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Phosphodiesterase activities in transgenic tobacco plants associated with the movement protein of tobacco mosaic virus

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Summary. Hydrolytic activities of leaf extracts from normal and transgenic plants, with (+MP) and without (−MP) the movement protein of tobacco mosaic virus, were examined. In the +MP transgenic plants, as compared with non-transgenic and −MP plants, higher hydrolytic activities were found on the following substrates: bis-(nitrophenyl)-phosphate (BPNPP, phosphodiesterase), p-nitrophenyl-(phenyl)-phosphate (PNPPP, nucleotide-phosphodiesterase) and thymidine-3′-monophosphate p-nitrophenyl ester (T_3MPP; 3′nucleotide phosphodiesterase.) The +MP plant lines, as compared with other transgenic plants, exhibited higher nucleotide-phospho-diesterase activity in the soluble as well as in the membrane fraction. Substrate concentration kinetic studies revealed the presence of a nucleotide-phospho-diesterase with a high substrate affinity in the +MP extracts in addition to the enzyme with a relatively low substrate affinity present also in the −MP transgenic plants. This "high affinity" enzyme could be removed from the soluble fraction by precipitation with anti-MP serum, indicating its possible association with the movement protein.

Key words: Movement protein – Phosphodiesterase – Transgenic plants

Introduction

During infection, plant viruses move from cell to cell through plasmodesmata, which are intracellular connection channels in plant tissue (Hull 1989). In infections by tobacco mosaic virus (TMV) this transfer ability is facilitated by the movement protein (MP), which is a product of the TMV-encoded I2-RNA during early stages of infection (Hunter et al. 1976; Watanabe et al. 1984; Hull 1989). The TMV-MP has been found to be localized to plasmodesmata in TMV-infected plants (Tomenius et al. 1987) as well as in plants transgenic for the MP (Atkins et al. 1991).

The TMV-MP alters the molecular size-exclusion limits of plasmodesmata in transgenic plants that express the MP gene (Wolf et al. 1989), increasing it by more than ten-fold as compared with normal plants. This result suggests that there is some type of process that enzymatically reduces the effectiveness of the plasmodesmata structural barrier. The changes in size-exclusion limits of the plasmodesmata correlate with the accumulation of MP (Doem et al. 1990). Thus, the effect of MP may be direct, as proposed by Citovsky et al. (1990), or it may exert hydrolytic activities on the plasmodesmata structure either by its own activity or by inducing host-cell hydrolytic functions.

In the present study an attempt was made to compare some hydrolytic activities in leaves of transgenic tobacco plants that express the MP gene with those which have the intermediate plasmid but lack the MP gene.

Materials and methods

Plants

The following transgenic plant lines were grown in the greenhouse as described (Doem et al. 1990) and the leaves were used for experiments: lines 274 and 277, which express the MP gene of TMV (274 harbors a single gene copy; C. M. Doem, personal communication), 306 H which harbors the intermediate plasmid, without the MP gene, line 024 which expresses the GUS

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gene [*Nicotiana tabaccum* cv. Xanthi transformed with the plasmid MON530 expressing the β-glucuronidase (*GUS*) gene, (T. Fujiwara and R. N. Beachy, unpublished)], and line 3646 which expresses the TMV coat protein (Powell-Abel et al. 1986).

Enzyme extracts and protein fractionation

Leaves from tobacco plants grown under identical conditions were pulverized under liquid nitrogen. The powder was ground with a mortar and pestle on ice in the presence of nonsoluble, polyvinyl-polypyrrolidone (PVP, 0.5 g/g leaves). To the paste, Tris-HCl buffer, 100 mM, pH 7.5, was added (5 ml/g leaves) and ground on ice. After centrifugation (10,000 *g*, 15 min), the supernatant was used for enzyme activity assays. For the preparation of the membrane fraction, leaves were treated as described above but soluble PVP was used and the pellet was washed twice with 2% Triton × 100 and twice with the buffer, then resuspended in the original volume of buffer and used for further examination.

