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In vivo phosphorylation of the 30-kDa protein of tobacco mosaic virus

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The 30-kDa protein of tobacco mosaic virus, which is involved in cell-to-cell movement function, is phosphorylated in tobacco protoplasts. To investigate which portion of the protein is phosphorylated we inoculated several truncated 30-kDa protein mutants into protoplasts and determined whether or not those truncated proteins are phosphorylated. The results showed that amino acid residues 234–261 of the 30-kDa protein are required for this phosphorylation.

30-kDa protein; Phosphorylation, Cell-to-cell movement, In vitro transcript; Tobacco mosaic virus, Tobacco protoplast

1. INTRODUCTION

It is widely accepted that plant viruses encode a gene (or genes) for the cell-to-cell movement function [1]. In the case of tobacco mosaic virus (TMV), it has been shown that the 30-kDa protein is involved in this function [2,3]. The 30-kDa protein is synthesized transiently at the early stage of infection [4,5] and destined to be localized in plasmodesmata [6,7], where this protein is believed to be involved in the cell-to-cell movement function. It has been shown that this protein has the ability to enlarge the exclusion limit size of the plasmodesmata [8] and also the capacity to bind single-stranded nucleic acids [9,10].

Atkins et al. [11] recently reported that this protein is phosphorylated in vivo, but in heterologous insect cells. Here, we confirm that this 30-kDa protein is phosphorylated in plant cells using the protoplast system. As a first step to investigate the phosphorylated portion of the 30-kDa protein, we constructed several truncated 30-kDa protein mutants, inoculated them into protoplasts and investigated whether or not these proteins were phosphorylated in vivo.

2. MATERIALS AND METHODS

Tobacco suspension culture cells (BY-2) were used throughout these experiments [12]. Protoplasts of tobacco BY-2 cells (1×10^6) were inoculated with TMV tomato strain L RNA (0.2 μg) or with in vitro transcribed RNA (0.2 μg equivalent [13]) using the electroporation method [13]. Mock-inoculated or viral RNA-inoculated protoplasts were cultured in phosphate-free modified Murashige-Skoog medium supplemented with 50 mM PIPES-KOH (pH 6.5) [12] at a concentration of 1×10^5 protoplasts/ml. Carrier-free [^{32}P]orthophosphate was added to each 1 ml of culture medium at 200 μCi/ml (7.4 MBq/ml) 1 h postinoculation and incubated for 8 h. The cultures were collected in microcentrifuge tubes and spun at 3,000 rpm for 3 min. The supernatant was removed and protoplasts were harvested. Protoplasts (1×10^5) were then disrupted by 40 μl of RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 2 mM phenylmethylsulfonyl fluoride and 100 mM β-glycerophosphate, and boiled for 3 min. The resulting lysates were diluted 20-fold with 0.8 ml of TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), 1 mM phenylmethylsulfonyl fluoride and 100 mM β-glycerophosphate. Antiserum (3 μl) against the 30-kDa protein [14] or preimmune serum was added to each preparation. The mixtures were kept at 0°C overnight. Thirty μl of a 10% suspension of protein A-Sepharose (Pharmacia) were added to each mixture. Each mixture was then subjected to moderate rotation for 2 h at 4°C. The Sepharose beads were washed three times with fresh TBS containing 0.02% SDS and once with fresh TBS. The washed Sepharose was boiled with 50 μl of Laemmli's sample buffer for 2 min before being applied onto an analytical 0.1% SDS/4–20% gradient-polyacrylamide gels (Daiichi Chemical, Japan). In some experiments the gels were fixed, stained with Coomassie brilliant blue G-250 and dried. Radioactive bands were detected by autoradiography. Accumulation of the 30-kDa protein in protoplasts was checked by Western-blotting and immunodetection analysis with anti-30-kDa protein antibody [14] as previously described [15].

Transcription of pLFW3 produces an infectious transcript corresponding to the wild-type TMV RNA [16]. It expresses the intact 264 amino acids of the 30-kDa protein. Using standard DNA cloning techniques, we removed restriction fragments from the virus sequence inserted in pLFW3 [17]. Three deleted clones (pLQD261, pLQD233 and pLQD9/142) were constructed, which correspond to nucleotide deletions 5689–6187 (*Sac*I introduced–*Nsi*I), 5605–6187 (*Aat*I–*Nsi*I), or 4936–5331 (*Ssp*I–*Ssp*I), respectively. pLQNF was described previously [2]. Transcription was performed as described [13]. The transcripts and corresponding proteins are referred to by deleting the prefix 'p' from their template plasmid names, i.e. LFW3, LQD261, LQD233, LQD9/142 and LQNF, respectively (Fig. 1).

