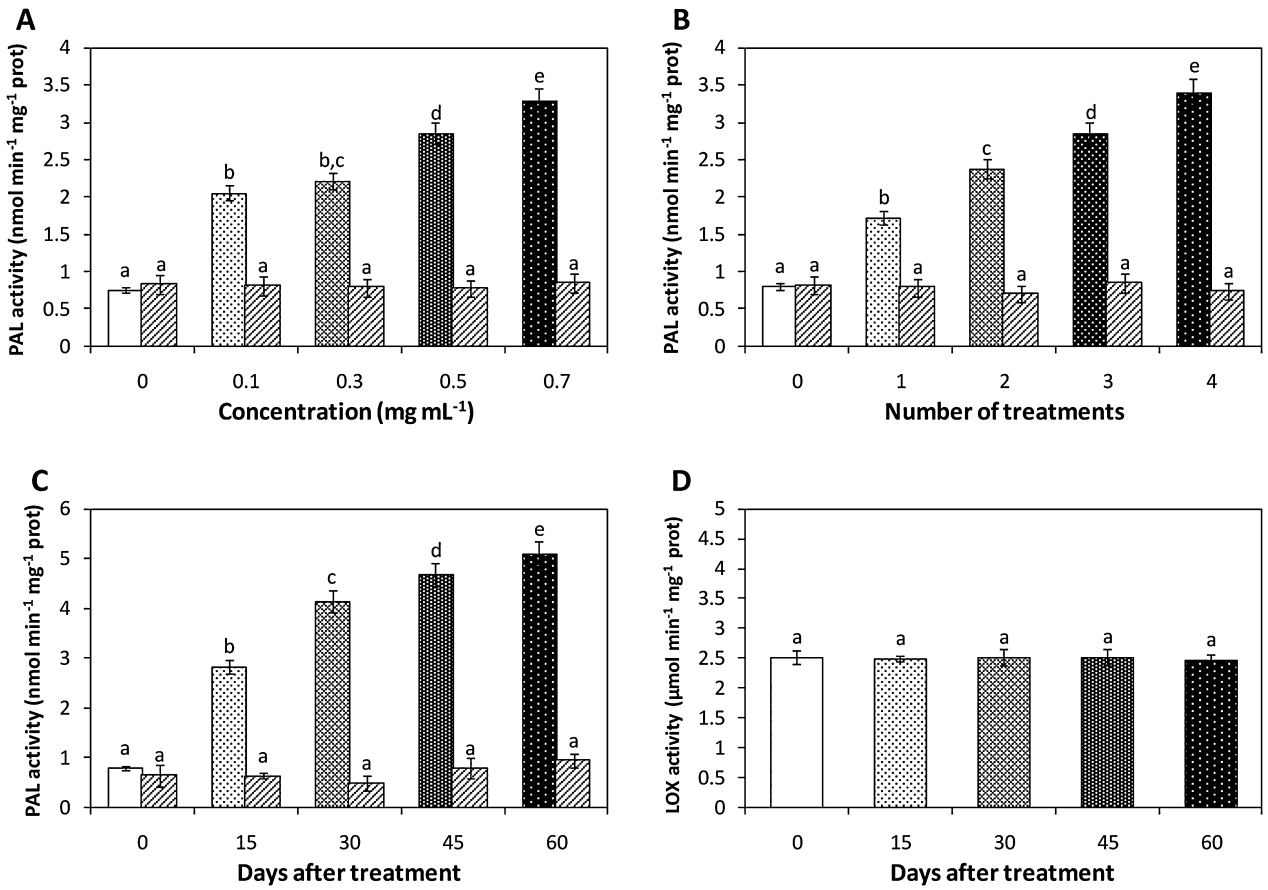

Fig. 3 Phenylalanine ammonia lyase (PAL) activity in leaves of Xhanti^{NN} tobacco plants treated with increasing concentrations of Poly-Ga (A), with increasing numbers of treatments (B) and cultivated for increasing times after treatment (C). Lipoxygenase (LOX) activity in plants treated with Poly-Ga and cultivated for increasing times after treatment (D). Bars correspond to the mean values obtained from seven plants ± SD and the right-hand bars correspond to control plants. PAL activity is expressed as nanomoles per minute per milligram of protein and LOX activity is expressed in micromoles per minute per milligram of protein. Different letters indicate significant differences ($P < 0.05$).

