MPMI Vol. 8, No. 5, 1995, pp. 658–665.

Evidence for Proteolytic Processing of Tobacco Mosaic Virus Movement Protein in *Arabidopsis thaliana*

Richard K. Hughes, Marie-Christine Perbal, Andrew J. Maule, and Roger Hull

Department of Virus Research, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, U.K.
Received 5 January 1995. Accepted 1 June 1995.

Two ecotypes of *Arabidopsis thaliana* were transformed with the gene encoding tobacco mosaic virus (TMV) movement protein (P30). P30 accumulated largely in a subcellular fraction containing cell wall components and as a soluble protein. The protein migrated in denaturing gels with an M_r of 30K, significantly faster than P30 (M_r approximately 34K) accumulating after expression in transgenic tobacco, *Escherichia coli* or *Spodoptera frugiperda* cells, or after virus multiplication in tobacco. The P30 from *A. thaliana* infected with TMV for 14 days comigrated with that from *E. coli,* but that from *A. thaliana* infected for 49 days was of the smaller size. The use of antisera specific for the N- or C-termini of P30 showed that in *A. thaliana* P30 was proteolytically processed at the N-terminus, a region essential for P30 function. The failure of these plants to complement a TMV P30 mutant indicated that processed P30 was nonfunctional, although the processing was not so rapid that it prevented the development of systemic infections with wild type TMV. The absence of detectable P30 phosphorylation in *A. thaliana* demonstrated that phosphorylation was not essential for movement protein function and suggested that this species may use proteolytic cleavage of the N-terminus as an alternative strategy to tobacco for deactivating P30.

Additional keywords: protein phosphorylation, protein processing, transgenic plants.

The 30-kDa protein (P30) encoded by tobacco mosaic virus (TMV) is essential for cell-to-cell spread (Deom et al. 1987) of the virus (for review, see Deom et al. 1992). Several biological activities have been identified for P30, including the binding to single-strand nucleic acids (Citovsky et al. 1990) and localization in tobacco plants to plasmodesmata (Atkins et al. 1991b) with an increased size exclusion limit (Waigmann et al. 1994; Wolf and Lucas 1994). The functional domains of P30 responsible for conferring these activities to the protein have been determined from a comparison of the behavior of wild-type and mutant forms of P30 (see Waigmann et al. 1994). The analysis of P30 with small deletions near the N-terminus of the protein has shown this region to be essential for maintaining the function of P30 in tobacco plants (Gafny et al. 1992). It has also been implied that the N-terminal region of P30 is capable of modulating activities associated with the C-terminus of the protein (Waigmann et al. 1994).

Although the calculated size of P30 from the predicted amino acid sequence (Goelet et al. 1982) is 30 kDa, the protein behaves anomalously under denaturing gel conditions (see Oliver et al. 1986; Citovsky et al. 1990). Also, when expressed in some eukaryotic expression systems, P30 is phosphorylated (Atkins et al. 1991a, 1991b; Watanabe et al. 1992). While the effect of P30 phosphorylation on the biological activity of the protein is unknown, it has been suggested that this modification could act as a mechanism to sequester P30 in the cell walls and inactivate P30 function (Citovsky et al. 1993).

Despite the wide host range of TMV, there are few studies reporting the behavior of P30 in hosts other than tobacco. *Arabidopsis thaliana* is an ideal organism to study plant–virus interactions since the genetics of this plant are the best characterized of all plant species and a large number of mutants are available. In addition, its susceptiblity to TMV (Sosnova and Polak 1975) allows the behavior of P30 to be examined in this important host. The production of *A. thaliana* plants expressing P30 would also allow an analysis of the functions of P30 in the absence of other viral gene products.

In this report, we show that P30 is not phosphorylated in *A. thaliana*. We propose that *A. thaliana* has an alternative mechanism for inactivating the function of P30 to that suggested for tobacco. This mechanism involves post-translational cleavage of the protein at the N-terminus.

RESULTS

Production of P30 transgenic *A. thaliana*.

A. thaliana ecotypes Landsberg *erecta* (L-*er*) and Columbia (Col-O) were transformed with the P30 gene from the U1 strain of TMV. Successful transformation was confirmed by resistance to kanamycin (selectable marker) and by amplification of an 819-bp fragment, corresponding to the complete P30 gene, from R_1 individuals (data not shown). The predicted amino acid sequence of these fragments was identical to the published sequence (Goelet et al. 1982) although a base substitution (G→A), giving a silent change in the codon for

Present address of R. K. Hughes: Department of Applied Genetics, John Innes Centre.

Corresponding author: R. Hull; E-mail: hull@bbsrc.ac.uk

proline, was found at nt 4977. In *A. thaliana*, the codon usage of CCA for proline is more frequent than CCG (Wada et al. 1991). Transcription of the gene was confirmed by amplification of the same 819-bp fragment from cDNA made to mRNA isolated from R_1 plants (data not shown). Following Western blot analysis, transformed plant line 1 (L-*er*) and line 3 (Col-O) were selected from 3 (L-*er*) and 7 (Col-O) lines, respectively, as lines giving the highest accumulation of P30.

P30 protein expression and subcellular distribution in transgenic *A. thaliana*.