3. RESULTS

Mock-inoculated or TMV RNA-inoculated protoplasts were labeled with [^{32}P]orthophosphate during the

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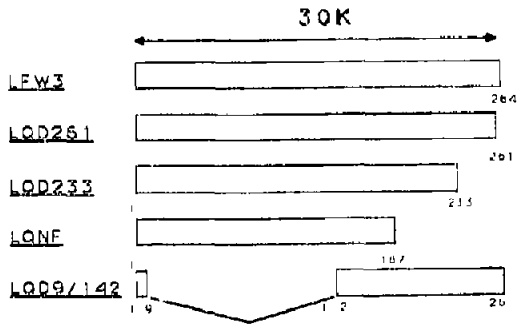

Fig. 1. Truncated 30-kDa proteins used in this experiment

period at which the majority of 30-kDa protein is synthesized, as shown by our previous data [4]. Protoplasts were disrupted and proteins were analyzed in an SDS polyacrylamide gel. A band specific of TMV-infected protoplasts could be detected by autoradiography (Fig. 2, large arrowhead). Immunoprecipitation experiments confirmed that this protein is indeed the 30-kDa protein. Anti-30-kDa protein antibodies could immunoprecipitate the protein but preimmune serum could not (Fig. 2; lane +anti-30-kDa). Bacterial alkaline phosphatase (Takara Shuzo, Japan) treatment, which is known to remove phosphate groups from phosphorylated amino acids, produced fading of the band (Fig. 2; lane BAP). Consequently we could detect phosphorylation of the 30-kDa protein in vivo using plant cells.

Another radioactive band with a slower electrophoretic mobility and specific of TMV-inoculated protoplasts could be seen (Fig. 2, infected, lane sample; small arrowhead). Anti-130-kDa/180-kDa protein antibodies [18] could not immunoprecipitate this band (data not shown). We have not characterized this band further.

Next we wanted to determine which portion of the 30-kDa protein is phosphorylated. Several truncated 30-kDa protein mutants were engineered: LQD261, LQD233, LQNF and LQD9/142 that would direct the synthesis of related 30-kDa proteins conserving amino acid residues 1–261, 1–233, 1–187 (plus 8 out-of-frame amino acids: His-Gly-Thr-Phe-Gly-Arg-Ser-Cys [2]) and 1–9/142–264, respectively (Fig. 1). These proteins are hereafter referred to by the name of their originating transcript; e.g. LQD261 proteins. We inoculated these transcripts into protoplasts and analyzed the accumulation/phosphorylation pattern of the truncated proteins. Immunodetection analyses showed that the LFW3, LQD261 and LQD233 proteins accumulated to a comparable level; in contrast, the LQNF and LQD9/142 proteins accumulated to much lower levels (Fig. 3A; arrowheads), even if one considers the fact that this antibody reacts somewhat better with the C-terminal portion of the protein than with the N-terminal portion [14]. This result shows that internal truncation or deletion of more than 77 amino acids from the C-terminus of the 30-kDa protein causes some instability in vivo, since these mutants are equally replicated (data not shown). The phosphorylation pattern of these proteins is shown in Fig. 3B (arrowheads). The LFW3 and LQD261 proteins are equally phosphorylated; but the LQD233 and LQNF proteins are barely phosphorylated, if at all. Fig. 3B also shows that the LQD9/142 protein was phosphorylated to a considerable level.