Poly-Ga induced the accumulation of PPCs

In order to determine whether PAL activation induced the accumulation of PPCs with potential antiviral activities, free and conjugated PPCs, such as salicylic acid (SA), DHBA, GA, vanillic acid (VA), CA, CHL, SCO, ESC, QUE and RUT, were detected and quantified by high-performance liquid chromatography (HPLC) in control plants and in plants treated with Poly-Ga and cultivated for 0, 15, 30, 45 and 60 days after treatment. The levels of total SA, GA, CA, SCO and ESC progressively increased in plants treated with Poly-Ga and cultivated for 15, 30, 45 and 60 days after treatment (Fig. 5), whereas the level of PPCs in control plants did not change (data not shown). The level of total VA initially increased and then remained stable, and the levels of total DHBA and RUT increased only at the end of the experiment time. The level of total QUE increased and then decreased during the experimental procedure (Fig. 5). In addition, the levels of free GA and CHL were higher than their conjugated forms, the levels of conjugated SA, DHBA, CA, SCO, ESC and RUT were higher than their free forms and the level of free VA was similar to the level of its conjugated form (Fig. 5). Furthermore, greater increases in total PPCs were registered for DHBA, GA, VA, CHL, SCO and RUT (Table 1). Thus, most of the analysed PPCs with potential antiviral activity, in free and conjugated form, accumulated in plants treated with Poly-Ga.

DISCUSSION

Poly-Ga induced systemic and long-term protection against TMV

In this work, we showed that the oligo-sulphated galactan Poly-Ga induced a dose-dependent, treatment number-dependent and long-term protection against TMV in tobacco plants, mimicking a vaccination effect. In this sense, vaccination in animals is induced by the infiltration of an antigen derived