Using antiserum 137 (raised to a synthetic peptide corresponding to the C-terminus of P30) for Western analysis, P30 from cell wall-enriched fractions purified from both lines of 56-day-old transgenic *A. thaliana* plants was detected as a protein of M_r 30K (Fig. 1, lanes 4 and 6). P30 protein of identical size was detected in fractions from young (7 or 14 day old) plants (data not shown). This product was not detected in untransformed tissue (Fig. 1, lanes 3 and 5); the band of M_r 60K, found in transformed and untransformed tissues, represented a non-specific immunoreaction. In plants of the transgenic tobacco line 277, P30 was detected as a protein migrating more slowly (M_r 34K) than the P30 from *A. thaliana* (M_r 30K; Fig. 1, compare lanes 2 and 4). Presumably, this slower-migrating protein was the phosphorylated form of the P30 detected previously from the same transformed tobacco line and from TMV-infected tobacco plants (Atkins et al. 1991a, 1991b; Citovsky et al. 1993; Deom et al. 1987). In contrast, P30 produced by recombinant baculovirus expression in *S. frugiperda* cells (Atkins et al. 1991a), was detected as two closely migrating products (Fig. 1, lane 7) of M_r 34K (comigrating with tobacco-expressed P30; lane 2) and M_r 33K (comigrating with *E. coli*-expressed P30; lane 8). Presumably, these proteins corresponded, respectively, to the phosphorylated and nonphosphorylated forms of P30. On the same gel, P30 expressed in *A. thaliana* migrated significantly faster (equivalent to a M_r difference of 4K) than any of these products. The difference in mobility was unlikely to be attributable to proteolytic cleavage during extraction since the same sized product was detected when extracting P30 using a mixture of protease inhibitors (data not shown). From a comparison of the intensities of the immunoreaction with P30 products on a Western blot, the level of accumulation of P30, on a per gram fresh weight basis, was very similar for the cell wall-enriched fractions from transgenic tobacco or from the transgenic lines of *A. thaliana* (Fig. 1, lanes 2, 4, and 6). P30, comigrating with *E. coli*- or tobacco-expressed P30, was not detected in cell wall-enriched fractions of 7- or 14-day-old transgenic *A. thaliana* L-*er* plants (data not shown).

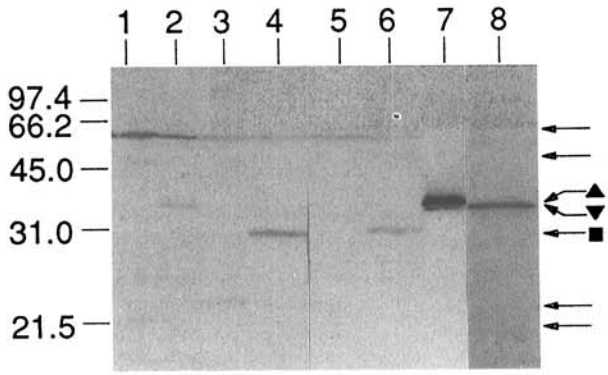

Fig. 1. Western blot analysis of P30 in transgenic *Arabidopsis thaliana*. Cell wall-enriched fractions were purified from 56-day-old *A. thaliana* or tobacco plants. P30 purified from recombinant baculovirus (Ac.TK6) expression in *Spodoptera frugiperda* (Atkins et al. 1991a) and after overexpression from pETP30 in *E. coli* (Citovsky et al. 1990), were electrophoresed in parallel. Proteins were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and probed with antiserum 137. Lane 1, tobacco nontransgenic line 306; lane 2, tobacco transgenic line 277; lane 3, *A. thaliana* L-*er* (untransformed); lane 4, *A. thaliana* L-*er* (transgenic line 1); lane 5, *A. thaliana* Col-O (untransformed); lane 6, *A. thaliana* Col-O (transgenic line 3); lane 7, P30 from baculovirus expression in *S. frugiperda* cells; lane 8, *E. coli*-expressed P30. The positions of P30 detected after expression in *A. thaliana* (■), *E. coli* (▼) and *S. frugiperda* (▲, ▼) and of nonspecific cross-reacting products detected also in fractions from untransformed plants (←), are indicated. M_r markers are (in kDa): phosphorylase B (97.4), serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), and trypsin inhibitor (21.5).

To determine the subcellular location of transgenically expressed P30 in *A. thaliana*, tissues from L-*er* line 1 plants were fractionated as described in Materials and Methods and analyzed by Western blotting using antiserum 137 (Fig. 2). P30 accumulated in approximately equal amounts in the soluble (S30; lane 2) and cell wall-enriched fractions (lane 4), was just detectable in the microsomal (PE30) fraction (lane 1) but was not detectable in the organellar (PE1) fraction (lane 3). In addition to P30, several nonspecifically reacting proteins were differentially enriched by the fractionation (arrows in Fig. 2).

N-terminal posttranslational processing of P30 in transgenic *A. thaliana*.

When a Western blot of a cell wall-enriched fraction from mature transgenic *A. thaliana* L-*er* plants was analyzed using antiserum R2 (raised to the GST-fusion protein carrying a peptide corresponding to the N-terminal 32 amino acids of P30), no P30 was detected (Fig. 3, lane 3), although parallel analysis with antiserum 137 identified a protein of M_r 30K from the same sample (Fig. 3, lane 1). On the same Western blots with either antiserum, a cell wall-enriched fraction from transgenic tobacco revealed P30 as a protein of M_r 34K (Fig. 3, lanes 2 and 4).

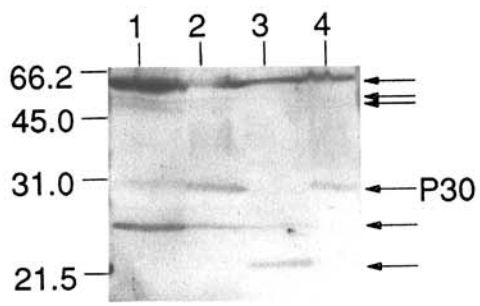

Fig. 2. Western blot analysis of the subcellular distribution of P30 in transgenic *Arabidopsis thaliana*. Fractions containing putative P30 were purified from 56-day-old *A. thaliana* L-*er* plants. Proteins were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose, and probed with antiserum 137. Lane 1, PE30 fraction; lane 2, S30 fraction; lane 3, PE1 fraction and lane 4, cell wall-enriched fraction. The positions of P30, and of nonspecific cross-reacting products detected also in fractions from untransformed plants (←), are indicated. M_r markers are as indicated for Figure 1.

Accumulation of P30 in TMV-infected *A. thaliana*.

Although P30 transgenically expressed in *A. thaliana* appeared to be proteolytically processed at the N-terminus, TMV can give rise to systemic infections in this host. Therefore, either the N-terminally processed P30 can be functional in *A. thaliana* or the processing represents the product of P30 inactivation. To test this, untransformed *A. thaliana* plants were infected with TMV strain U1 and the accumulation of P30 compared with TMV U1 infected tobacco. Figure 4A shows a Western analysis of cell wall-enriched fractions taken from *A. thaliana* plants (L-*er* and Col-O) 49 days postinoculation (dpi) with TMV U1 (lanes 5 to 8), or uninfected plants of the same age (lanes 1 to 4). P30 from TMV-infected nontransgenic plants (lanes 5 and 7) comigrated with that expressed in uninoculated transgenic plants (lanes 2 and 4). Two immunologically cross-reacting proteins of M_r 24K and M_r 55K (e.g., lanes 5 and 8) were also detected in this experiment. The smaller protein was probably a contaminant usually seen in other subcellular fractions from healthy tissue that reacted nonspecifically with antiserum 137 (see Fig. 2).

