Phosphorylation of tobacco mosaic virus cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase

Vitaly Citovsky,¹ B. Gail McLean, John R. Zupan, and Patricia Zambryski²

Department of Plant Biology, University of California, Berkeley, California 94720 USA

In host plants, cell-to-cell spread of tobacco mosaic virus (TMV) presumably occurs through intercellular connections, the plasmodesmata. TMV movement is mediated by a specific virus-encoded single-strand nucleic acid-binding protein, P30. The mechanism by which P30 operates is largely unknown. Here, we demonstrate that P30 expressed in transgenic plants is a phosphoprotein. We have developed an assay for in vitro phosphorylation of purified P30 by plant cell wall fractions and have localized the phosphorylation sites to amino acid residues Ser-258, Thr-261, and Ser-265. Interestingly, the P30 phosphorylation sites do not correspond to any known consensus phosphorylation sites for protein kinases. While P30 binding to single-stranded DNA (ssDNA) was shown to involve Thr-261, phosphorylation of this residue does not appear to play a role in binding activity. The protein kinase activity contained in the cell wall fractions was developmentally regulated, expressed predominantly in leaves. Within a leaf, this protein kinase activity increased with leaf maturation and correlated with the reported development of secondary plasmodesmata, sites of P30 accumulation. We suggest that phosphorylation may represent a mechanism for the host plant to sequester P30 following its localization to cell walls.

[*Key Words:* TMV movement; plasmodesmata; protein kinase; phosphorylation; protein–nucleic acid binding.]

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In plants, viruses presumably spread from infected to adjacent healthy cells through intercellular connections, the plasmodesmata. Cell-to-cell movement of plant viruses is an active process mediated by specific virus-encoded movement proteins (for review, see Atabekov and Taliansky 1990; Citovsky and Zambryski 1991; Maule 1991; Deom et al. 1992). The best characterized movement protein is the P30 protein of tobacco mosaic virus (TMV). Three biological activities have been attributed to P30: (1) localization to plasmodesmata (Tomenius et al. 1987; Ding et al. 1992); (2) increase in plasmodesmal permeability (Wolf et al. 1989); and (3) cooperative binding to single-strand nucleic acids (Citovsky et al. 1990, 1992a). On the basis of these observations, P30 was proposed to form complexes with the transported genomic TMV RNA and to target these complexes to and through the enlarged plasmodesmata channels (Citovsky and Zambryski 1991).

The molecular mechanism by which P30 operates is still obscure. By analogy with many biological macromolecules, one possibility for control of P30 activity is via post-translational modification, such as phosphorylation. P30 was shown to be phosphorylated when ex-

pressed from a baculovirus vector in insect cells (Atkins et al. 1991). However, the potential ability of host plants to phosphorylate P30 has not been demonstrated directly nor have the P30 phosphorylation sites been identified. Furthermore, the effect of P30 phosphorylation on the biological activity of this protein is unknown.

Here, we demonstrate that P30 is phosphorylated at carboxy-terminal serine (S-258 and S-265) and threonine (Thr-261) residues by a protein kinase contained in the plant cell wall fraction. This study also shows that the cell wall-associated serine/threonine-specific protein kinase is developmentally regulated, with higher activity in older leaves; in immature leaves, this protein kinase activity correlates with the basipetal (tip-to-base) maturation of the leaf. This pattern of P30 phosphorylation correlates with previously reported basipetal accumulation of this protein in leaf cell walls (Ding et al. 1992), suggesting that phosphorylation may represent a mechanism for the host plant to sequester P30 in the mature tissue.

Results

Phosphorylation of P30 by cell wall-enriched fractions of tobacco leaves

Potential phosphorylation of P30 was studied following expression in transgenic plants of *Nicotiana tabacum*,

¹Permanent address: Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794 USA.

²Corresponding author.

the natural host of TMV. Such plants have been shown to produce a functional P30 (Deom et al. 1987, 1990). Protein extracts made from the mature leaves of P30 transgenic plants used here demonstrated that P30 is found predominantly in the cell wall-enriched fraction and minimally in the soluble fractions (not shown; see also Deom et al. 1990). To determine whether P30 expressed in transgenic plants is a phosphoprotein, a P30-containing cell wall-enriched fraction of transgenic tobacco leaves was incubated in the presence of [γ - 32 P]ATP. Figure 1A shows radiolabeling of a 34-kD protein present in cell walls of the P30 transgenic plant (lane 2) but not in a wild-type plant (lane 1). Western blot analysis demonstrated that the radiolabeled 34-kD band comigrated with P30 in the same cell wall-enriched fraction (not shown). These results suggest that P30 expressed in transgenic plants is a phosphoprotein.

Using transgenic plants, however, it was not possible to determine whether P30 was autophosphorylated or was a substrate for a plant protein kinase. To address this question, we used a reconstruction system in which purified P30 (produced in *Escherichia coli*) was added to cell wall-enriched fractions of wild-type tobacco leaves. Figure 1B shows that while P30 alone incubated in the presence of [γ - 32 P]ATP was not phosphorylated (lane 9), incubation of purified P30 with plant cell wall-enriched fractions resulted in phosphorylation of this protein (lane 1).