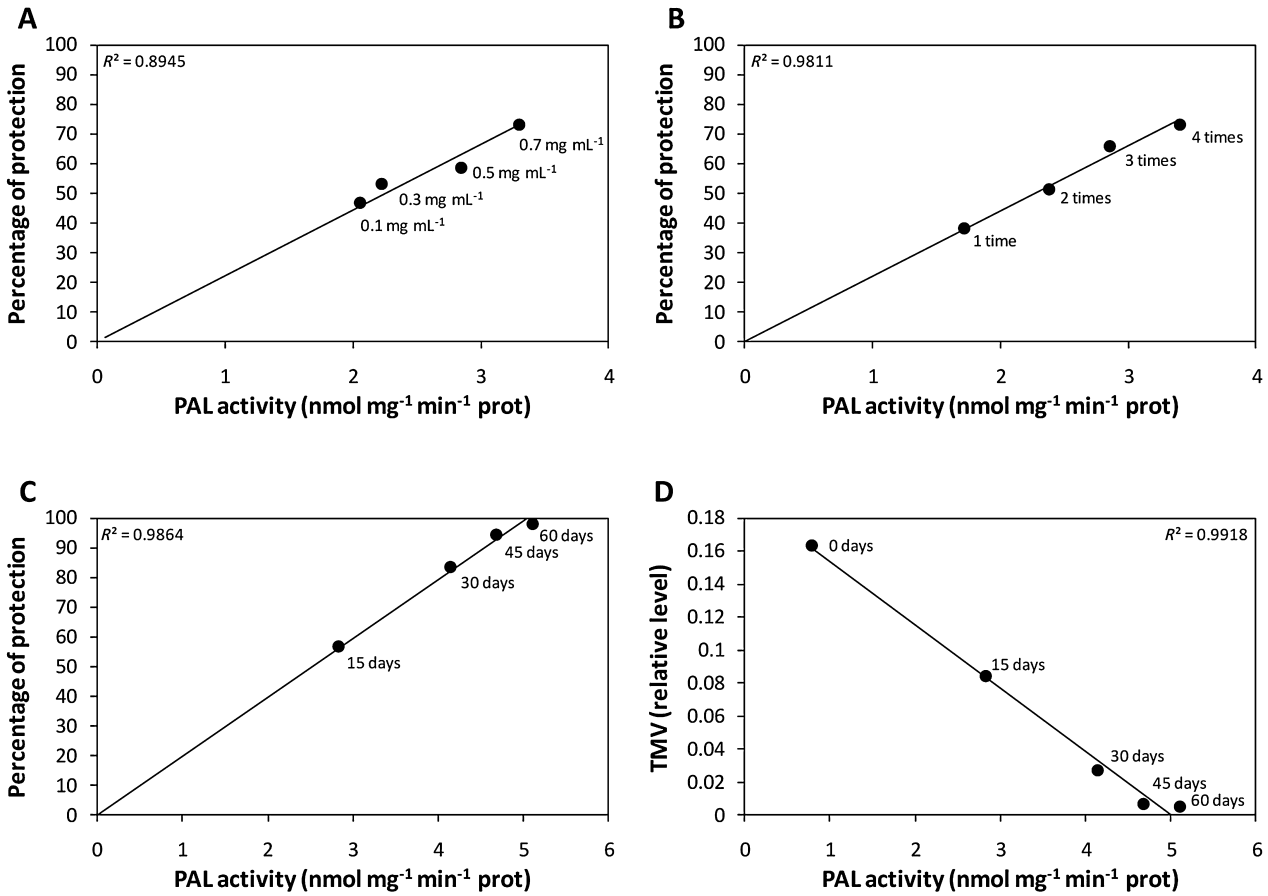

Fig. 4 Correlation between protection against tobacco mosaic virus (TMV) infection and phenylalanine ammonia lyase (PAL) activity in plants treated with increasing concentrations of Poly-Ga (A), with increasing numbers of treatments (B) and cultivated for increasing times after treatment (C). Correlation between the decrease in the relative levels of TMV-capsid protein (CP) transcript and the increase in PAL activity in plants treated with Poly-Ga and cultivated for increasing times after treatment (D). Protection corresponds to the percentage decrease in the number of necrotic lesions, and PAL activity is expressed in nanomoles per minute per milligram of protein.

from a pathogen which activates innate immunity, which, in turn, stimulates cellular immunity. In animals, innate immunity is activated by antigens corresponding to microbe-associated molecular patterns (MAMPs), such as proteins, oligosaccharides, lipoproteins, lipooligosaccharides and nucleic acids derived from pathogens (Kawai and Akira, 2010). These MAMPs bind to plasma membrane TLRs and/or intracellular nucleotide-binding and oligomerization domain (NOD) receptors present in dendritic cells, macrophages and immunocompetent tissues. The interaction between a MAMP and its receptor triggers a signal transduction cascade that activates nuclear factor-κB (NF-κB) transcriptional regulation factor, which, in turn, activates genes coding for interleukins and interferon. The latter inflammatory signals induce the proliferation of T lymphocytes and B lymphocytes, conferring specificity and amplifying the immune response. In contrast, plants only have an innate immune system and lack the cellular system to amplify the immune response. Innate immunity in plants also depends on MAMPs and plasma membrane-specific receptors (Boller and Felix, 2009). In plants, MAMPs are molecules derived from pathogens, such as bacterial flagellin, bacterial translation elongation factor Ef-Tu, bacterial LCOs (nod factors), fungal HGs, fungal COSs, among others (Boller and Felix, 2009; Chinchilla *et al*., 2007; Kaku *et al*., 2006). The binding of MAMPs to their specific receptors triggers a signal transduction cascade that activates defence genes, leading to the synthesis of PR proteins with antibacterial and antifungal properties, and to the accumulation of phytoalexins with antiviral activity (Aslam *et al*., 2009; Kishi-Kaboshi *et al*., 2010; Zhang *et al*., 2007). As mentioned above, Poly-Ga induced a dose-dependent, treatment number-dependent and long-term antiviral protection, mimicking a vaccination effect, but the mechanisms that determine the number of dose-dependent effect in plants remain to be determined.