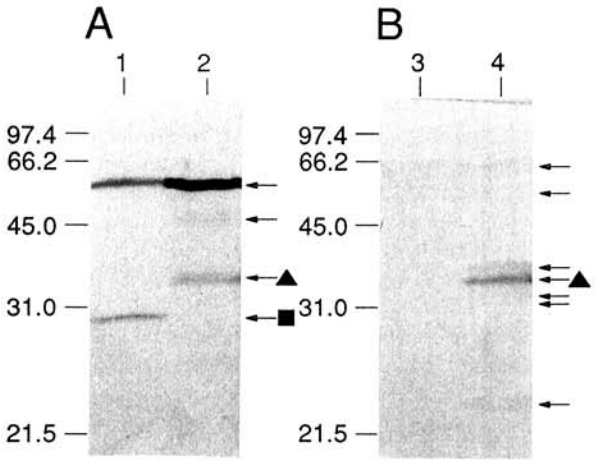

Fig. 3. Western blot analysis of N-terminal processing of P30 in transgenic *Arabidopsis thaliana*. Cell wall-enriched fractions were prepared from 56-day-old *A. thaliana* L-*er* or tobacco plants. Proteins were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose, and probed with antiserum 137 (A) or antiserum R2 (B). Lanes 1 and 3, *A. thaliana* (transgenic line 1); lanes 2 and 4, tobacco (transgenic line 277). The positions of P30 detected after expression in *A. thaliana* (■) and tobacco (▲), and of nonspecific cross-reacting products detected also in fractions from untransformed plants (←), are indicated. M_r markers are as indicated for Figure 1.

This analysis of TMV-infected *A. thaliana* at 49 dpi indicated that the P30 produced by the virus was processed in the same way as the transgenic P30. It might be concluded, therefore, that either the processed P30 is active as a MP or that it is an inactive form that accumulates at late stages of infection. To address the possibility that nonprocessed, functional P30 might be required at times earlier than 49 dpi, P30 accumulation was analyzed in plants infected for only 14 days (Fig. 4B). At this earlier time, TMV-infected *A. thaliana* accumulated a form of P30 (Fig. 4B, lane 5) that was similar, but slightly faster in mobility than, P30 produced in TMV-infected tobacco (Fig. 4, lane 2), and the same mobility as P30 expressed in *E. coli* (Fig. 4, lane 4). It is therefore likely that during the early stages of infection of *A. thaliana* by TMV an unprocessed form of P30 is available to assist virus movement. At later stages (49 dpi) in the infection, perhaps when the major phases of virus invasion were complete, only processed P30 could be detected (Fig. 4, lane 6). P30, derived from infection of untransformed *A. thaliana* L-*er* plants with the Cg strain of TMV (Li et al. 1983), behaved identically with P30 from TMV U1, in all respects (data not shown).

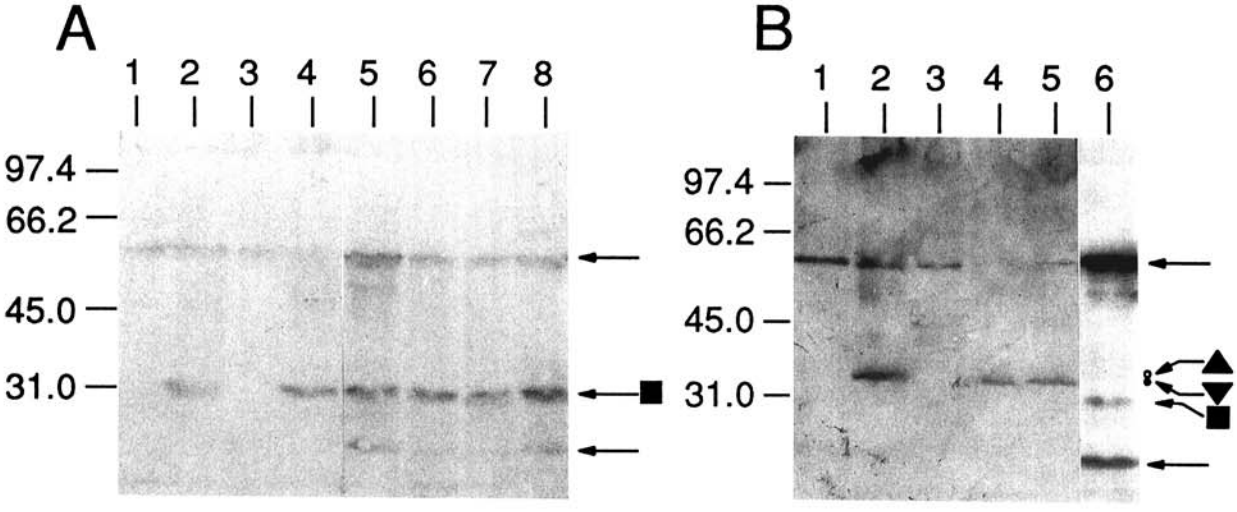

Fig. 4. Western blot analysis of P30 accumulation in tobacco mosaic virus (TMV) U1-infected *Arabidopsis thaliana* and tobacco. **A,** Transformed or nontransformed *Arabidopsis thaliana* plants were infected with TMV U1 and maintained for 49 days (lanes 5 to 8), or left uninoculated (lanes 1 to 4) Proteins from cell wall-enriched fractions were separated on a 12.5% SDS-PAGE gel, transferred to nitrocellulose and probed with antiserum 137. Lane 1, 5; Col-O (untransformed); lane 2, 6; Col-O (transgenic line 3); lane 3, 7; L-*er* (untransformed); lane 4, 8; L-*er* (transgenic line 1). **B,** Proteins from cell wall-enriched fractions from uninoculated tobacco (nontransgenic line 306; lane 1), TMV U1-infected tobacco (nontransgenic line 306; lane 2), uninoculated *A. thaliana* L-*er* (untransformed; lane 3), and TMV U1-infected *A. thaliana* L-*er* (untransformed) harvested 14 dpi (lane 5) or 49 dpi (lane 6) were analyzed as described for A and compared with *E. coli*-expressed P30 (lane 4). The positions of P30 detected after expression in *A. thaliana* (processed ■, nonprocessed), tobacco (▲), and *E. coli* (▼), and of nonspecific cross-reacting products also detected in fractions from healthy plants (←), are indicated. In B, the markers (O,●) are used to highlight the gel shift that was observed between P30 in lane 2 (O) and the comigrating P30s in lanes 4 and 5 (●). M_r markers are as indicated for Figure 1.