The supernatant proteins were subjected to ammonium sulfate fractionation by adding appropriate volumes of a cold, saturated solution of the salt (pH 7.5) in the Tris-HCl buffer, followed by centrifugation. The pellets were resuspended in 1/4 of the original crude extract volume of the buffer and dialyzed against the same buffer.

Examination of enzyme activities

The following substrates were used for the respective enzyme activities: (1) Alkaline and acid phosphatase, p-nitrophenyl phosphate (PNPP). (2) Phosphodiesterase, Bis (p-nitrophenyl) phosphate (BisPNPP). (3) Nucleotide phosphodiesterase, p-nitro-phenyl-phenyl-phosphonate (PNPPP). (4) Nucleotide phosphodiesterase-5′-specific, thymidine 5′-monophosphate p-nitrophenylester (T_5MPP). (5) Nucleotide phosphodiesterase-3′specific, thymidine 3′-monophosphate p-nitrophenylester (T_3MPP). All materials were purchased from Sigma.

Routinely a final volume of 0.5 ml the following compounds were added: 100 mM Tris-HCl pH 7.5, (for alkaline phosphatase, pH 9.0, and for acid phosphatase citrate buffer, pH 5.0): 5 mM substrate, (dissolved in buffer or ethanol, 70%); 20 mM $MgSO_4$, and 0.1 ml of the enzyme source. After incubation at 37 °C, 0.1 ml was removed at various time intervals and mixed with 2 ml NaOH 0.1 N and absorbance was measured at 400 nm. An extinction coefficient of 18 O.D. for 1 mM was used for calculations. A control for non-enzymatic decomposition of the substrate was run for each experiment. Protein concentration was determined with the Pierce BCA protein assay reagents.

Antigen-antibody precipitation

One milliliter of enzyme source (crude or ammonium sulfate-precipitated fractions) was mixed with 2.5 µl of a rabbit antibody to TMV-MP and 100 µl of protein-A-Sepharose (CL-413, Sigma), incubated on ice with occasional mixing for 1 h. After centrifugation at 15,000 *g* for 20 min, the supernanant was removed and the pellet was resuspended in the original amount of buffer. Both fractions were used for enzyme activities. All experiments were repeated at least three times but for some of them only data of one experiment are presented (see Table 2).

Results

Table 1 describes the activities of the enzyme preparations on PNPP (acid), which represents the acid-phosphatase activity, and on BisPNPP and PNPPP, which represent phosphodiesterase and nucleotide-phosphodiesterase, respectively.

The comparison of extracts from non-transgenic plants with those from transgenic plants which lack the movement protein (−MP), and with transgenic plants which express the MP gene (+MP), reveals that there is a significant increase in each of the examined enzymes caused by transgenicity per se. This increase occurs in soluble, as well as in pellet-bound, activities (Table 1).

Both −MP and +MP transgenic plants harbor a kanamycin-resistance gene (as a marker), which is expressed by a neomycin phosphotransferase (NPTII, Umezawa et al. 1967). This enzyme phosphorylates a variety of aminoglycosides (Davies et al. 1971). Phosphorylation (inactivation?) of such intracellular pre-existing compounds may trigger a biochemical response which is expressed by the dramatic increase in the acid-phosphatase activity.

The main differences between the two types of transgenic plants, i.e., −MP and +MP, were noted as an increase in the two phosphodiesterases, but not in acid phosphatase, in the +MP extracts. The acid-phosphatase activity values fluctuated in various experiments and no consistent differences could be demonstrated between −MP and +MP plant extracts (data not shown).