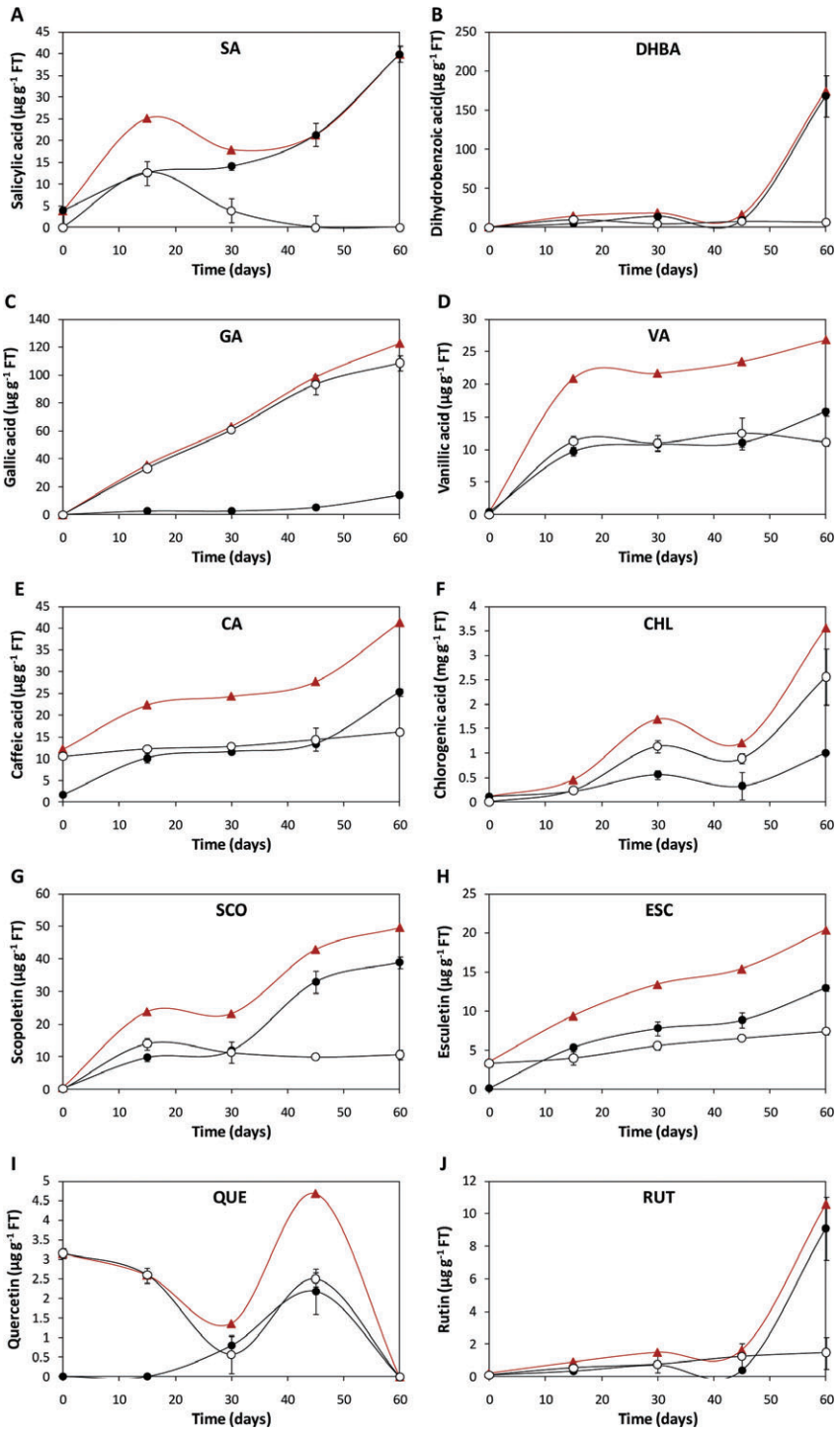

Fig. 5 Level of free, conjugated and total phenylpropanoid compounds (PPCs) corresponding to salicylic acid (A), dihydroxybenzoic acid (B), gallic acid (C), vanillic acid (D), caffeic acid (E), chlorogenic acid (F), scopoletin (G), esculetin (H), quercetin (I) and rutin (J) in tobacco plants treated with Poly-Ga and cultivated for increasing times after treatment. The level of PPCs is expressed as micrograms per gram of fresh tissue (FT) except for CHL which is expressed as milligrams per gram of FT. Mean values of free PPCs (open circles) and conjugated PPCs (black circles) were obtained from three independent triplicates ± SD. Total PPCs (free PPCs + conjugated PPCs) are indicated by red triangles.

Table 1 Level of free, conjugated and total phenylpropanoid compounds (PPCs) in control plants and in plants treated with Poly-Ga cultivated for 60 days after treatment.