In vitro phosphorylation of *E. coli*-expressed P30.

The comigration of *E. coli*-expressed P30 with P30 from *A. thaliana* analyzed 14 days after infection with TMV (Fig. 4B), suggested that P30 may not be phosphorylated in *A. thaliana*. A kinase activity with the capacity to phosphorylate P30 in vitro has been identified in cell wall fractions from uninfected tobacco (Citovsky et al, 1993). To investigate whether *A. thaliana* contained a similar kinase activity, enzyme preparations were prepared from the cell walls of uninfected, nontransformed tobacco (line 306) and from uninfected, untransformed *A. thaliana* L-*er* plants, and used to phosphorylate *E. coli*-expressed P30. Kinase activity was detected by the incorporation of radiolabel from α^{32}P-ATP into proteins of M_r 34K observed by autoradiography of the blotted protein gel (Fig. 5A) and verified as P30 by Western analysis (Fig. 5B). Incubation of *E. coli*-expressed P30 with the tobacco kinase preparation resulted in incorporation of radiolabel into a protein of approximate M_r 34K (Fig. 5, lane 2) that comigrated (Fig. 5, lane 8) with P30 extracted from the P30-transgenic tobacco line 277 (Fig. 5, lane 10). This latter protein could also be phosphorylated (Fig. 5, lane 4) to a lesser extent by the tobacco kinase suggesting that it was incompletely phosphorylated in vivo. Incubation of *E. coli*-expressed P30 with the *A. thaliana* kinase preparation also showed a radiolabeled protein of approximate M_r of 34K (Fig. 5, lane 3) but the Western analysis did not show a comigration with the tobacco-derived phosphorylated form of P30 (Fig. 5, compare lane 9 with lanes 8 and 10). To confirm that this difference in mobility did not reflect differences in protein sample quality for electrophoresis, both kinase-treated samples (Fig. 5, lanes 8 and 9) were combined (Fig. 5, lane 7) and electrophoresed in parallel; two closely migrating proteins were detected by western analysis (Fig. 5, lane 7).

The contradictory indications for a P30-specific kinase activity from *A. thaliana* were resolved in control reactions where both kinase preparations were incubated in the absence of P30 (Fig. 5, lanes 5,6). Whereas the tobacco enzyme failed to produce a radiolabeled protein of M_r 34K (Fig. 5, lane 5), a protein of that mobility was apparent from the *A. thaliana* extract (lane 6) indicating that *A. thaliana* cell wall preparations could phosphorylate an endogenous protein of M_r 34K, but not P30.

The use of a soluble protein fraction from tobacco (mature leaves) and *A. thaliana* (young or mature leaves) as a source of putative kinase, or the inclusion of 4 mM spermidine in assays using *A. thaliana* extracts, also did not result in the phosphorylation of *E. coli*-expressed P30 (data not shown).

Functionality of processed P30 in *A. thaliana*.

The presence of P30 of M_r 30K and M_r 33K in TMV-infected *A. thaliana* did not demonstrate whether the M_r 30K protein could function in virus movement. In a more direct test for the functionality of the processed P30, transformed *A. thaliana* L-*er* plants were challenged with a TMV P30 mutant virus. A frameshift mutant (TE1) of TMV U1 has been shown to be complemented in a P30 transgenic tobacco line to give a full systemic infection (Gera et al. 1995). TE1 and wild-type TMV U1 were inoculated to transformed *A. thaliana* L-*er* plants and to untransformed and transgenic tobacco line 277 plants. Replication and local and long-distance movement of TMV in these infections were assessed by RT-PCR of the coat protein gene. Replication and systemic infection occurred in all the combinations of host and virus, except transformed *A. thaliana* and TE1 (data not shown).

DISCUSSION

P30 expressed in healthy transgenic *A. thaliana* plants was processed by cleavage of an N-terminal portion of the protein. This processing was efficient with only processed P30 being detectable in young or old tissues. TMV U1-encoded P30 was similarly processed to a product with the same electrophoretic mobility as the transgenically expressed protein. The N-terminal region of P30 has been identified as being essential for maintaining the movement function of this protein (Gafny et al. 1992) and the inability to complement the movement of the TMV U1 P30 mutant in transgenic *A. thaliana* plants strongly suggests that processed P30 was nonfunctional in supporting virus movement. The synthesis of a full-length P30 cDNA from transgenic plants indicated that the N-truncated protein did not result from defective gene expression.

The expression of inactive forms of P30 may be expected to confer some level of resistance of the plant to the virus (see Lapidot et al. 1993; Malyshenko et al. 1993); however, similar levels of TMV U1 coat protein were expressed in systemically infected leaves of P30-transgenic and untransformed *A. thaliana* plants (data not shown). Moreover, nonprocessed, functional P30, could be detected in plants 14 days after infection. These observations can probably be explained by differences in the steady-state levels of nonfunctional and functional forms of P30 and the time course of their production. In healthy transgenic plants, P30 would be produced continu-