Table 1. Hydrolytic activities (release of p-nitrophenyl: µmol h^{-1} mg^{-1} of protein) of fractions extracted from leaves of normal, −MP, and +MP lines of tobacco plants on PNPP, BisPNPP and PNPPP

Substrate	Supernatant			Pellet[a]			Pellet[b]		
	Control[d]	−MP	+MP	Control	−MP	+MP	Control	−MP	+MP
PNPP (acid)	1.2	9.20	5.80	0.20	1.2	0.8	0.3	2.20	2.4
BisPNPP	0.27	0.88	1.08	0.12	0.4	1.4	ND[c]	0.11	1.6
PNPPP	0.42	0.66	1.04	0.18	0.28	1.2	0.2	0.40	0.8

[a] First pellet after centrifugation at 10,000 *g* for 15 min
[b] Pellet washed with Triton × 100 and buffer
[c] Not detectable
[d] Non-transgenic plants
For details, see Materials and methods

Table 2. Phosphodiesterase activities in leaf extracts of various transgenic plants. Leaves of various ages were pulverized in liquid nitrogen, extracted with buffer and centrifuged at 10,000 *g* for 15 min to yield supernatant (sup) and pellet fractions. The pellet was resuspended in the original volume of the buffer and both fractions were examined for hydrolytic activities on BisPNPP and PNPPP. For details see Materials and methods

Substrate	Age of leaf[a] and cell fraction	μmol h^{-1} mg^{-1} protein of nitrophenol (SD)					
		Plant line		+Coat protein[b]	−MP	+Low MP[b]	+MP
		Control	+GUS[b]				
BisPNPP	Young sup	1.4 (0.4)	1.1	1.3	1.2 (0.15)	2.4	2.8 (0.2)
	pellet	1.6 (0.2)	1.3	1.8	0.60 (0.3)	5.0	3.0 (0.8)
	Old sup	0.26 (0.01)	0.32	0.25	0.85 (0.03)	0.87	1.15 (0.13)
	pellet	0.21 (0.12)	0.35	0.29	0.44 (0.34)	0.44	1.15 (0.36)
PNPPP	Young sup	0.23 (0.2)	ND	ND	0.75 (0.06)	1.8	1.9 (0.7)
	pellet	0.18 (0.1)	ND	ND	0.40 (0.08)	0.7	1.2 (0.05)
	Old sup	0.44 (0.02)	0.62	0.5	0.65 (0.05)	0.94	1.04 (0.08)
	pellet	0.20 (0)	0.20	0.13	0.38 (0.07)	0.88	1.07 (0.06)

[a] "Young" = leaf #1 and "Old" = leaf #6 (see Doem et al. 1990)
[b] Results of one experiment – others are averages of three experiments

We have found that there may be quantitative differences in the activities of the enzymes tested in extracts from leaves of different ages (data not shown). To this end, plants were grown in parallel and equivalent leaves from −MP and +MP plants were used for comparative studies. As presented in Table 2, data derived from experiments which compare some of the hydrolytic activities in transgenic plants harboring various introduced genes indicate that the increased nucleotide phosphodiesterase activity is associated with the presence of the MP, particularly in young leaves.

The significance of these results was further examined after treating the extracts with anti-MP antibody and precipitating antibody:antigen complexes with protein A. Table 3 demonstrates a significant loss of activity in the soluble fraction due to the precipitation process, particularly of nucleotide-phosphodiesterase (PNPPP) in the +MP plant extracts.

To eliminate possible effects on enzyme activities by compounds present in the soluble fraction of the crude extracts, the proteins were fractionated by ammonium sulfate and the active fractions (60–100% saturation) were subjected to MP-antibody precipitation. The soluble and pellet fractions were then examined for enzyme activities. Table 4 shows that after antibody:antigen precipitation the +MP plant extracts lose approximately 30% of the nucleotide phosphodiesterase activity in the supernatant while the −MP plants lose only approximately 10%. The activities could not be recovered in the pellet fraction.

Another approach to distinguish between the enzyme activities that are associated with the MP and those that are not was to determine enzyme affinities to various substrates by varying the concentration of the substrate.