	Control plants			Treated plants			
PPCs	Free*	Conjugated	Total	Free	Conjugated	Total	Fold change†
SA	4	0.0	4	40	0	40	10
DHBA	0.5	0.1	0.6	168	6	174	290
GA	0.02	0	0.02	14	109	123	6150
VA	0.4	0	0.4	16	11	27	68
CA	1.6	11	12.6	25	16	41	3
SCO	0.4	0.1	0.5	39	11	50	100
ESC	0.2	3.3	3.5	13	7	20	6
CHL	3	110	113	1000	2550	3550	31
QUE	0	3.2	3.2	0	0	0	0
RUT	0.1	0.1	0.2	9	1.5	10.5	52

CA, caffeic acid; CHL, chlorogenic acid; DHBA, dihydroxybenzoic acid; ESC, esculetin; GA, gallic acid; QUE, quercetin; RUT, rutin; SA, salicylic acid; SCO, scopoletin; VA, vanillic acid.

*The level of free, conjugated and total PPCs is expressed as micromoles per gram of fresh tissue.

†Fold change in total PPCs.

The activation of innate immunity induced by Poly-Ga suggests that oligosaccharides may reach the plasma membrane of tobacco leaf cells, where specific receptors for sulphated oligosaccharides may exist. With regard to the recognition of sulphated oligosaccharides in plants, it has been shown that nod factors, which are sulphated glucosamine oligosaccharides secreted by rhizobial bacteria, interact with receptors located in the plasma membrane of leguminous root cells (Lerouge *et al*., 1990; Madsen *et al*., 2003). Thus, plants not related to leguminous species may have receptors in foliar tissue recognizing sulphated oligosaccharides. The exitence of such receptors may explain the responses induced by sulphated oligosaccharides such as sulphated laminarin, sulphated fucans and Poly-Ga in tobacco plants (Klarzynski *et al*., 2003; Laporte *et al*., 2007; Ménard *et al*., 2004). The occurrence and cellular location of specific receptors recognizing sulphated oligosaccharides in plants remain to be determined.

Poly-Ga induced a sustained activation of PAL defence enzyme

With regard to the defence triggered by MAMP–receptor interaction, a common response in plants is the activation of PAL and LOX defence enzymes, which lead to the synthesis of PPCs and oxylipins with antimicrobial activities (Blée, 2004; La Caméra *et al*., 2004). In this work, we detected a sustained and progressive increase in PAL activity in plants treated with increasing concentrations of Poly-Ga, with increasing numbers of treatments and cultivated for different times after treatment. This contrasts with previous findings in tobacco plants treated with laminarin, sulphated fucans or with a fructooligosaccharide extracted from roots of the plant *Arctium lappa*, where PAL expression and activity were only transiently induced (Klarzynski *et al*., 2000, 2003; Wang F. *et al*., 2009). In addition, the increase in PAL activity correlates linearly with protection against TMV infection, suggesting that the activation of the phenylpropanoid pathway is involved in the antiviral effect induced by Poly-Ga.

Poly-Ga induced the accumulation of PPCs

Tobacco plants treated with Poly-Ga showed a progressive accumulation of several PPCs in their free and/or conjugated forms. Interestingly, free SA increased in plants treated with Poly-Ga and cultivated for increasing times after treatment (without infection), indicating that Poly-Ga induced a systemic acquired resistance in tobacco plants and that its accumulation may contribute to the antiviral effect (Vlot *et al*., 2009). In addition, the accumulation of free and conjugated PPCs correlates with the increase in protection against TMV, indicating that PPCs may contribute to the antiviral effect induced by Poly-Ga. In this sense, it has been shown that free and conjugated forms of PPCs can have antiviral activity against animal viruses by inhibiting their replication. For example, free GA, CA, CHL and ESC inhibit enterovirus, herpes virus and hepatitis virus infection (Chiang *et al*., 2002; Choi *et al*., 2010; Tian *et al*., 2009; Wang G. F. *et al*., 2009) and glycosides of GA, CA and QUE inhibit syncytial virus, hepatitis C virus and herpes virus infection (Kernan *et al*., 1998; Tian *et al*., 2009; Zuo *et al*., 2005). It is important to mention that the accumulation of compounds other than phenylpropanoids may also contribute to the antiviral effect induced by Poly-Ga in tobacco plants. In this sense, it has been shown that terpenoids and alkaloids extracted from plants have antiviral effects against TMV (Wu *et al*., 2007; Yan *et al*., 2010; Zhang *et al*., 2007). Thus, the accumulation of PPCs, as well as terpenes, terpenoids and/or alkaloids, with potential antiviral activity may explain the effect of Poly-Ga in tobacco plants.