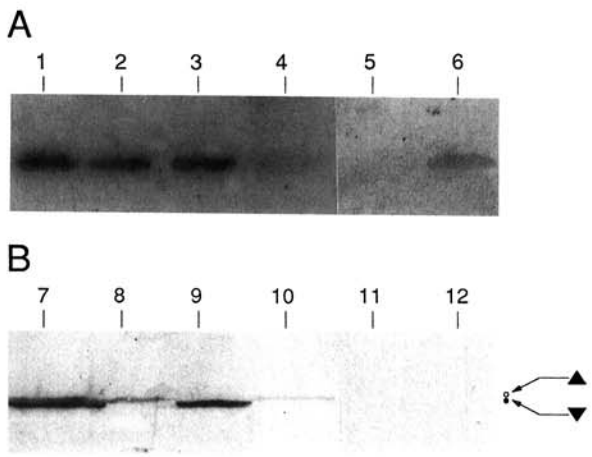

Fig. 5. Assay for cell wall-associated P30-specific kinase activity. Cell wall-enriched fractions were prepared from plant tissue and assessed for kinase activity by incorporation of ^{32}P into proteins of approximate M_r 34K. **A,** The autoradiograph of separated labeled proteins. The same samples were assessed for P30 specificity in a parallel gel by Western analysis with antiserum 137 **(B).** Kinase preparations from untransformed *Arabidopsis thaliana* L-*er* (lanes 3, 6, 9, and 12), nontransgenic tobacco line 306 plants (lanes 2 and 5), or transgenic tobacco line 277 (lane 4) were incubated with (lanes 2, 3, 8, and 9) or without (lanes 4 to 6 and 10 to 12) *E. coli*-expressed P30. The difference in relative mobility of P30 in lanes 8 and 9 was confirmed by combining the samples into the same lane (lane 1 and 7). The positions of phosphorylated (▲) and non-phosphorylated (▼) forms of P30 are indicated.

ously but at a lower level than virus-encoded P30 which is produced only transiently (Lehto et al. 1990). In tobacco, P30 accumulation has been detected predominantly in the early phases of virus multiplication (Blum et al. 1989; Lehto et al. 1990) and is followed by coat protein accumulation. Coat protein of TMV strain Cg accumulates in *A. thaliana* (Col-O) plants to near maximum levels at 14 days after infection (Ishikawa et al. 1991) which, by inference, suggests that P30 accumulates to maximum levels some time before this. It is likely that, in infected plants, the processing of P30 is not rapid enough to prevent the interaction of functional molecules with plasmodesmata, and therefore cannot prevent the spread of the virus. Alternatively, the mechanism may function to reduce the deleterious effects on the plant resulting from the long-term accumulation of functional P30.

The present evidence suggests that *A. thaliana*, in contrast to tobacco, does not produce a protein kinase able to use P30 as a substrate. The detection of nonphosphorylated but functional P30 after virus infection also suggests that phosphorylation of P30 is not necessary for movement protein function. We propose that proteolytic processing of P30 is an alternative mechanism to that described in tobacco (Citovsky et al. 1993) for inactivating P30 function. The processing is probably mediated by the action of a specific proteinase. Attempts, however, to detect the activity of a proteinase from *A. thaliana* which resulted in the cleavage of *E. coli*- or tobacco-expressed P30 to a product of M_r 30K, were inconclusive (unpublished results).

The inactivation of protein function by proteolytic processing following the action of a proteinase is well documented and proteins produced during stress are often processed in this manner (Koizumi et al. 1993; Parsell and Lindquist 1993; Stermer et al. 1994). P30 exerts a profound effect on carbon metabolism and photosynthate partitioning (Lucas et al. 1993) and the possibility for its formation of complexes with host cellular RNAs, though not investigated experimentally, must surely be recognized. Proteolytic processing in *A. thaliana* of a viral protein, whose accumulation in the cell could induce such stress conditions, may not therefore be surprising. The present work has also identified a potential problem when using TMV-*A. thaliana* as a model system for studying plant–viral interactions or when constructing P30-transgenic *A. thaliana* plants to analyze P30 functions.

MATERIALS AND METHODS

Plant material.

Tobacco (*Nicotiana tabacum* cv. Xanthi nn) line 277 transgenic for the P30 gene of TMV U1 and the control nontransgenic line 306 (Deom et al. 1987, 1990) were used in the present study. Tobacco plants were grown in glasshouse conditions where daylight was supplemented by sodium halide lamps (14-h photoperiod) at 20 to 22°C. Seeds of *A. thaliana*, stored at 4°C, were germinated under fluorescent lights on germination medium (Valvekens et al. 1988) with or without kanamycin (50 μg ml^{-1}) in a controlled growth room maintained at 20 to 22°C (16-h photoperiod). When four true leaves had developed (7 days) the seedlings were transferred to a controlled growth room maintained at 18 to 20°C, 60% RH, under fluorescent lights (10-h photoperiod). R_1 progeny of transgenic lines were used in this study.

A. thaliana transformation and selection of transformants.

The P30 gene was excised from pTM934 (Deom et al. 1987) by digestion with *Bam*HI and *Eco*RI. For transformation of *A. thaliana* Col-O plants this fragment was ligated with *Bam*HI/*Eco*RI-digested pJIT30 (Mullineaux et al. 1990). A 2.2-kbp fragment was then excised from the resulting plasmid, pTM30JI1, by digestion with *Kpn*I/*Xho*I and ligated with *Kpn*I/*Sal*I-digested pBIN19 (Bevan 1984) to give the plasmid pTM30JI2. For transformation of *A. thaliana* L-*er* plants the same *Bam*HI/*Eco*RI fragment from pTM934 was blunt-ended with DNA polymerase I, Klenow fragment and ligated with pSLJ642 from which the spectinomycin gene had been removed (*Cla*I/*Xba*I; DNA polymerase I, Klenow) to give the plasmid, pSLJ30K. pSLJ642 (kindly provided by J. D. G. Jones; unpublished data) is a pRK290 based binary vector, constructed from TRI′-NPT-ocs3′ and 35S-SPEC-ocs3′ expression cassettes (Jones et al. 1992). Plasmids pTM30JI2 and pSLJ30K were electroporated directly into *Agrobacterium tumefaciens* C58 (pGV3850) (Zambryski et al. 1983) as described by Nagel et al. (1990). Transformation of *A. thaliana* by *A. tumefaciens* was performed using the root explant transformation method of Valvekens et al. (1988) with minor modifications described by Balcells (1991) and Innes (1991). Transformed root tissue was incubated on kanamycin plates (50 μg ml^{-1}) and regenerated into plants. Seeds were harvested, germinated, and transformants were selected for kanamycin resistance. Those R_1 seedlings showing normal phenotype were allowed to develop and genomic DNA was purified from whole transformed and untransformed plants as described by Dellaporta et al. (1983). Transgenic lines expressing the P30 gene were selected by PCR-amplification of P30 DNA using the conditions described below for the detection of TMV sequences in plants. The P30-transgenic L-*er* line 1 and Col-O line 3 were selected for further study. The copy number of the chimeric gene or a comparison of its level of expression in these transgenic lines was not determined.

