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Short Communication

Dysfunctionality of a tobacco mosaic virus movement protein mutant mimicking threonine 104 phosphorylation

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Replication of tobacco mosaic virus (TMV) is connected with endoplasmic reticulum (ER)-associated membranes at early stages of infection. This study reports that TMV movement protein (MP)-specific protein kinases (PKs) associated with the ER of tobacco were capable of phosphorylating Thr^{104} in TMV MP. The MP-specific PKs with apparent molecular masses of about 45–50 kDa and 38 kDa were revealed by gel PK assays. Two types of mutations were introduced in TMV MP gene of wild-type TMV U1 genome to substitute Thr^{104} by neutral Ala or by negatively charged Asp. Mutation of Thr^{104} to Ala did not affect the size of necrotic lesions induced by the mutant virus in *Nicotiana tabacum* Xanthi nc. plants. Conversely, mutation of Thr to Asp mimicking Thr^{104} phosphorylation strongly inhibited cell-to-cell movement. The possible role of Thr^{104} phosphorylation in TMV MP function is discussed.

Cell-to-cell movement of tobacco mosaic virus (TMV) is mediated by a 30 kDa movement protein (MP) encoded by TMV RNA (reviewed by Carrington *et al.*, 1996; Lazarowitz & Beachy, 1999; Tzfira *et al.*, 2000). It has been shown that P30 accumulates in plasmodesmata of TMV-infected and MP-transgenic plants (Tomenius *et al.*, 1987; Atkins *et al.*, 1991; Oparka *et al.*, 1997; Heinlein *et al.*, 1998) to increase their permeability (Wolf *et al.*, 1991; Ding *et al.*, 1992). The TMV MP co-aligns with microtubules (McLean *et al.*, 1995; Heinlein *et al.*, 1995) and is tightly associated with ER-derived membranes of infected cells (Reichel & Beachy, 1998; Heinlein *et al.*, 1998). Furthermore, it has been reported that ER-enriched fractions from infected tobacco leaves contain TMV MP, RNA and replicase, implying that virus replication and protein synthesis take place in this compartment (Mas & Beachy, 1999). Importantly, it is known that P30 accumulates in the cell wall (CW) fraction of transgenic plants as a phosphoprotein (Citovsky *et al.*, 1993; Waigmann *et al.*, 2000) and that the CW-associated protein kinase(s) (PKs) can use MP as substrate. C-proximal residues Ser^{258}, Thr^{261} and Ser^{265} have been identified as phosphorylation sites *in vitro* (Citovsky *et al.*, 1993) and *in vivo* (Waigmann *et al.*, 2000). TMV encoding a mutant MP mimicking phosphorylation of these sites by negatively charged Asp substitution is unable to move from cell to cell in *Nicotiana tabacum* plants (Waigmann *et al.*, 2000). It has been proposed that C-terminal phosphorylation of TMV MP abolishes its ability to promote virus spread (Waigmann *et al.*, 2000).

Watanabe *et al.* (1992) reported that C-terminal residues 234–261 are required for TMV MP phosphorylation in tobacco protoplasts, although it is clear that in TMV-infected protoplasts MP can be phosphorylated at multiple internal phosphorylation sites (Haley *et al.*, 1995). In particular, two distinct domains (residues 61–114 and 212–231) can be substrates for PK(s) other than the CW-associated PK(s) mentioned above. Similarly, it has been shown that Ser^{37} and Ser^{238} of tomato mosaic tobamovirus (ToMV) MP can be phosphorylated in protoplasts. The presence of Ser at position 37 or phosphorylation of Ser^{37} is important for ToMV MP functionality (Kawakami *et al.*, 1999). Thus, the results from different groups of workers

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imply that TMV MP is phosphorylated *in vivo*, although it is hard to say in what cellular compartments MP phosphorylation occurs. It is important to emphasize that only the C-proximal sites of CW-associated TMV MP are found phosphorylated *in planta* (Waigmann *et al.*, 2000), while multiple internal sites of MP could be phosphorylated in infected protoplasts (Haley *et al.*, 1995; Kawakami *et al.*, 1999). Despite the apparent contradiction, the various results are not necessarily incompatible. Thus, TMV MP might be transiently phosphorylated when subjected to processes of phosphorylation/dephosphorylation at its internal sites by cytoplasmic PK(s), whereas only the C-proximal sites are selectively phosphorylated by CW-associated PKs.

