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Short-term polyamine response in TMV-inoculated hypersensitive and susceptible tobacco plants

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SUMMARY

The short-term polyamine response to inoculation, with tobacco mosaic virus (TMV), of TMV-inoculated NN (hypersensitive) and nn (susceptible) plants of *Nicotiana tabacum* (L.) cv. Samsun was investigated. Free and conjugated polyamine concentrations, putrescine biosynthesis, evaluated through arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activities, and putrescine oxidation, via diamine oxidase (DAO) activity, were analysed during the first 24 h from inoculation. Results were compared with those of mock-inoculated control plants. In NN TMV-inoculated plants undergoing the hypersensitive response (HR), free putrescine and spermidine concentrations had increased after 5 h compared with controls; polyamine conjugates also tended to increase compared with controls. In both virus- and mock-inoculated plants, ADC and ODC activities generally increased whereas DAO activity, which was present in controls, was detectable only in traces in inoculated tissues.

In TMV-infected susceptible plants, free putrescine and spermidine concentrations were lower at 5 h relative to controls, as were polyamine conjugates. No differences were revealed in ADC and ODC activities whereas DAO activity was not detectable. These results further support the hypothesis that polyamines are involved in the response of tobacco to TMV and that, only a few hours after inoculation, the response of hypersensitive plants is distinct from that of susceptible ones.

Key words: Arginine and ornithine decarboxylases, tobacco mosaic virus, *Nicotiana tabacum* L. (tobacco), putrescine, spermidine, hypersensitive response.

INTRODUCTION

In several higher plants, especially in the Solanaceae, polyamines have been shown to form mono-substituted and di-substituted basic amides with hydroxycinnamic acids (HCAs); these have been reported to be involved in the defence mechanism in several plant–pathogen interactions (Favali *et al.*, 1997; Torrigiani *et al.*, 1997; Walters & Mackintosh, 1997). The possibility now exists of manipulating polyamine metabolism in order to control plant diseases. Increases in HCAs occurred during the hypersensitive response (HR) to tobacco mosaic virus (TMV) of *Nicotiana sylvestris* plants which bear the resistance N′ gene, grown at 29 °C, whereas at 32 °C, when flowering was inhibited and TMV infection became systemic, the accumulation of HCAs was suppressed (Martin-Tanguy, Martin & Gallet, 1973). Furthermore, the application of HCAs to leaf discs of tobacco caused a 90% reduction in the number of local lesions induced by TMV (Martin-Tanguy *et al.*, 1976). A 20-fold increase in ODC activity in tobacco leaves undergoing HR has also been reported (Negrel, Vallée & Martin, 1984). Less is known about susceptible (nn) tobacco plants in which TMV produces a systemic infection (Staskawicz *et al.*, 1995).

Torrigiani *et al.* (1997) reported that in NN tobacco cv. Samsun, concentrations of free and conjugated polyamines increased towards the centre of the HR lesion; maximum accumulation of free polyamines occurred 3 d after inoculation, whereas conjugates peaked 2 d later. These increases were accompanied by enhanced ADC and, especially, ODC activities. By contrast, in nn susceptible plants, both conjugated putrescine and spermidine decreased. These findings support the contention that polyamine conjugates are involved in the establishment of the HR and thus in virus resistance.

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Because early variations in free polyamine pattern would suggest a possible involvement of such compounds in signal transduction pathways, and because HR is considered a form of programmed cell death (PCD, Greenberg, 1997), we focused our attention on the short-term events occurring during the HR with respect to polyamines. Changes in polyamine metabolism (free and conjugated polyamine levels, putrescine biosynthesis and oxidation) during the first 24 h after virus inoculation were investigated in both hypersensitive (NN) and susceptible (nn) Samsun plants.

MATERIALS AND METHODS

Plant material and TMV inoculum

Plants of TMV-susceptible (nn) and TMV-resistant (NN) *Nicotiana tabacum* L. cv. Samsun were grown in the glasshouse with a photoperiod of 8 h (1·87 W m^{-2}) at 25 °C, up to the vegetative stage when approx. 15 leaves were present (*c.* 2 months old).

The third mature leaf (counting from the base of the stem) of each plant (5 nn and NN) was inoculated with the same viral charge (200 μl of purified TMV suspension at 0·1 mg ml^{-1} 0·01 M sodium phosphate buffer, pH 7), in order to allow comparison between the polyamine response in nn and NN plants. Control plants (5 nn and NN) were mock-inoculated by treating the third mature leaf with carborundum and 200 μl of phosphate buffer. The whole third leaf from virus-inoculated and mock-inoculated plants was collected at 0, 5, 10 and 24 h after inoculation and samples were stored at −80 °C until use.

