Expression, subcellular location and modification of the 50 kDa protein encoded by ORF2 of the apple chlorotic leaf spot trichovirus genome

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A putative movement protein of molecular mass 50 kDa encoded by the ORF2 of the apple chlorotic leaf spot virus (ACLSV) genome was expressed in Escherichia coli using an expression vector and was then used to produce an antiserum. Immunoblot analysis using an antiserum raised against this protein showed that the ORF2 protein of ACLSV was detected in both cell wall and cell membrane fractions prepared from infected Chenopodium quinoa tissues. The ORF2 protein from infected tissues had a molecular mass of 52 kDa, larger than that of the full-length ORF2 protein (50 kDa protein) expressed in E. coli. Incubation of the 52 kDa protein with alkaline phosphatase resulted in a decrease in its apparent molecular mass from 52 kDa to 50 kDa, strongly suggesting that the ORF2 protein of ACLSV is phosphorylated in infected plant tissues.

Apple chlorotic leaf spot trichovirus (ACLSV) has very flexuous filamentous particles, approximately 600 to 700 nm in length and contains a polyadenylated plus-sense, ssRNA with a molecular mass of \(2.48 \times 10^6\) Da and a single coat protein of 22 kDa (Yoshikawa & Takahashi, 1988). With respect to its taxonomic position, ACLSV has recently been transferred to the newly established genus Trichovirus from the closterovirus group (Martelli et al., 1993). The complete nucleotide sequence of ACLSV RNA was determined for isolates from plum (ACLSV-P) (German et al., 1990) and apple (ACLSV-A) (Sato et al., 1993). The genomes of ACLSV-P and ACLSV-A consist of 7555 and 7552 nucleotides excluding a Y-terminal poly(A) tail, respectively, and contain three open reading frames (ORFs 1, 2 and 3). ORFs 1, 2 and 3 of ACLSV-A encode proteins with molecular masses of 216.5 kDa, 50.4 kDa and 21.4 kDa, respectively (Sato et al., 1993). The 216.5 kDa protein contains two consensus sequences associated with the RNA polymerase and NTP-binding helicase. The 21.4 kDa protein is the viral coat protein (Sato et al., 1993) and the 50.4 kDa protein was suggested to be the movement protein (German et al., 1990; Sato et al., 1993).

In order to evaluate the function of the 50.4 kDa protein of ACLSV, we expressed the protein in Escherichia coli, produced a specific antiserum, and analysed its expression, subcellular location and modification in infected leaves.

ACLSV (P-205), originally isolated from an apple tree (Yanase, 1974) was propagated by mechanical inoculation in Chenopodium quinoa Willd. and used throughout this study.

To express the ORF2 protein, we constructed plasmid pKK50K which expresses the full-length ORF2 protein without any change in the coding region. The ORF2 region was amplified by PCR using pBCLS38 as template and two primers: 5' AACCCGGGATGATGATAAGGGGTCA 3', corresponding to nt positions 5727 to 5744 of the ACLSV-A genome (Sato et al., 1993) and containing a SmaI site, and 5' AACTGCAGTCACACACTTGGCAGAAG 3', complementary to nt positions 7092 to 7100 and containing a PstI site. The PCR product was digested with SmaI and PstI and ligated to a pKK223-3 expression vector (Pharmacia) restricted with the same enzymes. The plasmid (pKK50K) was subsequently used to transform competent E. coli JM105.

Purification of ORF2 protein expressed in E. coli was as follows. The preculture of E. coli (1 ml) containing pKK50K was added to 100 ml fresh LB medium and grown at 37 °C to a cell density of 0.6 to 0.8 (A650). The culture was further grown for 3 to 4 h after the addition of IPTG to a final concentration of 1 mM. The cells were centrifuged at 19000 g for 15 min and the
pellet was resuspended in 20 ml 1 M-sucrose. After the first centrifugation stage described above, the pellets were resuspended in 20 ml 10 mM-EDTA and 2% Triton X-100 and incubated at 4 °C overnight. The suspension was centrifuged and the resulting pellet was dissolved in 3 ml of gel loading buffer (50 mM-Tris–HCl, pH 8.8, 10% glycerol, 2% SDS, 2% mercaptoethanol, 0.01% bromophenol blue) and incubated at 100 °C for 3 min. The protein preparation was electrophoresed in an SDS–15% polyacrylamide gel using a preparative disc gel apparatus (ATTO Corporation). The fractions containing the ORF2 protein were collected using a fraction collector and were dialysed against 0.025 M-sodium acetate, 1 mM-EDTA, 5 mM-DTT and 0.5 mM-PMSF.

An antiserum raised against the ORF2 protein was prepared in a rabbit by intramuscular injections of the purified ORF2 protein. The antiserum was absorbed with the ORF2 protein expressed in E. coli before use.

