Phosphorylation of the Movement Protein of *Cucumber Mosaic Virus* in Transgenic Tobacco Plants

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**Abstract.** The 3a protein of *Cucumber mosaic virus* is essential for the cell-to-cell movement of the viral RNA through plasmodesmata. We have introduced an epitope peptide before the stop codon of the 3a protein and cloned the tagged ORF into a binary vector for *Agrobacterium*-mediated transformation. The established transgenic tobacco lines produced the 3a protein, which was specifically detected with anti-3a and anti-epitope antisera. Metabolic labeling and subsequent immunoprecipitation revealed that [³²P]-orthophosphate was incorporated into the 3a protein. The phosphoamino acid analysis indicated that the 3a protein contained phosphoserine but not phosphothreonine or phosphotyrosine. This is the first demonstration of the 3a protein phosphorylation in planta.

**Key words:** *Cucumber mosaic virus*, movement protein, phosphorylation

**Introduction**

*Cucumber mosaic virus* (CMV) contains three positive-strand genomic RNAs (RNAs 1–3) in addition to a subgenomic RNA 4. RNA 3 encodes coat protein and a movement protein 3a, both of which are involved in the cell-to-cell movement of the virus and required for the systemic infection of the host plant [1]. The coat protein was also suggested to interact with a host factor leading to the inhibition of the virus systemic movement [2].

Movement proteins of plant viruses vary in their structures but they seem to share fundamental mechanisms for the translocation of the virus genome to neighboring cells through plasmodesmata. The movement protein of *Tobacco mosaic virus* (TMV) has been reported to associate with plasmodesmata, endoplasmic reticulum and microtubules [3]. Matsushita et al. [4] reported a possible interaction between the movement protein of *Tomato mosaic virus* (ToMV) and transcriptional coactivator KELP. Thus, the movement proteins should have multiple functional domains. It is possible that the proteins are functionally regulated through the changes in modification and/or the tertiary structure.

In both TMV and ToMV, phosphorylation status of the movement proteins has been analyzed in relation to their functions [5–12]. Although a few examples of phosphorylation were reported in other plant virus species such as *Potato leafroll virus* [13,14] and *Apple chlorotic leaf spot virus* [15], it has never been reported for CMV. In the present study, we expressed CMV 3a protein in transgenic tobacco lines to examine its phosphorylation status directly by metabolic labeling in planta with [³²P]-orthophosphate.

**Methods**

**Plasmid Construction**

The cDNA for the 3a protein of CMV(O) [16] was prepared from the plasmid pUCMVO3. A coding sequence for the influenza hemagglutinin (HA) epitope (YPYDVPDYA) was inserted before the stop
This cDNA fragment was subcloned into a binary vector pART27 [17] to construct pART27-CMVOMP-HA, which was used for the expression of the tagged 3a protein under the Cauliflower mosaic virus 35S promoter.

**Transgenic Plants**

Leaf disks from *Nicotiana tabacum* cv. Samsun NN were infected with *Agrobacterium tumefaciens* LBA4404 carrying pART27-CMVOMP-HA and cultivated in the presence of kanamycin to regenerate transgenic plants.

**Antibodies**

A rabbit antiserum for HA tag was purchased from MBL (code #561). To obtain anti-3a antiserum, a rabbit was immunized with a recombinant 3a protein expressed in *E. coli* by using the 3a cDNA derived from CMV(Y) [18] and pET His-tag expression vector (Novagen).

**Results and Discussion**

We have established 34 kanamycin-resistant tobacco lines among which 23 lines were positive for the expression of CMV 3a protein. As shown in Fig. 1, the two representative transgenic lines A19 and A28 produced 3a protein of approximately 35 kDa that reacted with both anti-HA and anti-3a antisera. Such a protein was absent in the control plants (G and NT).

Comparison with the known amount of the recombinant GST-fused 3a protein, we estimated that the A28 line produced approximately 2 μg of 3a protein per 1 g (wet weight) of leaf tissue.

The transgenic line A28 and the control line carrying GUS gene were subjected to metabolic labeling followed by immunoprecipitation. As shown in Fig. 2, the 3a protein in A28 was specifically precipitated with both anti-HA and anti-3a antisera and demonstrated to be labeled with [32P]-orthophosphate. The result of phosphoamino acid analysis (Fig. 3) indicated that the incorporation of [32P]-orthophosphate was due to protein phosphorylation at the serine residue.

Ding et al. [19] reported that the transgenically expressed 3a protein could support the cell-to-cell movement of microinjected CMV RNA, indicating that the 3a protein alone was sufficient for the short-distance movement. Thus, the functional implication of the 3a protein phosphorylation could be considered independently of other CMV gene products. This is the first demonstration of the CMV 3a protein phosphorylation *in planta*. The phosphorylation assay

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**Fig. 1.** Detection of 3a protein in transgenic tobacco lines. Leaf tissues (25 mg wet weight/lane) were homogenized in liquid nitrogen with SDS sample buffer. The crude extracts were analyzed by Western blotting using anti-HA (left) and anti-3a (right) antibodies, which were visualized with biotinylated secondary antibody, avidin, and biotinylated peroxidase. Solid and open arrows indicate the positions of 3a protein expressed in tobacco and *E. coli*, respectively. A19 and A28: transgenic tobacco lines carrying 3a gene, G: transgenic tobacco line carrying GUS gene, NT: non-transgenic tobacco, C: 10 ng of GST-fused 3a protein with HA tag (39 kDa). Molecular mass (kDa) markers are shown on the right.