Polyamine analysis and enzyme assays

Samples of 1·0–1·5 g were analysed for free and conjugated polyamines by homogenizing them in three volumes of cold 5 % trichloroacetic acid (TCA (w/v)) and centrifuging for 10 min at 20000 ***g***. Free polyamines and TCA-soluble conjugated polyamines in aliquots of the supernatant (0·3 ml) were hydrolysed, detected by dansyl-procedure on TLC plates and analysed by comparing with standard polyamines as previously described (Torrigiani *et al.*, 1997).

All extraction procedures for ADC and ODC were carried out in an ice bath. Samples (0·5–1·0 g) were homogenized in five volumes of 100 mM Tris-HCl, pH 8·5, containing 50 μM pyridoxalphosphate. CO_2 released from labelled-ornithine and arginine was measured following the procedure previously optimized for tobacco (Altamura *et al.*, 1993) and described by Torrigiani *et al.* (1997). The concentration of 2 mM unlabelled substrate was chosen in consideration of the K_m values reported for other higher plants (Slocum, 1991). Protein content was measured by the protein-dye binding method according to Bradford (1976), using bovine serum albumin as standard.

DAO (EC 1.4.3.6) activity was assayed by a radiometric method that measures the [^{14}C]Δ^1-pyrroline formed from [^{14}C]putrescine (Okuyama & Kobayashi, 1961). Samples were homogenized in 100 mM potassium phosphate buffer, pH 8, containing 2 mM dithiothreitol, sonicated and centrifuged at 26000 ***g*** for 30 min. Aliquots (0·2 ml) of supernatant were incubated, after a 45 min preincubation at room temperature, at 37 °C for 30 min with 7·4 kBq of [^{14}C]putrescine (4·03 GBq $mmol^{-1}$, NEN), 30 μg of catalase and 1 mM putrescine; the reaction was stopped by adding 0·2 ml of 2 % sodium carbonate and [^{14}C]pyrroline was immediately extracted in 2 ml of toluene by vortexing for 10 s. After a brief centrifugation, aliquots (0·5 ml) of the lipophilic phase were withdrawn and added to 4 ml of scintillation liquid (Ready Gel®, Beckman); the radioactivity was determined with a Beckman® LS 1800 scintillation counter.

The statistical significance of the differences between means was analysed using Student's *t*-test.

RESULTS

Hypersensitive plants

In NN mock-inoculated tobacco leaves (controls) polyamine concentrations changed little during 24 h (Fig. 1*a*). By contrast, in TMV-inoculated leaves, significant increases ($P < 0·05$) in free putrescine and spermidine titres were already observed after 5 h compared with controls; this difference was maintained later since polyamine levels in inoculated samples were always significantly ($P < 0·05$ at 10 h and $P < 0·01$ at 24 h) higher (up to 2·5-fold at 24 h when lesions were not yet visible) than in their controls (Fig. 1*a*).

Whereas in control and inoculated leaves TCA-soluble conjugated polyamines did not vary much up to 10 h, by 24 h they had reached concentrations significantly higher ($P < 0·01$; 12-fold for putrescine and 3-fold for spermidine) than in mock-inoculated tissue, and about 4-fold (putrescine) and 10-fold (spermidine) higher than at zero time (Fig. 1*b*). In both classes of polyamines (free and conjugated), putrescine reached the highest concentration at 24 h; spermine was undetectable or present in traces.

Enhanced polyamine levels in TMV-inoculated leaves, however, were not paralleled by corresponding increases in ADC or ODC activities except at 24 h (Fig. 2) when the former significantly ($P < 0·05$) differed from controls. Both in controls and TMV-inoculated leaves, ODC was higher than ADC activity at 10 and 24 h (Fig. 2). In controls, ADC activity doubled by 10 h but then returned to the