Results from SDS–PAGE showed that a protein band corresponding to the expected size of the ORF2 protein (50 kDa protein) was expressed only in a culture containing IPTG (Fig. 1a, lane 4). The 50 kDa protein that was over-expressed in E. coli was recovered as insoluble inclusions and purified by SDS–15% PAGE using a preparative disc gel apparatus as described above (Fig. 1a, lane 5). Immunoblot analysis showed that the antiserum that was raised against the purified 50 kDa protein reacted specifically with the protein extracted from E. coli cultured in the presence of IPTG (Fig. 1b).

In order to examine the subcellular location of the 50 kDa protein, subcellular extracts of healthy and infected C. quinoa leaves (upper leaves showing symptoms 8 days after inoculation) were prepared essentially as described by Eggen et al. (1988). Freshly harvested tissues (0.3 g) were homogenized in 0.3 ml of buffer G (50 mM-Tris–acetate, pH 7.4, 10 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT and 0.5 mM-PMSF). The homogenates were centrifuged at 1000 g for 15 min. The pellets were washed twice in buffer G and suspended in 0.3 ml of buffer S (50 mM-Tris–acetate, pH 8.0, 50 mM-potassium acetate, 5 mM-DTT, 0.5 mM-PMSF and 25% glycerol) (cell wall fraction, CW). The supernatants after the first centrifugation were adjusted to 20% (v/v) glycerol and then centrifuged for 30 min at 30000 g. The resulting pellets were suspended in 0.3 ml of buffer S (cell membrane fraction, CM) and the supernatants were used as soluble (S) fractions. Samples from each fraction suspended in an equal volume were electrophoresed in an SDS–8% or 12.5% polyacrylamide gel and then analysed by immunoblotting using the antiserum raised against the ORF2 protein expressed in E. coli as described previously (Yoshikawa et al., 1992).

Immunoblot analysis showed that a major band with an apparent molecular mass of 52 kDa was detected in total protein, CM and CW fractions along with one or two more diffuse, adjoining, fainter bands extracted from infected tissues (Fig. 2, lanes 2, 4 and 5). No reaction was detected with any fraction from healthy tissues (Fig. 2, lanes 6 to 9).

To follow the accumulation of the ORF2 protein in infected tissues, inoculated leaves of C. quinoa were fractionated into CM, CW and S fractions at different times after inoculation and then analysed by immunoblotting. In CM and CW fractions, the 52 kDa protein was first detected 3 days after inoculation, a time which coincides with the appearance of chlorotic lesions in inoculated leaves, and was consistently detected from 4 to 7 days after inoculation (Fig. 3c). This experiment was repeated three times and similar results were obtained on each occasion.

As shown in Fig. 2, the ORF2 protein extracted from infected C. quinoa tissues migrated as diffuse bands with an additional major band that had a molecular mass of 52 kDa, larger than the molecular mass (50 kDa) of the protein expressed in E. coli. To confirm that there was a real difference in these electrophoretic mobilities, rather than a difference arising from effects such as migration interference by plant proteins, the
Fig. 2. Immunoblot analysis of the proteins in subcellular fractions prepared from ACLSV-infected (lanes 2 to 5) or healthy (lanes 6 to 9) *C. quinoa* tissues using an antiserum raised against the ORF2 protein expressed in *E. coli* cells. Lanes 1 and 10, ORF2 protein expressed in *E. coli*; lanes 2 and 6, total proteins; lanes 3 and 7, soluble fractions; lanes 4 and 8, cell membrane fractions; lanes 5 and 9, cell wall fractions.

Fig. 3. Time course of accumulation of the ORF2 proteins in the membrane fraction (a) and the cell wall fraction (b), and of the coat protein in the soluble fraction (c) prepared from ACLSV-inoculated leaves. Samples from leaves taken 1 to 7 days after inoculation were electrophoresed in SDS-12.5 % polyacrylamide gels and analysed by immunoblotting using antisera against the ORF2 protein (a, b) or raised against purified ACLSV (c). Lanes 1 and 9, ORF2 protein expressed in *E. coli* (a, b) and ACLSV coat protein (c); lanes 2 to 8, samples from leaves 1, 2, 3, 4, 5, 6 and 7 days after inoculation, respectively.

Fig. 4. Immunoblot analysis of the ORF2 proteins expressed in *E. coli* cells and ACLSV-infected tissues using an antiserum raised against the ORF2 protein. Lanes 1 and 6, ORF2 protein from *E. coli*; lane 2, proteins extracted from infected tissues; lane 3, proteins extracted from healthy tissues; lane 4, a mixture of the ORF2 protein expressed in *E. coli* and proteins from infected tissues; lane 5, a mixture of ORF2 protein expressed in *E. coli* and proteins from healthy tissues.

Fig. 5. Effects of AP on the electrophoretic mobilities of the ORF2 protein extracted from ACLSV-infected *C. quinoa* leaves. Lanes 1 and 9, ORF2 proteins expressed in *E. coli*; lanes 2 to 6, ORF2 proteins from infected tissues incubated with AP for 0, 5, 10, 15 and 30 min at 37 °C, respectively; lane 7, ORF2 protein from infected tissues incubated for 30 min at 37 °C without AP; lane 8, ORF2 protein from infected tissues without incubation.