The TMV genome is accepted widely to be translocated from cell to cell as an MP–RNA complex. Moreover, it has been reported that TMV MP is an efficient repressor of *in vitro* translation and phosphorylation of MP prevents its translation-repressing ability (Karpova *et al.*, 1999). Possible roles of viral MP phosphorylation in regulation of TMV genome expression have been discussed recently by Lee & Lucas (2001).

Microsomal fractions from leaves of *N. tabacum* var. Samsun were isolated by sucrose gradient centrifugation, as described by Mas & Beachy (1999), and analysed for PK activity using preparations of bacterially expressed TMV U1 $(His)_6$-MP as a substrate for labelling in the presence of [γ-^{32}P]ATP. Purification of $(His)_6$-MP was carried out as described by Karpova *et al.* (1997). The level of MP phosphorylation activity varied along the sucrose gradient with maximums in fractions 2–4 and 12–15 (Fig. 1a). The presence of endoplasmic reticulum (ER) lumenal-binding protein (BiP), an ER membrane resident protein (Reichel & Beachy, 1998), was revealed in fractions 2 and 11–13 by Western blotting (Fig. 1b). These data indicate that MP-specific PK activity was at a maximum in ER-containing fractions of the sucrose density gradient (Fig. 1a, b). However, there was no BiP in fractions 4 and 15 that phosphorylate the MP. Thus, the PK(s) that phosphorylate the MP may not reside exclusively in the ER.

The influence of different divalent metal cations on the MP-specific ER-associated PK activity was examined in a series of experiments. Mg^{2+} and particularly, Mn^{2+} were found to stimulate MP phosphorylation, whereas no stimulation was detected in the presence of Ca^{2+} (Fig. 1c). There was no PK activity when no metal cation was added to the reaction. The number and molecular masses of the MP-specific PKs in ER-associated fractions were determined by gel PK assays (Zhang & Klessig, 1997). The recombinant TMV MP and myelin basic protein (MBP), a universal substrate for mitogene-activated PKs, were compared using the assay. MP or MBP were co-polymerized with acrylamide and 20 μl of each fraction was loaded on a gel. Two or three major bands, with a