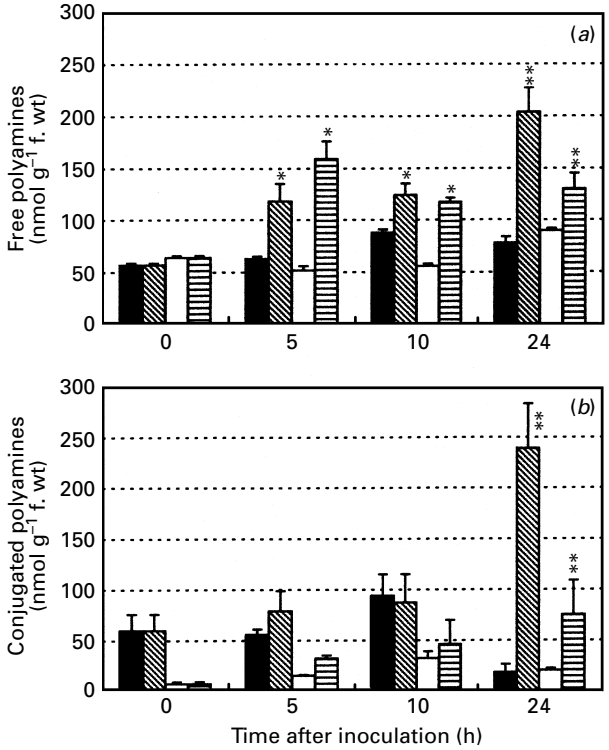

Figure 1. Pattern of free (*a*) and conjugated (*b*) putrescine (Pu, ■ ▨) and spermidine (Sd, □ ▤) in mock-inoculated (■ □) and TMV-inoculated (▨ ▤) hypersensitive (NN) tobacco plants during the first 24 h after inoculation. Bars represent SD ($n = 4$). Asterisks indicate values significantly different (* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$) from those of their respective controls.

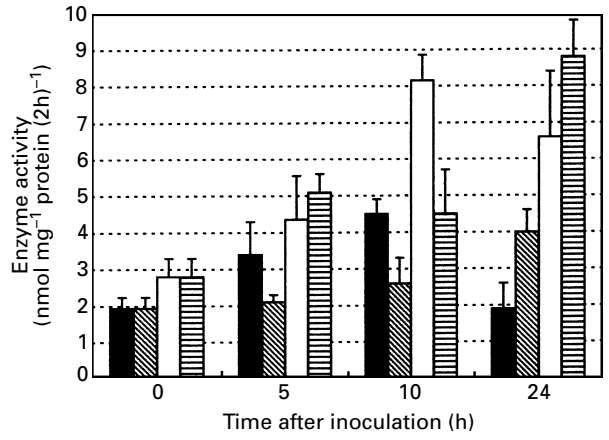

Figure 2. Pattern of arginine decarboxylase (ADC, ■ ▨) and ornithine decarboxylase (ODC, □ ▤) activities ($^{14}CO_2$ evolution) in mock-inoculated (C, ■ □) and TMV-inoculated (V, ▨ ▤) hypersensitive (NN) tobacco plants up to 24 h from inoculation. Bars represent SD ($n = 5$).

initial level while ODC activity tended to increase 3 to 4-fold compared to zero time. In inoculated samples, both enzyme activities showed increasing activity with time, especially ODC which rose about 3-fold compared with zero time.

DAO activity (measured in the presence of 1 mM unlabelled putrescine) did not vary with time in