50 kDa protein expressed in *E. coli* was mixed with the total protein extracted from *C. quinoa* tissues and analysed by immunoblotting. Fig. 4 clearly shows that the ORF2 protein extracted from infected *C. quinoa* has a lower electrophoretic mobility than that expressed in *E. coli*. This suggests that the ORF2 protein undergoes post-translational modification.

We investigated the possibility of two forms of post-translational modification, i.e. phosphorylation and glycosylation. The ORF2 protein was extracted from ACLSV-infected *C. quinoa* leaves as follows. Fresh tissue (2 g) was homogenized in 3 ml of buffer G and 3 ml of gel loading buffer and incubated for 3 min at 100 °C. The preparation was electrophoresed in an SDS-7.5 % polyacrylamide gel using a preparative disc gel apparatus. Fractions containing the ORF2 protein were pooled and dialysed against 0.025 M-Tris-acetate, pH 7.4. The preparation was freeze-dried and suspended in 1.5 ml 0.025 M-Tris-acetate, pH 7.4. For the phosphorylation assay, protein samples (8 μl) extracted from infected tissues were incubated with 1 unit alkaline phosphatase (AP) (Boehringer Mannheim) at 37 °C for 5, 10, 15 and 30 min. The samples were then analysed by immunoblotting as described above.

To examine glycosylation, the samples were analysed by affinity blotting with biotin-labelled concanavalin A (ConA), soybean lectin (SBA), *Ulex europaeus* lectin (UEA-I) and wheat germ lectin (WGA) as described by Faye & Chrispeels (1985).

Fig. 5 shows that incubation of the 52 kDa protein extracted from infected tissues with AP for 5, 10, 15 and 30 min resulted in a decrease in its molecular mass from...
52 kDa to 50 kDa (Fig. 5, lanes 3–6) so that the protein from infected tissues comigrated with the 50 kDa protein expressed in E. coli. In contrast, incubation without AP did not alter its mobility (Fig. 5, lane 7). This result strongly suggests that the ORF2 protein is phosphorylated in infected tissue.

The possibility that glycosylation of the 52 kDa protein was occurring was examined by affinity blotting with biotin-labelled lectins (ConA, SBA, UEA-1 and WGA). The protein was not detected by any lectins used (data not shown), suggesting that it is not modified by glycosylation.

The ORF2 protein of ACLSV is suggested to be a movement protein based on its amino acid sequence similarity with movement proteins of other plant viruses (German et al., 1990; Melcher, 1990; Mushegian & Koonin, 1993; Sato et al., 1993). As reported in the present paper, ACLSV ORF2 protein was mainly detected in CM and CM fractions extracted from infected tissues, in agreement with the subcellular locations of other plant virus movement proteins (Albrecht et al., 1988; Godefroy-Colburn et al., 1986; Kormelink et al., 1994; Moser et al., 1988; Osman & Buck, 1991). The transient accumulation of movement proteins has been reported for several plant viruses. In tobacco mosaic virus (TMV)- or alfalfa mosaic virus (AMV)-infected tobacco plants, the movement proteins appeared to be expressed transiently in membrane fractions, but accumulated more stably in cell walls (Bern et al., 1986; Godefroy-Colburn et al., 1986; Moser et al., 1988). Our results on the accumulation of the ORF2 protein indicated that it was first detected 3 days after inoculation, and then persisted until the end of the experiment (7 days after inoculation) in CM as well as CW fractions.

Evidence of post-translational modification of the movement proteins of several plant viruses has either been indicated or demonstrated. For example, the 32 kDa protein of AMV and the gene I product of cauliflower mosaic virus were suggested to be glycosylated because they have several potential glycosylation sites in their amino acid sequences (Albrecht et al., 1988; Godefroy-Colburn et al., 1986). On the contrary, the 30 kDa protein of TMV expressed using a baculovirus expression vector was phosphorylated, but not modified by N-linked glycosylation in Spodoptera frugiperda cells (Atkins et al., 1991). Recently, Citovsky et al. (1993) demonstrated that the 30 kDa protein of TMV was phosphorylated by cell wall-associated protein kinase. Our results also suggest that the ORF2 protein of ACLSV is phosphorylated in infected tissues. However, there was no evidence that the ORF2 protein was glycosylated. It would be very interesting to know whether phosphorylation is a common feature of the movement proteins of plant viruses and what significance it has in relation to their functions in host plant cells.

Together with data obtained from the comparisons of its amino acid sequence with other plant virus movement proteins, the subcellular location and phosphorylation of the ORF2 protein of ACLSV that we report in this paper provide further evidence that the ORF2 protein of this trichovirus may act as a movement protein.

We would like to thank Dr R. H. Converse for his critical reading of the manuscript. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

References


(Received 24 October 1994; Accepted 20 January 1995)