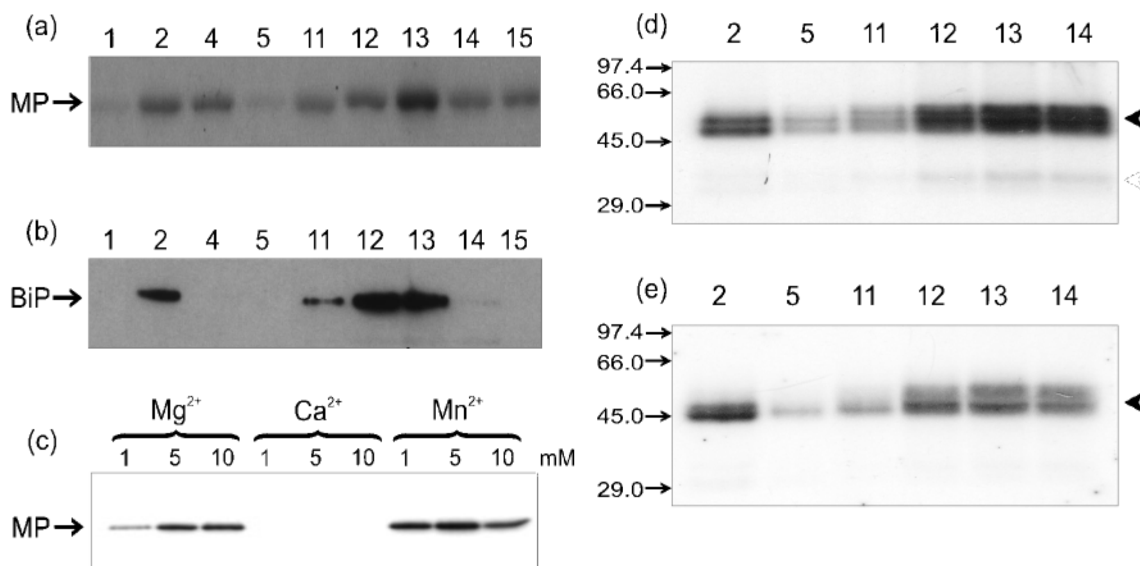

Fig. 1. Characterization of MP-specific ER-associated PK activity. (a) PAGE of TMV MP phosphorylated *in vitro* by ER-enriched sucrose gradient fractions. Identification of the MP band was performed in a series of separate Western blot assays. (b) Western blot analysis of BiP in the same fractions. Lane numbers correspond to the number of the sucrose gradient fraction. (c) Electrophoresis of TMV MP phosphorylated *in vitro* by a sucrose gradient fraction with high PK activity in the presence of divalent cations. Cation concentrations (mM) are indicated over the lanes. (d, e) Gel PK assays with recombinant TMV MP (d) and MBP (e) as substrates. Lane numbers correspond to sucrose density gradient fractions. Positions of TMV MP (a, c), BiP (b) and standard marker proteins (in kDa) (d) are indicated. The closed arrowhead indicates the position of kinases larger than 45 kDa; the open arrowhead denotes a minor component of 38 kDa.

molecular mass somewhat higher than 45 kDa, were revealed by this approach in ER-associated fractions when TMV MP (Fig. 1d) or MBP (Fig. 1e) was used as a substrate. It is possible that the multiple bands revealed in the ER-containing fractions by gel PK assay represent different isoforms or degradation products of MP/MBP-specific PK(s). The similarity of molecular masses (45–50 kDa) of the ER-associated MP-specific PKs described above and of mitogen-activated protein kinases Ntf4 and Ntf6 (Wilson *et al.*, 1995) might reflect a relationship. Remarkably, the activity of PKs described in this study and that of Ntf4 and Ntf6 PKs was stimulated by Mn^{2+} and Mg^{2+}. A lower molecular mass minor component (molecular mass of 38 kDa) was revealed as a minor band in the gel PK assays (Fig. 1d, open arrowhead). Matsushita *et al.* (2000) report that the cytoplasmic 38 kDa plant casein kinase II (CKII) is capable of phosphorylating ToMV MP and it is possible that the minor 38 kDa component detected by gel PK assay (Fig. 1d) represents CKII. Radioactive bands were not observed when the control samples (no exogenous protein added as a substrate) were analysed.

To localize the sites phosphorylated by the ER-associated PK activity in TMV MP, $(His)_6$-MP was phosphorylated *in vitro* in the presence of a microsomal fraction, [γ-^{32}P] ATP and 1 mM $MnCl_2$. After additional purification of phosphorylated protein on Ni–NTA resin in the presence of 6 M guanidium/HCl, pH 8·0 (Qiagen), according to the manufacturer's protocol, and immobilization on thiopropyl–Sepharose 6B (Sigma), the MP was digested by sequence-grade trypsin (Sigma) and the resulting peptides were analysed by two different approaches. First, two-dimensional peptide mapping was applied to separate the phosphopeptides (Fig. 2a). Analyses were performed on HTLE-7002 equipment in accordance with the manufacturer's protocol (CBS Scientific). Several ^{32}P-labelled tryptic peptides could be seen in the phosphopeptide map (Fig. 2a), which is consistent with TMV MP being phosphorylated by ER-associated PK at multiple sites. The five most prominent spots (Fig. 2a, numbers 1–5), corresponding presumably to major ^{32}P-labelled tryptic peptides, were subjected to phospho-amino acid analysis; it was demonstrated that ^{32}P was incorporated into spots corresponding to phosphoserine in peptides 2–5 and to phosphothreonine in peptide 1 (data not shown). Second, the ^{32}P-labelled phosphopeptides were separated by HPLC and isolated in sufficient amounts for partial amino acid sequencing (first five amino acids were analysed in each peptide). In the present work, we have focused on characterization of peptide 1, which was found to contain Thr^{104} in the N-terminal sequence ADEAT. Indirect evidence for the importance of Thr^{104} in TMV MP activity was provided by analyses of functional reversions of Thr^{104} dysfunctional mutants (Deom & He, 1997; Boyko *et al.*, 2002). It is also noteworthy that Thr^{104} is conserved in MPs encoded by different ToMV (Koonin *et al.*, 1991).