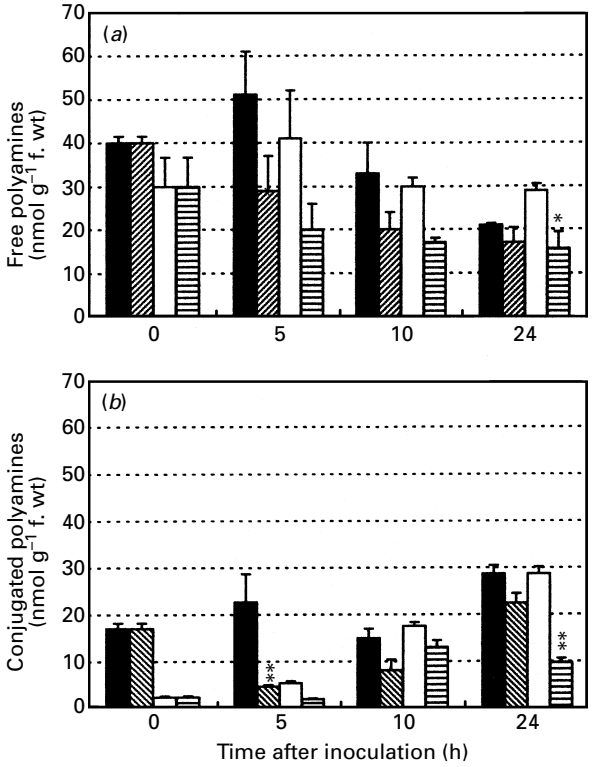

Figure 3. Pattern of free (*a*) and conjugated (*b*) putrescine (Pu, ■ ▨) and spermidine (Sd, □ ▤) in mock-inoculated (C, ■ □) and TMV-inoculated (V, ▨ ▤) susceptible (nn) tobacco plants during the first 24 h after inoculation. Bars represent SD ($n = 4$). Asterisks indicate values significantly different (* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$) from those of their respective controls.

mock-inoculated samples (zero time: 1·04, 5 h: 1·19, 10 h: 0·90, 24 h: 0·96 nmol mg^{-1} protein (30 min)$^{-1}$), whereas it was barely detectable in infected leaves.

Susceptible plants

In mock-inoculated nn plants (controls), free putrescine and spermidine concentrations tended to decrease (by up to 50% for putrescine) from 0 to 24 h (Fig. 3*a*). In infected leaves, free putrescine and spermidine followed a similar downward trend with time but their titres were always lower than in their respective controls reaching, at 24 h, values *c.* 50% of those at zero time; at that time spermidine levels were significantly ($P < 0{\cdot}05$) lower than in controls.

In control plants, concentrations of TCA-soluble conjugated putrescine and, especially, spermidine were smaller than those of free polyamines, except at 24 h (Fig. 3*b*). In infected plants at 5 h, conjugated putrescine titres were significantly lower ($P < 0{\cdot}01$) than in respective controls, whereas at 24 h the concentration of conjugated spermidine was significantly lower than in controls ($P < 0{\cdot}01$). As in NN plants, neither free nor conjugated spermine was detected.

Both ADC and ODC activities were present in susceptible leaves (Fig. 4); the latter was significantly higher ($P < 0{\cdot}01$) than the former at 0 and 5 h (about

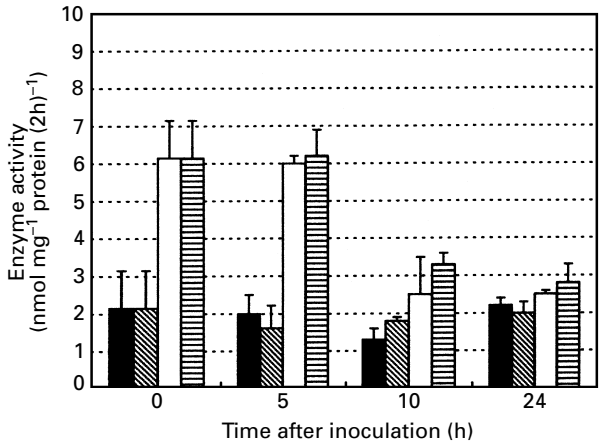

Figure 4. Pattern of arginine decarboxylase (ADC, ■ ▨) and ornithine decarboxylase (ODC, □ ▤) activities ($^{14}CO_2$ evolution) in mock-inoculated (C, ■ □) and TMV-inoculated (V, ▨ ▤) susceptible (nn) tobacco plants up to 24 h from inoculation. Bars represent SD ($n = 5$).

3-fold). Both in controls and inoculated samples, ADC activity did not change significantly during the 24 h (Fig. 4) whereas ODC activity decreased *c.* 50 % after 10–24 h compared with zero time. No significant differences in enzyme activity were observed between mock- and inoculated leaves. Only traces of DAO activity were detected in control and inoculated tissues.

DISCUSSION

We report contrasting short-term polyamine responses to TMV in hypersensitive (NN) and susceptible (nn) tobacco plants. Previous findings indicated that in the HR to TMV in NN tobacco plants polyamine concentrations increased in inoculated leaves compared with controls (Torrigiani *et al.*, 1997); in particular, a strong accumulation of free and conjugated polyamines was observed in the hypersensitive lesion.The present short-term analysis reveals increasing concentrations which are already detectable after 5 h and reach a maximum after 24 h for both free and conjugated polyamines. The latter, compared with results reported earlier (Torrigiani *et al.*, 1997), might be explained on the basis of the viral concentration used. Here, a TMV-inoculum of 0·1 mg ml^{-1} was used with the aim of inducing hypersensitive lesions more extensively. This might have enhanced polyamine accumulation thus making it possible to detect changes only 5 h after inoculation. In the previous work, free and conjugated polyamines reached total levels (putrescine plus spermidine) at 24 h of *c.* 80–90 nmol g^{-1} f. wt (Torrigiani *et al.*, 1997), but here final concentrations of *c.* 300 nmol g^{-1} f. wt were reached. It is also worth noting that a 'dilution' effect of the infected tissue (which later displays visible lesions) by healthy tissue could occur; thus, it is reasonable to suppose that local concentrations of polyamines were even greater than they appear. Indeed, polyamine accumulation appears to be an early response to TMV inoculation in NN plants and amounts seem to depend upon the extent of viral charge.