Viruses.

Wild-type TMV strain U1 (tobacco mosaic virus, common strain) or TMV strain Cg (Li et al. 1983) or a movement-deficient mutant of TMV U1 (TE1; Gera et al. 1995) containing a frameshift mutation in the P30 gene, were propagated in *Nicotiana tabacum* cv. Xanthi nn (line 277 for TE1) or *A. thaliana* plants. Wild-type virus was purified by the method of Gooding and Hebert (1967). Viral RNA was purified from particles by digestion with proteinase K (Boehringer Mannheim) according to the manufacturer's instructions. Tobacco leaves were mechanically inoculated with virus in 30 mM sodium phosphate, pH 7.0, using Carborundum as an abrasive. Virus was introduced into the leaves of *A. thaliana* by piercing the upper epidermis with a fine needle (26G, 0.45 × 13 mm) several times at 7 to 10 days after germination. In cell-to-cell movement experiments, the proximal half of a single leaf was inoculated as above and the other half was analyzed by Southern blot hybridization (Southern 1975) for the presence of TMV U1 coat protein cDNA, made from RNA.

Detection of TMV sequences in leaf tissue.

Total RNA was extracted from leaf tissue as described by Verwoerd et al. (1989) with the following modifications. After precipitation with lithium chloride and ethanol, the pellets

were redissolved in 10 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$ containing 20 µg ml^{-1} DNAse (RNAse-free) and incubated at 37°C for 1 h. First-strand cDNA, generated by reverse transcription, was used as a template for PCR amplification of P30 cDNA. The reaction (20 µl) consisted of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl; 4 mM $MgCl_2$; 0.01% (w/v) gelatin; 1 mM dATP, dTTP, dCTP, and dGTP; 12 µg of RNAse inhibitor per ml (human placenta, Pharmacia Biotech.); 20 pmoles primer 1 (5′ CAGTCATTAAAACGAATCCGATTCGGCG 3′; complementary to nt 5688 to 5709 of TMVU1); and Moloney murine leukemia virus reverse transcriptase (200 U). After 1 h at 37°C the reaction was heated to 94°C for 1 min and supplemented with 80 µl of buffer consisting of 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 20 pmoles primer 1, 40 pmoles primer 2 (5′ ACGTAGATGGCTCTAGTTGTTAAAGG 3′, nt 4903 to 4922) and Taq DNA polymerase (2.5 U). An 819-bp fragment consisting of 807 bp of P30 cDNA flanked by 6 bp at each end was detected after electrophoresis in 1% (w/v) agarose gels containing ethidium bromide. Potential contamination of RNA preparations from transgenic lines with genomic DNA was analysed by omission of reverse transcriptase from the reactions. TMV coat protein cDNA (480 bp fragment) was detected as described above with the following primers: 5′ ATGTCTTACAGTATCACTACTCCATCTC 3′ (nt 5712 to 5739) and, 5′ TCAAGTTGCAGGACCAGAGGTCCAAACC 3′ (complementary to nt 6164 to 6191) to prime first-strand cDNA synthesis. Fragments containing putative TMV sequences were blotted in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto Hybond-N membrane and hybridized at 65°C overnight with cDNA probes prepared by the random priming method of Feinberg and Vogelstein (1983). Probes were prepared using P30 DNA amplified by PCR from pTM934 (Deom et al. 1987) or from coat protein cDNA amplified by RT-PCR from TMVU1 RNA purified from virus particles; the fragments were purified by electroelution from the agarose gels prior to labeling.

Cloning and sequencing of P30 DNA from transgenic *A. thaliana*.

P30 DNA fragments from transgenic *A. thaliana* line 1 (L-*er*) and line 3 (Col-O) were blunt-ended with DNA polymerase I, Klenow fragment and ligated with *Sma*I digested pUC19. Sequencing of the entire inserts was carried out by the dideoxy chain termination method (Sanger et al. 1977) using the Sequenase Version 2.0 DNA sequencing kit (U.S. Biochemical, Cleveland, OH).

Purification and detection of P30 mRNA.

Total mRNAs were purified from total RNA by binding them to streptavidin-paramagnetic particles using the PolyATract mRNA isolation system as described by the manufacturer (Promega). Between 100 and 600 µg of total RNA was used to prepare mRNA for use as a template in an RT-PCR reaction to detect P30 cDNA by replacing primer 1 with oligo$(dT)_{12\text{-}18}$ to prime first strand cDNA synthesis. P30 cDNA was then amplified by PCR using primers 1 and 2.

Production of P30 in *S. frugiperda* or *E. coli*.

P30 produced by recombinant baculovirus (Ac.TK6) expression in *S. frugiperda* (Sf21) cells was purified using the procedure of Atkins et al. (1991a). P30 produced by overexpression in *E. coli* BL21(DE3)pLysE cells (pETP30, Citovsky et al. 1990) was purified using the procedure of Citovsky et al. (1991) for purifying cauliflower mosaic virus P1.

Production of antiserum to N-terminus of P30.

To express the N-terminal amino acids of TMV U1 P30, a 120-bp fragment corresponding to nt 4903 to 4999 of TMV U1 sequence flanked by *Eco*RI sites was amplified from pTB2 (Hilf and Dawson 1993) by PCR using the conditions described above to obtain P30 cDNA and ligated to *Eco*RI digested pGEX3X (GST gene fusion system, Pharmacia Biotech.). The primers were: 5′ CTCGGAGAATTCTATGGCTCTAGTTGTTAAAGG 3′ and 5′ CAGTCAGAATTCTTTACAGGGTAAACATCG 3′ corresponding to nt 4903 to 4922 and complementary to nt 4979 to 4999 of TMVU1 sequence, respectively (*Eco*RI sites underlined). The vector was transformed into competent *E. coli* JM83 cells. Recombinants were examined for fusion protein expression by SDS-PAGE and the fusion protein of M_r 30.2K was purified by affinity chromatography on glutathione Sepharose-4B to >90% purity as judged by Coomassie blue staining in SDS-PAGE gels according to Pharmacia Biotech. This was used directly for raising polyclonal antibodies in rabbits. Male rabbits (New Zealand white) were injected subcutaneously with 15 µg of fusion protein in 0.5 ml of PBS/Freunds complete adjuvant (1:1). Boosters of 15 µg of fusion protein in 0.5 ml of PBS/Freunds incomplete adjuvant (1:1) were introduced at biweekly intervals. Preimmune and immune sera were tested for their antigenicity towards P30 in Western blots. The immune serum was designated R2.