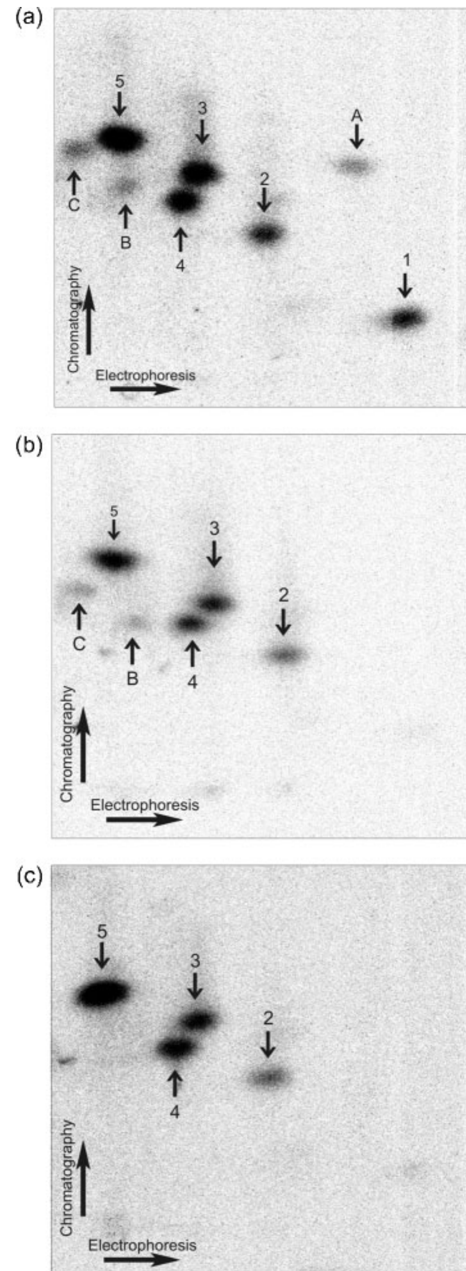

Fig. 2. Two-dimensional mapping of ^{32}P-labelled tryptic peptides of wild-type and mutant TMV MPs phosphorylated by ER-associated PK activity. (a) Wild-type MP, (b) T^{104}A and (c) T^{104}D mutant MPs. The five most prominent spots are indicated by numbers and the remaining minor spots by letters. The directions of electrophoresis and chromatography are indicated by arrows at the bottom left-hand corner.

To study the importance of Thr^{104} for function, point mutations were introduced into the recombinant $(His)_6$-MP gene to replace Thr^{104} in bacterially expressed MPs with (i) alanine, which prevents phosphorylation, or (ii) aspartate, which is believed to mimic protein phosphorylation (Waigmann *et al.*, 2000). The two mutant MP forms obtained were designated as T^{104}A and T^{104}D, respectively.

To examine the phosphorylation patterns of mutated MPs, the preparations of bacterially expressed recombinant $T^{104}A$ and $T^{104}D$ proteins were phosphorylated as described above and analysed by two-dimensional peptide mapping. Fig. 2(b, c) show that substitution of Thr^{104} by either of the amino acids Ala or Asp led to the disappearance of