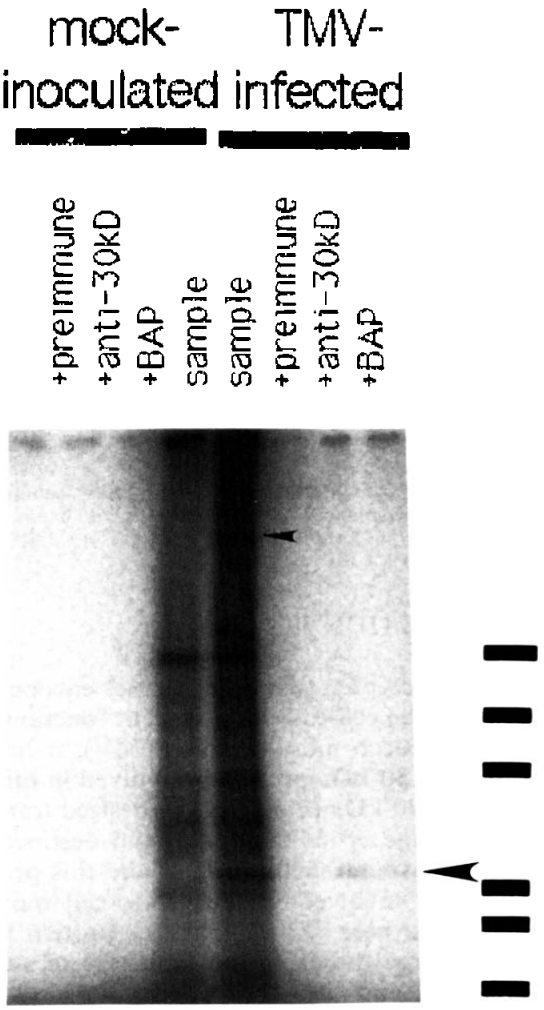

Fig. 2. Detection of the in vivo phosphorylation of the 30-kDa protein (large arrowhead). Mock-inoculated or TMV-RNA infected protoplasts were ^{32}P-labeled in vivo. Proteins were analyzed on a 0.1% SDS/4–20% polyacrylamide gradient gel directly (lanes 'sample') or after immunoprecipitation. BAP lanes show the immunoprecipitated preparations after bacterial alkaline phosphatase treatment. The small arrowhead also indicates a TMV-infection-specific band. Bars at the right indicate the positions of protein molecular weight markers (Amersham, Rainbow markers; 92.5, 69, 46, 30, 21.5, 14.3 kDa).

4. DISCUSSION

We present here evidence of the in vivo phosphorylation of the 30-kDa protein that was previously reported