In conclusion, the oligo-sulphated-galactan Poly-Ga induced a dose-dependent, treatment number-dependent and long-term protection against TMV in tobacco plants, mimicking a vaccination effect. The antiviral effect of Poly-Ga was determined, at least in part, by a sustained activation of PAL defence enzyme,

leading to the accumulation of PPCs with potential antiviral activity.

EXPERIMENTAL PROCEDURES

Preparation of Poly-Ga

Poly-Ga was prepared as described by Zúñiga *et al*. (2006).

Plant culture and treatments

Xhanti^{NN} and Xhanti^{nn} tobacco plants were cultivated in flowerpots with a mixture of compost–vermiculite (3 : 2) in a growth chamber using a light/dark period of 16 h/8 h, light intensity of 100 μmol/m^2/s at 22 °C, and watered every 3 days.

For assays with increasing concentrations of Poly-Ga, Xhanti^{NN} tobacco plants were sprayed with water on the upper and lower faces of all the leaves (control plants, $n = 7$; initial height, 15 cm) or with an aqueous solution of Poly-Ga at concentrations of 0.1, 0.3, 0.5 or 0.7 mg/mL ($n = 7$ for each concentration of Poly-Ga; initial height, 15 cm), once a week for 2 weeks, and cultivated for 15 days after treatment.

For assays with increasing numbers of Poly-Ga treatments, Xhanti^{NN} tobacco plants were sprayed with water on the upper and lower faces of all the leaves (control plants, $n = 7$; initial height, 15 cm) or with an aqueous solution of Poly-Ga at a concentration of 0.5 mg/mL, once a week for 1, 2, 3 or 4 weeks ($n = 7$ for each number of treatments; initial height, 15 cm), and cultivated for 15 days after treatment.

For assays with increasing times after treatment, Xhanti^{NN} tobacco plants were sprayed with water on the upper and lower faces of all the leaves, once a week for 2 weeks, and cultivated for 0, 15, 30, 45 or 60 days after treatment (control plants, $n = 7$; initial height, 15 cm) or sprayed with an aqueous solution of Poly-Ga at a concentration of 0.5 mg/mL, once a week for 2 weeks, and cultivated for 0, 15, 30, 45 or 60 days after treatment ($n = 7$ for each time after treatment; initial height, 15 cm).

For infection with TMV and the detection of necrotic lesions, Xhanti^{NN} control plants and plants treated with increasing concentrations of Poly-Ga, for increasing numbers of treatments and cultivated for increasing times after treatment (see above) were infected with TMV in a single leaf located in the middle part of each plant, and the numbers of necrotic lesions were counted in the infected leaf.

For infection with TMV and the detection of the TMV-CP transcript level, Xhanti^{nn} control tobacco plants and treated plants cultivated for increasing times after treatment (see above) were infected with TMV in a single leaf located in the middle part of each plant; the apical leaves with mosaic symptoms were collected after 20 days and the total leaves from two plants were pooled to obtain triplicate samples.

For the detection of PAL activity, the total leaves from each Xhanti^{NN} control plant and the total leaves from each plant treated with increasing concentrations of Poly-Ga, with increasing numbers of treatments and cultivated for increasing times after treatment (see above), and not infected with TMV, were collected. PAL activity was detected in each plant of the control ($n = 7$) and treated groups ($n = 7$ for each treatment).

For the detection of PPCs, the total leaves from each Xhanti^{NN} control plant and the total leaves from each plant cultivated for increasing times after treatment (see above), and not infected with TMV, were collected, and the total leaves from two plants were pooled to obtain triplicate samples.

Infection with TMV

Xhanti^{NN} tobacco plants were infected in a single leaf in the middle part of each plant with 100 μL of TMV-U 1 viral suspension (2 μg of protein) using carborundum, and necrotic lesions were counted 5 days after infection. Xhanti^{nn} tobacco plants were infected in a single leaf in the middle part of each plant using the latter procedure, and mosaic symptoms were observed 20 days after infection.