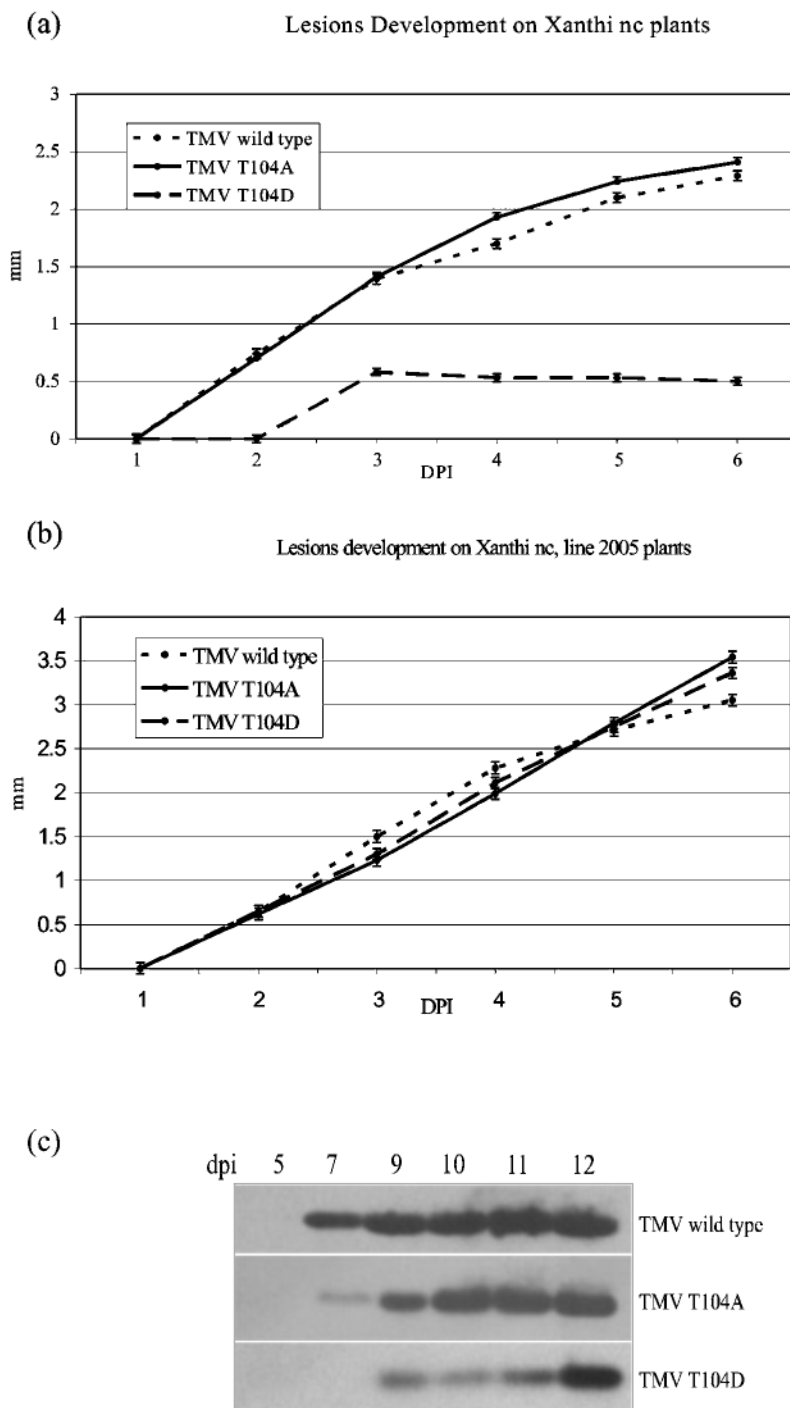

Fig. 3. Dysfunctionality of TMV MP caused by substitution of Thr^{104} by Asp ($T^{104}D$ TMV) is complemented in MP-transgenic plants. Development of local lesions induced by wild-type, $T^{104}D$ and $T^{104}A$ TMV MP mutants on leaves of (a) *N. tabacum* cv. Xanthi nc. and (b) MP-transgenic *N. tabacum* cv. Xanthi nc., line 2005, plants. Mean values (with SE bars) of local lesions diameter (mm) calculated for not less than 50 local lesions are presented. (c) Western blot analysis of TMV coat protein from the upper leaves of *N. tabacum* var. Samsun plants infected with wild-type TMV and MP mutants $T^{104}D$ and $T^{104}A$. Days post-inoculation (DPI) are indicated above the lanes.

one major and one minor spot (Fig. 2a, labelled 1 and A) from the phosphopeptide map. The major spots, 2–5 (Fig. 2), were still present in the phosphopeptide maps of the mutant MPs. These results indicate that (i) Thr^{104} can be phosphorylated *in vitro* by ER-associated PK(s) in wild-type MP and (ii) the level of phosphorylation of phosphopeptides 2–5 was not decreased by replacement of Thr^{104} with either of the amino acids. It is noteworthy that two minor spots (Fig. 2a, b, labelled B and C) observed in phosphopeptide maps of wild-type and T^{104}A MP were missing from phosphorylated T^{104}D MP (Fig. 2c). One can speculate that substitution of Thr^{104} by Asp may change the MP conformation so that these two sites are not exposed to phosphorylation. Finally, the ER-associated PKs responsible for T^{104}A, T^{104}D and wild-type MP phosphorylation were examined by gel PK assay. The number and apparent molecular masses of PKs revealed were similar in the experiments when wild-type and mutant MPs were used as substrate (data not shown). Therefore, no particular ER-associated PK was responsible for the Thr^{104} phosphorylation only.