Table 3. The effect of anti-MP antibodies on hydrolytic activities of extracts from leaves. To 0.5 ml of supernatants of extracts from leaves of −MP and +MP plants, 25 μl of MP-antibodies and 100 μl of Protein-A-agarose were added and incubated on ice for 60 min with occasional mixing. The suspensions were centrifuged at 15,000 *g*, for 15 min. The supernatant was kept and the precipitate was resuspended in the original volume (0.5 ml). The original leaf extracts, the supernatants (sup) and the resuspended precipitates (0.1 ml) were examined for hydrolytic activities on BisPNPP and PNPPP as described in the Materials and methods

Enzyme source	μmol/h per assay		% of original extract	
	BisPNPP	PNPPP	BisPNPP	PNPPP
+MP	–	0.210	–	100
+MP	0.720	0.280	100	100
−MP+antibody: sup	–	0.190	–	90
+MP+antibody: sup	0.580	0.170	81	61
−MP+antibody: pellet	–	0.025	–	12
+MP+antibody: pellet	0.045	0.060	6	25

Figure 1 depicts a typical biphasic Lineweaver-Burk curve which indicates the presence of a low-affinity enzyme in both +MP (A) and −MP (B) plant extracts, and of a high-affinity enzyme which seems to be present only in +MP extracts. The apparent Km values for soluble enzyme fractions, and for Triton washed pellets from +MP and −MP plants using three substrates, are listed in the legend of the figure. None of the extracts examined exhibited any measurable activity on thymidine-5′-p-nitrophenyl phosphate (5′-specific nucleotide phosphodi-

Table 4. The effect of antibody precipitation on hydrolytic activities in ammonium sulfate precipitates (ASP). To a protein fraction, obtained after adding ammonium sulfate between 60% and saturation, TMV-antibodies and protein-A were added. After fractionating, the hydrolytic activities on BisPNPP, PNPPP and T_3MPP were examined. Reactants as described in Table 3. No activities were found in the antibody precipitates

Enzyme source	Plant line	BisPNPP		PNPPP		T_3MPP	
		O.D.$_{400}$/h	%	O.D.$_{400}$/h	%	O.D.$_{400}$/h	%
Ammonium sulfate	−MP	0.420	100	0.400	100	0.240	100
Precipitate (ASP)	+MP	0.370	100	0.360	100	0.210	100
ASP + antibody	−MP	0.350	84	0.360	90	0.240	100
Supernatant	+MP	0.260	70	0.240	68	0.150	71

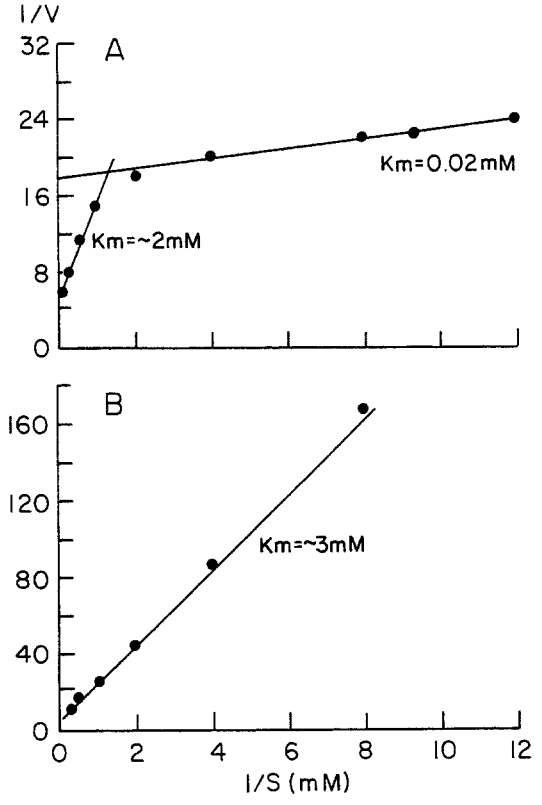

Fig. 1 A, B. A Lineweaver-Burk plot for the hydrolytic activities of supernatant extracts from +MP (**A**) and −MP (**B**) plants at various concentrations of T_3MPP. Reaction mixtures, as described under Materials and methods, were incubated with various concentrations of T_3MPP at 37 °C for 90 min. V = O.D.$_{400} \times 10^{-3}$/h. The range of Km values in the supernatant were for BisPNPP, 0.9–1.0 and 0.66–0.7 (0.05–0.07) mM; for PNPPP, 0.1–0.2 and 0.12–0.17 (0.02–0.04) mM, and for T_3MPP, 3.0 and 2.0–3.0 (0.02–0.06) mM, in −MP and +MP respectively. In the pellets the Km values were for BisPNPP, 0.5–1.0 and 0.5–0.7 mM; for PNPPP, 0.4–0.5 and 0.2–0.5 mM; for T_3MPP, 1.0–2.0 and 2.0–4.0 mM, in −MP and +MP respectively