Subcellular fractionation of P30 from plants.

All operations were carried out at 4°C. Plant tissue (tobacco leaves or whole *A. thaliana* plants, minus roots) were frozen in liquid nitrogen, ground to a fine powder and homogenized in buffer A (1 ml per gram of tissue) consisting of 100 mM Tris-HCl, pH 7.5, containing 10 mM KCl, 5 mM $MgCl_2$, 0.4 M sucrose, 10% (v/v) glycerol and 10 mM β-mercaptoethanol. In some extractions a mixture of protease inhibitors was also included: phenylmethylsulphonyl fluoride (0.2 mM), leupeptin (1 µM), and pepstatin (1 µM). The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 1,000 × *g* for 10 min to give a pellet (PE1) and a supernatant (S1). PE1 was the organellar fraction. The residue on the Miracloth was washed in buffer A containing 2% (v/v) Triton X-100 (4 ml g $tissue^{-1}$) and centrifuged at 3,500 × *g* for 10 min. This procedure was repeated a further four times. The pellet was resuspended (20 µl g $tissue^{-1}$) in urea sample buffer (75 mM Tris-HCl, pH 6.8, containing 4.5% [w/v] SDS, 9 M urea, 1.1 M β-mercaptoethanol and 0.05% [w/v] bromophenol blue); boiled for 10 min; and centrifuged at 12,000 × *g* for 5 min to remove insoluble material. This was the cell wall-enriched fraction. The supernatant (S1) was centrifuged at 30,000 × *g* for 30 min to give a supernatant (S30) and a pellet (PE30). PE30 was redissolved in urea sample buffer (20 µl g $tissue^{-1}$), boiled for 5 min, and centrifuged at 12,000 × *g* for 5 min. PE30 was the microsomal (membrane-enriched) fraction. S30 was combined with 5× SDS-PAGE loading buffer (250 mM Tris-HCl, pH 6.8, containing 0.5 M dithiothreitol, 10% [w/v] SDS, 50% [v/v] glyc-

erol, and 0.5% [w/v] bromophenol blue; this was the soluble fraction).

Detection of P30 expression.

Extracted proteins were transferred from denaturing gels to nitrocellulose filters and probed with antisera to P30 protein (Towbin et al. 1979). Two purified antisera were used to probe Western blots. Antiserum 137 (Deom et al. 1987) was raised against a synthetic peptide corresponding to amino acids 255 to 267 (C-terminus) of P30. Antiserum R2 is described above. Both antisera were purified by cross adsorption with acetone powder obtained from tobacco line 306 (leaf tissue) and untransformed *A. thaliana* L-*er* (whole plants minus roots). P30 antiserum on blots was detected using goat anti-rabbit IgG-alkaline phosphatase conjugate and the chromogenic substrate nitroblue tetrazolium. Marker proteins (Biorad), stained with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid/sulfosalicylic acid in blots, were used to construct a regression line from a $\log_{10} M_r$ versus R_f plot to determine the M_r of unknown proteins.

In vitro phosphorylation assay of *E. coli*-expressed P30.

Cell wall-enriched or soluble fractions containing putative protein kinase were prepared from entire leaves of untransformed *A. thaliana* L-*er* or tobacco line 306 plants according to Citovsky et al. (1993). Mature fully expanded tobacco leaves (12.5 cm) or *A. thaliana* leaves (immature young leaves, 4 mm, or mature fully expanded leaves, 15 mm) were used as a potential source of protein kinase. In vitro phosphorylation assays on *E. coli*-expressed P30 were performed as described by Citovsky et al. (1993) with the exceptions that proteins were transferred onto nitrocellulose filters and radiolabeled proteins were visualized by autoradiography of the nitrocellulose filters. An identical gel was run in parallel, blotted, and probed with antiserum 137 as described above for the detection of P30. In some experiments with *A. thaliana* extracts, the polyamine spermidine (4 mM) was included in the assay mixture since this has been shown to improve the phosphorylation of some plant proteins (Ye et al. 1994).

ACKNOWLEDGMENTS

We would like to thank Tim Wells for the growth and maintenance of tobacco plant material, Roger Beachy for providing seeds of transgenic tobacco line 277 and the control nontransgenic line 306, John Donson and Satoshi Naito for providing, respectively, virus preparations of TE1 (movement-deficient mutant of TMV U1) and the Cg strain of TMV, Carl Deom, Philip Mullineaux, Jonathan Jones, and Vitaly Citovsky for providing pTM934, pJIT30, pSLJ642, and pETP30 respectively, and Simon Deakin for assistance in the production of antiserum R2. We also thank Andrew Davis, Nigel Hannant, and Peter Scott for photography, Pedro Bustamante, Amanda Francis, Corrin Wallis, and Robert Briddon for helpful discussions, and Jeffrey Davies for constructive criticism of the manuscript. This research was supported by a European Union grant (BRIDGE project BIOT CT-90-0156) to RH and a John Innes Foundation studentship grant to M-CP. The work was carried out under MAFF license No. PHF 1419/811/113.

LITERATURE CITED

Atkins, D., Roberts, K., Hull, R., and Bishop, D. H. L. 1991a. Expression of the tobacco mosaic virus movement protein using a baculovirus expression vector. J. Gen. Virol. 72:2831-2835.

Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P., and Beachy, R. N. 1991b. The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. J. Gen. Virol. 72:209-211.

Balcells, L. 1991. Valvekens protocol: A guide to high efficiency transformation. Pages 1-8 in: *Arabidopsis*: The Compleat Guide. D. Flanders and C. Dean, eds. Published as part of UK AFRC *Arabidopsis* Programme.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711-8721.