Preparation of protein extracts

Protein extracts were prepared as described by Laporte *et al*. (2007).

Detection of PAL and LOX activities

PAL activity was determined in 1 mL of reaction mixture containing 100 mM phosphate buffer, pH 7.0, 13 mM phenylalanine and 100 μg of protein extract at 40 °C. The increase in absorbance, caused by cinnamic acid accumulation, was monitored at 290 nm for 90 min. PAL activity was calculated using the extinction coefficient of cinnamic acid ($\varepsilon = 17.4$ mM^{-1} cm^{-1}).

LOX activity was determined in 1 mL of reaction mixture containing 100 mM phosphate buffer, pH 7.0, 0.4 mM linoleic acid and 100 μg of protein extract. The increase in absorbance caused by the accumulation of conjugated dienes was monitored at 234 nm for 10 min. LOX activity was calculated using the extinction coefficient of conjugated dienes ($\varepsilon = 25$ mM^{-1} cm^{-1}).

Quantification of TMV-CP transcripts by real-time RT-PCR

Total RNA was extracted from tobacco leaves (0.5 g) using a FavorPrep Plant Total RNA Kit (Favorgene, Ping Tung, Taiwan) and quantified with a Quanti-iT Ribogreen RNA Assay Kit (Invitrogen, Eugene, OR, USA). The relative level of transcripts coding for TMV-CP was detected, as well as the 18S RNA level as an

internal control. PCR primers used for CP amplification were as follows: Forward-CP, 5′-CTGCCGAAACGTTAGATGCTACT-3′; Reverse-CP, 5′-TCCGGTTCCTCTGATCAATTCT-3′. Those for 18S RNA amplification were as follows: Forward-18S, 5′-TTCTTTGT ACCTTTTGCTGGCTTAT-3′; Reverse-18S, 5′-CTCTGGTCCTTCTTTA TACAACAAAC-3′. RT-PCRs were performed using the Sensimix One-Step Kit (Quantace, London, UK), 5 μg of total RNA, 10 μM of each PCR primer, 2 mM $MgCl_2$ and a real-time thermocycler Rotor gene 6000 (Corbett, Research, Sydney, Australia). The RT step was performed for 30 min at 49 °C, the inactivation step for 10 min at 95 °C and the PCR amplification used 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. Fragments amplified by RT-PCR were detected by fluorescence using SYBR GREEN I included in the amplification kit. RT-PCRs were performed in triplicate from three independent replicates. Sample values were averaged, normalized using the ΔΔCT method and the mean value of the control was subtracted from the mean value of the treated sample to determine the fold change in the treated sample. The relative transcript level was expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Analysis of PPC level by HPLC

Tobacco leaves (1 g) were frozen in liquid nitrogen and pulverized in a mortar with a pestle. To detect free PPCs, 5 mL of 100% methanol were added, the mixture was incubated in darkness for 24 h at room temperature, centrifuged at 7400 *g* for 15 min and the supernatant was recovered. To detect conjugated PPCs, 5 mL of 100% ethanol and 5 mL of 1 M NaOH were added, the mixture was incubated in darkness for 24 h at room temperature, neutralized to pH 7.0 with HCl (1 M), centrifuged at 7400 *g* for 15 min and the pellet was solubilized in 1 mL of 100% methanol.

Free PPCs in the methanol fraction (20 μL) were analysed by HPLC using an Agilent HPLC equipment model 1110 (Agilent Technologies, Santa Cruz, CA, USA) and a reversed phase C-18 column (length, 15.5 cm; inner diameter, 4.6 mm; particle size, 5 μm) coupled to a photodiode array detector. PPCs were eluted with a linear gradient from 0% to 60% (v/v) of acetonitrile in 5% (v/v) trifluoroacetic acid for 20 min at a flow rate of 1 mL/min. PPCs were detected at 254, 280, 314 and 360 nm using the absorption spectra of pure commercial standards (Sigma, St. Louis, MO, USA), and quantified using a calibration curve prepared with pure standards at concentrations ranging from 0 to 1 mg/mL.

Statistical analysis

Significant differences were determined by two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test (*T*). Differences between mean values were considered to be significant at a probability of 5% ($P < 0.05$) (Zar, 1999).

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