esterase). The high affinity enzyme was present only in the soluble fraction of the +MP plants (see Fig. 1). The Km values of the high affinity (low Km) enzyme in extracts from both plants for each substrate appear to be similar, regardless of the differences in the Km values of the low affinity enzyme for the same substrates. A Lineweaver-Burk plot for the soluble and pellet fractions of the antibody-treated enzyme from a +MP plant extract (on PNPPP) revealed that the high affinity enzyme activity disappeared from the soluble as well as from the antibody-bound pellet. The low affinity Km value in this pellet increased approximately 20-fold (from 0.15 to 3.0 mM; data not shown), indicating a possible disturbed interaction between the pelleted enzyme and substrate, probably caused by the enzyme link to the antibody and protein-A-agarose.

Discussion

The movement protein appears in the soluble fraction in leaf extracts at the early stage of plant infection and is localized at later stages in the cell wall and membrane fractions. In transgenic plants that express the MP gene the plasmodesmata undergo a change that results in an increase in size-exclusion limits (Wolf et al. 1989; Doem et al. 1990) perhaps as a result of enzyme activities, particularly hydrolytic ones. In preliminary experiments we have examined hydrolytic activities of various plant extracts on the following substrates; N-CBZ-glycine-PNP-ester and N-CBZ-L-tyrosine-PNP-ester (protease-type activity); PNP-sulfate (sulfatase-type activity) and PNP-N-acetyl-β-D-glucosaminide (glucanase-type activity). No significant differences of hydrolytic activities on these substrates were evident between −MP and +MP plant extracts (data not shown).

The findings demonstrated that, beyond the significant increase in hydrolytic activities caused by the overall transgenic process, there are distinctive hydrolytic activities associated with the presence of the MP. The enzyme(s) seem(s) to hydrolyze phospho-diester bonds and exert(s) a noticeable specificity toward nucleotide-phospho-diesters. The enzyme is inactive on the 5′-nucleotide-like substrate and active on 3′-nucleotide-like substrate (T3′MPP), pointing to an RNase or a nucleotide-cyclase type activity. The low affinity for T_3MPP, as compared with the affinity for the non-specific nucleotide phosphodiesterase substrate (PNPPP), indicates a nucleotide

specificity other than thymidine-3′-monophosphate. It is of interest to mention recent data concerning the possible role of cyclic-AMP in the transport of TMV in tobacco plants (Atabekov et al. 1989; Atabekov and Taliansky 1990). These authors have shown that exogenous dibutyryl c-AMP and papaverin (increasing the c-AMP levels in animal cells) rescue the transport of the temperature-sensitive mutant, LS1, from cell to cell at non-permissive temperatures. The nucleotide-phosphodiesterase may hydrolyse cAMP specifically or non-specifically causing an impairment of membrane-linked activities and thereby altering the function of the cell-to-cell channels. We were unable to demonstrate whether the MP is active per se or if its presence enhances this particular hydrolytic activity. The precipitation of a substantial portion of this enzyme by the MP-specific antibody indicates a structural relationship between the enzyme and the MP. Moreover, the presence of this activity in the tightly membrane-bound fraction points to the possible association of the activity with the MP, which is also located in this fraction. The lack of the high substrate affinity enzyme (low Km values) in the membrane fraction may result from the integration of the enzyme into the membrane, similar to the phenomenon found for the enzyme bound to Protein-A-sepharose (see above).

The nucleotide-diesterase activity seems to be associated with the presence of the MP as demonstrated by its presence in +MP transgenic plants as well as by its interaction with the MP-specific antibody.

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