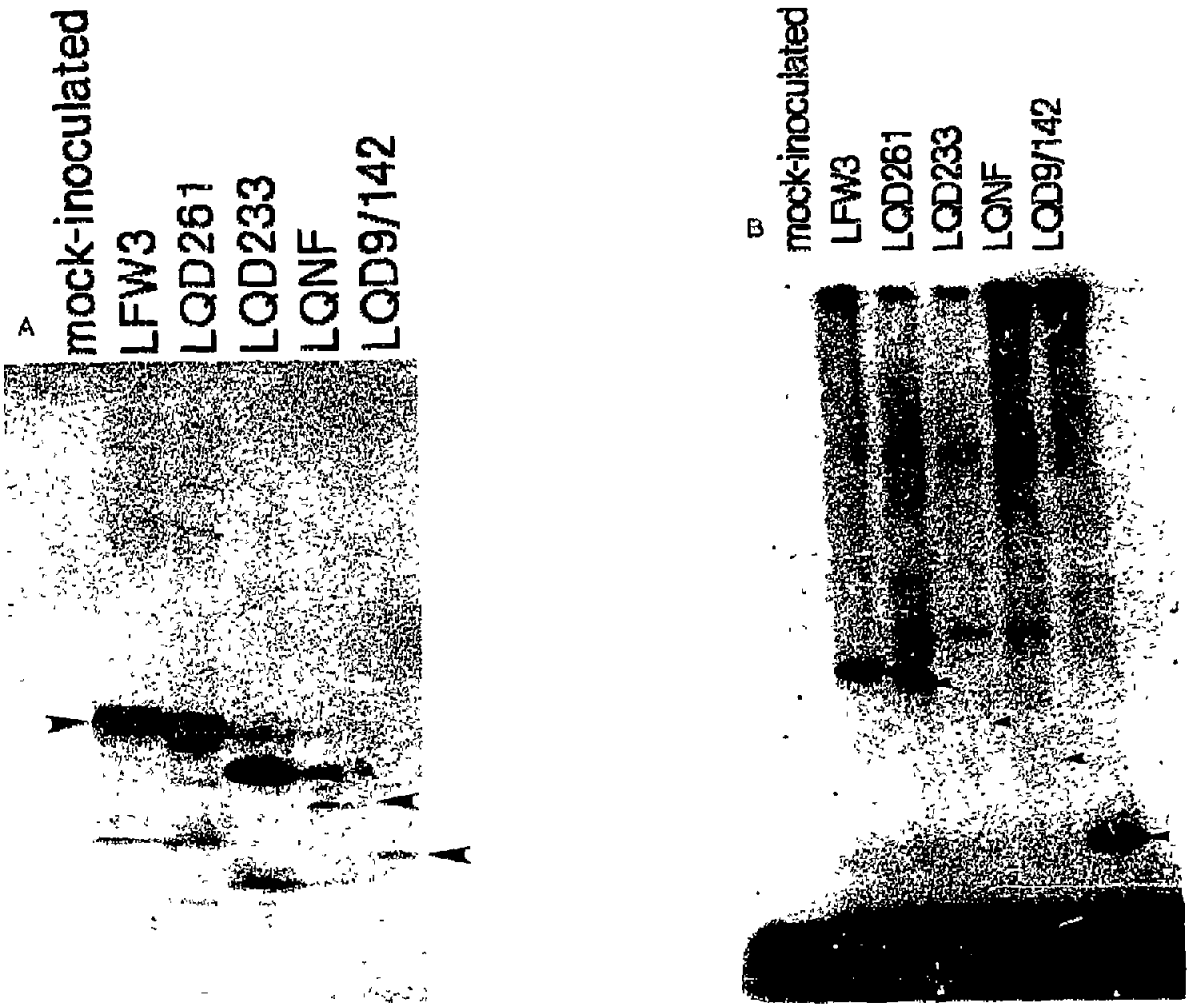

Fig. 3. (A) Western-blotting and immunodetection of the truncated 30-kDa proteins by anti-30-kDa protein antiserum. Protein samples equivalent to 2×10^4 protoplasts were electrophoresed and analyzed. (B) Phosphorylation pattern of the truncated 30-kDa proteins. Each protein sample of protoplasts equivalent to 2×10^4 was subjected to immunoprecipitation with anti-30-kDa protein antiserum and analyzed as in Fig. 1. Arrowheads indicate the positions of each protein.

[11,19]. While protoplasts do not retain the integrity of plasmodesmata, our protoplast system has some advantages for the analysis of phosphorylation. The phosphorylation detected here might be important for fine analysis of the 30-kDa protein in relation to the function and understanding of the molecular interactions between the 30-kDa protein and host plant cells. To test the relation of the function of the protein and its modification, we must compare in planta and protoplast experiments in the future.

It was shown that LQD261 and LQD233 are competent in cell-to-cell movement [20], while LQNF [2] and LQD9/142 (unpublished data) are incompetent. Analysis of LQD233 showed that C-terminal truncation by 31 amino acids of the 30-kDa protein lowered considerably but did not eliminate the ability to promote virus cell-to-cell movement [20]. Other groups showed similar results [21,22]. The data presented here might indicate that these amino acid residues 234–261 of the 30-kDa protein contain the target site(s) of phosphorylation. Combining the available informations, one possibility is that phosphorylation is not directly related to the cell-to-cell movement function of the 30-kDa protein. On the other hand, rather conversely, malfunction of the LQD233 protein in cell-to-cell movement might indicate another possibility that host plants modulate/enhance the cell-to-cell movement function through this phosphorylation by an unrevealed kinase.

Atabekov and Taliansky [23] mentioned the possibility that the 30-kDa protein might be phosphorylated in plants by a cAMP-dependent kinase. This conclusion was based on experimental data suggesting that the thermosensitive defect in cell-to-cell movement of the Ls1 strain could be complemented by addition of exogenous cAMP [23]. It would be interesting to see whether cAMP has some effects on the function of the 30-kDa protein via phosphorylation.

As a whole, it is shown that the truncation of amino

acids 234 to 261 of the 30-kDa protein was unfavorable for in vivo phosphorylation. The simplest model is that the target site(s) for this phosphorylation is (are) located at amino acid residues 234–261 of the 30-kDa protein. The amino acid residues 234–261 of this protein are mapped in regions B and C [24]. There are juxtaposed basic amino acids in the region B. It is reported that the cAMP-dependent kinase of animals or yeasts phosphorylates serine residues in a context of the motif 'XRRXSX' [25]. In the 30-kDa protein, there are no amino acid sequences which fit completely with this motif, while our preliminary phosphoamino acid analyses of the intact 30-kDa protein showed that serine residues are phosphorylated. We do not know at present which serine residue(s) in the 30-kDa protein is (are) phosphorylated since no direct amino acid analysis has as yet been performed.

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