An opposite trend in polyamine accumulation was observed in nn tobacco plants inoculated with the same viral charge. Both free and conjugated polyamine titres tended to decrease, starting from 5 h after inoculation, thus supporting previous findings (Torrigiani *et al.*, 1997). Susceptible plants seem to be unable to synthesize and/or accumulate polyamines in response to TMV infection, at least within the first 24 h.

The different patterns of polyamine accumulation in hypersensitive plants compared with the susceptible ones cannot be explained on the basis of the respective putrescine biosynthetic activities which do not substantially change compared with controls. It is evident that at 24 h much higher polyamine titres (one order of magnitude) are accumulated in inoculated NN plants than in nn plants, in spite of the fact that biosynthetic activities are similar. Interestingly, in NN control plants the pattern of ADC activity, but not that of ODC, correlates with that of free and conjugated putrescine accumulation; in inoculated NN tissues, by contrast, it is ODC activity that correlates with the free and conjugate accumulation pattern. This lends support to the idea (Burtin *et al.*, 1989), that ODC activity is responsible for the putrescine utilized in conjugate synthesis. In NN plants putrescine biosynthesis (ADC plus ODC activities) tends to increase with time, the opposite occurs in nn plants further indicating, in the latter, a *genetic* tendency to synthesize less polyamines.

Because the viral concentration in the inoculated leaf after 24 h is practically that of the viral inoculum (Cheo, 1971), the observed decrease in free polyamine titres in nn plants cannot be explained on the basis of the number of viral intrinsic polyamine molecules, 19 per each viral particle (Rabiti *et al.*, 1994; Torrigiani *et al.*, 1995).

Moreover, ADC activity might not be directly related to putrescine accumulation since it has been reported that transgenic tobacco plants over-expressing oat ADC accumulate agmatine (Burtin & Michael, 1997), which is the first product of arginine decarboxylation, instead of putrescine (Masgrau *et al.*, 1997).

Present data suggest that a decrease in putrescine oxidizing activity (DAO) could account for polyamine accumulation. This hypothesis needs to be further, and extensively, verified although a role for the cell wall localized DAO activity in defence mechanisms has been already reported in a chickpea cv. resistant to fungal infections (Angelini *et al.*, 1993).

Little is known about putrescine- and spermidine cinnamoyl-transferases which are responsible for

conjugate synthesis; they have been described in tobacco under different *in vitro* culture conditions (Meurer-Grimes, Berlin & Strack, 1989; Negrel, Javelle & Paynot, 1991). An effect on such enzymes could result from the gene-for-gene interaction in NN plants, leading to an enhancement of their activity, which would not occur in nn plants.

In conclusion, it is reasonable to assume that: (i) the differences in ADC/ODC activities could not be detected due to the 'dilution' effect by healthy tissue; (ii) differences in polyamine accumulation could result from differential DAO- or transferase activities; (iii) changes in S-adenosylmethionine decarboxylase activity could also be involved and be responsible for polyamine accumulation through the synthesis of spermidine and subsequently of putrescine, via the acetylation pathway or other, as yet unknown, reverse pathways (Torrigiani *et al*., 1993; Del Duca, Beninati & Serafini-Fracassini, 1995).

Finally, present results are relevant to the understanding of programmed cell death (PCD) in plant–pathogen interactions. In animal cells overexpression of ODC and consequent spermidine accumulation, as well as exogenous spermidine supply, are reported to induce apoptosis which is a particular form of PCD (Poulin, Pelletier & Pegg, 1995). Tissues undergoing HR exhibit several features typical of PCD (Mittler & Lam, 1996) but little is known about the involvement of polyamines in this process in plants. Present data, together with a few earlier reports, suggest that in plants activation of polyamine metabolism could also have a role in triggering PCD.

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