To elucidate the functional importance of Thr^{104} for TMV cell-to-cell movement, mutations were introduced into the MP gene of a full-length TMV U1 cDNA copy to substitute Thr^{104} by Ala or Asp in modified MP. The mutant viruses referred to as TMV T^{104}A and TMV T^{104}D, respectively, were compared by inoculation of indicator plants reacting to TMV infection by production of local lesions (*N. tabacum* cv. Xanthi nc.) or systemic symptoms (*N. tabacum* var. Samsun, *N. benthamiana*). Opposite halves of the same leaf were inoculated and mean values for at least 10 inoculated leaves were compared. The specific infectivity levels (number of the local lesions produced by 1·5 μg RNA on Xanthi nc. leaves) of wild-type and T^{104}A transcripts were very similar, as was the size of lesions produced by T^{104}A and wild-type RNA (Fig. 3a). In contrast, the specific infectivity of T^{104}D RNA transcripts dramatically decreased (13 ± 5 and 86 ± 17 lesions per half-leaf were induced by T^{104}D and wild-type TMV, respectively). It should be emphasized that only tiny local lesions were produced by T^{104}D mutant (Fig. 3a), suggesting that the Thr to Asp substitution at position 104 strongly inhibited virus cell-to-cell movement. However, our results do not rule out that the MP produced by mutant T^{104}D is less stable than wild-type and T^{104}A MPs.

It is important to note that the difference in development of local lesions induced by the wild-type TMV and T^{104}D mutant was abolished when Xanthi nc. line 2005 plants transgenic for TMV MP gene were inoculated (Fig. 3b). Therefore, the movement deficiency of T^{104}D MP could be complemented *in trans* by MP produced in transgenic plants. In addition, wild-type and T^{104}A TMV induced a severe mosaic on *N. tabacum* var. Samsun, whereas TMV T^{104}D mutant caused only a mild mosaic on tobacco plants. Fig. 3(c) shows that accumulation of TMV T^{104}D in upper systemically infected leaves of *N. tabacum* var. Samsun plants was clearly delayed, whereas the time-course of accumulation of TMV T^{104}A and wild-type TMV in upper leaves was similar. In order to test the stability of the T^{104}A and T^{104}D mutations, the progeny of the mutant viruses was isolated from *N. benthamiana* plants. No reversions were detected by sequencing cDNA of T^{104}A and T^{104}D MP genes obtained by RT-PCR. No symptom differences could be detected on *N. tabacum* var. Samsun and Xanthi nc. plants inoculated with primary RNA transcripts or with the progeny of mutant viruses.

In conclusion, our *in vivo* experiments have shown that: (i) replacement of Thr^{104} in TMV MP with neutral Ala did not cause significant changes in cell-to-cell movement of TMV, indicating that phosphorylation of Thr^{104} was not essential for MP functions; (ii) substitution of Thr^{104} by a negatively charged Asp residue led to a strong inhibition of the local lesion development in Xanthi nc. tobacco. This inhibition could be eliminated in Xanthi nc. plants transgenic for MP gene. Presuming that this substitution functionally mimics phosphorylation, we suggest that Thr^{104} phosphorylation renders TMV MP dysfunctional. If this is the case, it seems logical to hypothesize that Thr^{104} phosphorylation *in vivo* represents a defence mechanism that protects the plant from virus infections. It should be mentioned that inactivation of the MP by the Asp^{104} mutation may not be directly due to mimicry of phosphorylation but due to the change of the MP conformation. It is evident that our data do not provide direct evidence that MP is in fact phosphorylated at Thr^{104} during infection. Alternatively, it may be phosphorylated only transiently *in vivo*. Experiments on examination of *in vivo* Thr^{104} phosphorylation are in progress.

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