Blum, H., Gross, H. J., and Beier, H. 1989. The expression of the TMV-specific 30-kDa protein in tobacco protoplasts is strongly and selectively enhanced by actinomycin. Virology 169:51-61.

Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. 1990. The P30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. Cell 60:637-647.

Citovsky, V., Knorr, D., and Zambryski, P. 1991. Gene I, a potential cell-to-cell movement locus of cauliflower mosaic virus, encodes an RNA-binding protein. Proc. Natl. Acad. Sci. USA. 88:2476-2480.

Citovsky, V., McLean, B. G., Zupan, J. R., and Zambryski, P. 1993. Phosphorylation of tobacco mosaic virus cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase. Genes Dev. 7:904-910.

Dellaporta, S. L., Woods, J., and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1:19-21.

Deom, C. M., Oliver, M. J., and Beachy, R. N. 1987. The 30-kilodalton gene product of tobacco mosaic virus potentiates virus movement. Science 237:389-393.

Deom, C. M., Schubert, K. R., Wolf, S., Holt, C. A., Lucas, W. J., and Beachy, R. N. 1990. Molecular characterisation and biological function of the movement protein of tobacco mosaic virus in transgenic plants. Proc. Natl. Acad. Sci. USA. 87:3284-3288.

Deom, C. M., Lapidot, M., and Beachy, R. N. 1992. Plant virus movement proteins. Cell 69:221-224.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Gafny, R., Lapidot, M., Berna, A., Holt, C. A., Deom, C. M., and Beachy, R. N. 1992. Effects of terminal deletion mutations on function of the movement protein of tobacco mosaic virus. Virology 187:499-507.

Gera, A., Deom, C. M., Donson, J., Shaw, J. J., Lewandowski, D. J., and Dawson, W. O. 1995. Tobacco mosaic tobamovirus does not require concomitant synthesis of movement protein for vascular transport. Mol. Plant-Microbe Interact. 5:784-787.

Gooding, G. V., and Hebert, T. T. 1967. A simple technique for purification of tobacco mosaic virus in large quantities. Phytopathology 57:1285.

Hilf, M. E., and Dawson, W. O. 1993. The tobamovirus capsid protein functions as a host-specific determinant of long-distance movement. Virology 193:106-114.

Innes, R. 1991. Transformation of *Arabidopsis thaliana* using Valvekens protocol: Improved root harvest. Pages 9-12 in: *Arabidopsis*: The Compleat Guide. D. Flanders and C. Dean, eds. Published as part of UK *Arabidopsis* Program.

Ishikawa, M., Obata, F., Kumagai, T., and Ohno, T. 1991. Isolation of mutants of *Arabidopsis thaliana* in which accumulation of tobacco mosaic virus coat protein is reduced to low levels. Mol. Gen. Genet. 230:33-38.

Jones, J. D. G., Shlumukov, L., Carland, F., English, J., Scofield, S. R., Bishop, G. J., and Harrison, K. 1992. Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Res. 1:285-297.

Koizumi, M., Yamaguchi-Shinozaki, K., Tsuji, H., and Shinozaki, K. 1993. Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. Gene 129:175-182.

Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J., and Beachy, R. N. 1993. A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. Plant J. 4:959-970.

Lehto, K., Bubrick, P., and Dawson, W. O. 1990. Time course of TMV 30K accumulation in intact leaves. Virology 174:290-293.

Li, Z-Y., Uyeda, I., and Shikata, E. 1983. Crucifer strain of tobacco mosaic virus isolated from garlic. Mem. Fac. Agric., Hokkaido Univ.

13:542-549.
Lucas, W. J., Olesinski, A., Hull, R. J., Haudenshield, J. S., Deom, C. M., Beachy, R. N., and Wolf, S. 1993. Influence of the tobacco mosaic virus 30-kDa movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants. Planta 190:88-96.
Malyshenko, S. I., Kondakova, O. A., Nazarova, J. V., Kaplan, I. B., Taliansky, M. E., and Atabekov, J. G. 1993. Reduction of tobacco mosaic virus accumulation in transgenic plants producing non-functional viral transport proteins. J. Gen. Virol. 74:1149-1156.
Mullineaux, P. M., Guerineau, F., and Accotto, G. P. 1990. Processing of complementary sense RNAs of *Digitaria* streak virus in its host and in transgenic tobacco. Nucleic Acids Res. 18:7259-7265.
Nagel, R., Elliot, A., Masel, A., Birch, R. G., and Manners, J. M. 1990. Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. FEMS Microbiol. Lett. 67:325-328.
Oliver, M. J., Deom, C. M., De, B. K., and Beachy, R. N. 1986. *In vitro* transcription and translation of cloned cDNAs encoding the 30-kDa protein gene of TMV. Virology 155:277-283.
Parsell, D. A., and Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27:437-496.
Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
Stermer, B. A., Bianchini, G. M., and Korth, K. L. 1994. Regulation of HMG-CoA reductase in plants. J. Lipid Res. 35:1133-1140.
Sosnova, V., and Polak, Z. 1975. Susceptibility of *Arabidopsis thaliana* (L.) Heynh. to infection with some plant viruses. Biol. Plant. 17:156-158.
Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.
Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.
Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA 85:5536-5540.
Verwoerd, C. T., Dekker, B. M. M., and Hoekema, A. 1989. A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17:2362.
Wada, K., Wada, Y., Doi, H., Ishibashi, F., Gojobori, T., and Ikemura, T. 1991. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. 19S:1981.
Waigmann, E., Lucas, W. J., Citovsky, V., and Zambryski, P. 1994. Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. Proc. Natl. Acad. Sci. USA. 91:1433-1437.
Watanabe, Y., Ogawa, T., and Okada, Y. 1992. *In vivo* phosphorylation of the 30K protein of tobacco mosaic virus. FEBS Lett. 313:181-184.
Wolf, S., and Lucas, W. J. 1994. Virus movement proteins and other molecular probes of plasmodesmal function. Plant Cell Environ. 17:573-585.
Ye, X. S., Avdiushko, S. A., and Kuc, J. 1994. Effects of polyamines on *in vitro* phosphorylation of soluble and plasma membrane proteins in tobacco, cucumber and *Arabidopsis thaliana*. Plant Sci. 97:109-118.
Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M., and Schell, J. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. 2:2143-2150.