The protein content in the cell wall-enriched preparation (Fig. 2A, lane 3) was significantly lower (<0.1%) than that of the soluble fraction of tobacco leaves (Figure 2A, lane 2). Under the conditions used (multiple homog-

Figure 1. Phosphorylation of P30 by plant cell wall-enriched fractions. (A) Phosphorylation of P30 expressed in transgenic tobacco. (Lane 1) Cell wall-enriched fraction of a mature wild-type tobacco leaf; (lane 2) cell wall-enriched fraction of a mature P30 transgenic tobacco leaf. (B) Phosphorylation of P30 deletion mutants by a wild-type leaf cell wall-enriched fraction. (Lane 1) P30 (wild-type, 268-amino-acid residues); (lane 2) P30 del 1 (deleted residues 65–86); (lane 3) P30 del 2 (deleted residues 88–113); (lane 4) P30 del 3 (deleted residues 111–125); (lane 5) P30 del 4 (deleted residues 130–185); (lane 6) P30 del 5 (deleted residues 185–225); (lane 7) P30 del 7 (deleted residues 225–268); (lane 8) no P30; (lane 9) P30, no cell walls. The numbers at left indicate the molecular masses (in thousands of daltons) of protein standards. For detailed description of the del mutants, see Citovsky et al. (1990, 1992a).

Figure 2. Soluble and cell wall-enriched fractions of tobacco leaves. (A) Protein content. (Lane 1) Total cell protein extract of a mature tobacco leaf; (lane 2) soluble fraction; (lane 3) cell wall-enriched fraction. Lanes 1 and 2 were stained with Coomassie brilliant blue R-250, and lane 3 was stained with silver; each lane represents fractions obtained from the same amount of leaf tissue. (B) Protein kinase activity. Soluble (lane 1) or cell wall-enriched fractions (lane 2) were incubated with P30, and protein phosphorylation was analyzed as described in Materials and methods. The numbers at left indicate the molecular masses (in kD) of protein standards. The arrowhead indicates the position of P30.

enizations and washes in the presence of high concentrations of Triton X-100; see Materials and methods), membranes of plant cell organelles, such as chloroplasts and nuclei, are lysed (Saxena et al. 1985; Mozner and Kloth 1991). P30 phosphorylation was negligible using the soluble preparation of tobacco leaves (Fig. 2B, lane 1; compare to P30 phosphorylation obtained with cell walls in lane 2). Thus, P30 phosphorylation is unlikely to be caused by a contamination of the cell wall preparation with detergent-soluble proteins. These results indicate the presence of a protein kinase that is tightly associated with the plant cell wall and that can use P30 as substrate.

Mutational analysis of P30 phosphorylation sites

A series of P30 deletion mutants (Fig. 1B) was used to delineate the region containing the sites of phosphorylation. Purified P30 mutants were incubated with wild-type tobacco leaf cell wall-enriched fractions to identify the smallest deletion that blocked phosphorylation. Figure 1B shows that a P30 mutant (del 7) with 43 carboxy-terminal amino acids deleted was unable to be phosphorylated (lane 7). Conversely, internal deletions, which covered most of the P30 sequence, did not affect phosphorylation (lanes 2–6). Our previous studies (Citovsky et al. 1992a) showed that del 7 retains the full single-stranded DNA (ssDNA) and RNA-binding activity of the intact P30, indicating that this mutation does not cause substantial changes in the protein conformation. Thus, the results in Figure 1B suggest that P30 phosphorylation sites are located within the carboxy-terminal part of the protein.

Phosphoamino acid analysis (Fig. 3A) identified serine and threonine as the sites of phosphorylation for both

Figure 3. Characterization of P30 phosphorylation sites. (A) Phosphoamino acid analysis of P30 from the transgenic plant (lane 1) and P30 phosphorylated by a wild-type plant cell wall-enriched fraction (lane 2). Phosphoamino acids were resolved using TLC; the arrows at left indicate positions of unlabeled phosphoamino acid standards. (B) Phosphorylation of P30 substitution mutants by wild-type plant cell wall-enriched fraction. (Lane 1) P30; (lane 2) P30 sb-1; (lane 3) P30 sb-2; (lane 4) P30 sb-3; (lane 5) P30 sb-4; (lane 6) P30 sb-5; (lane 7) P30 sb-6; (lane 8) P30 sb-7; (lane 9) no P30. The numbers at right indicate the molecular masses (in kD) of protein standards.

P30 expressed in transgenic plants (lane 1) and P30 purified from *E. coli* and phosphorylated by the wild-type cell wall-enriched fractions (lane 2). The carboxy-terminal part of P30 contains two clusters of serine and threonine residues [between positions 212–231 and 247–268; Fig. 4A]. To study the potential role of the carboxy-terminal serine/threonine clusters, two substitution mutants in these regions were constructed. The sb-1 mutant targeted the first cluster and contained Ser-212 and Thr-214 substituted with Ala and Ser-218, Ser-226, Ser-227, and Ser-231 substituted with Gly; the sb-2 mutant targeted the second cluster and contained Ser-247 substituted with Gly and Ser-258, Thr-261, Ser-265, and Ser-267 substituted with Ala [Fig. 4A]. Both of the substitution mutants were tested for their ability to be phosphorylated following incubation with cell wall-enriched fractions of tobacco leaves. Figure 3B shows that the sb-1 mutant was phosphorylated (lane 2), similarly to the wild-type P30 (lane 1). Conversely, phosphorylation of sb-2 was blocked completely (lane 3). These results further localize the sites for P30 phosphorylation to Thr-261 and one or more of the four carboxy-terminal serines (at positions 247, 258, 265, and 267) in the second cluster.

To identify precisely the positions of P30 phosphoamino acids, we restored individual serine and threonine residues in the sb-2 mutant. Five single-amino-acid substitution mutants were constructed, sb-3, sb-4, sb-5, sb-6, and sb-7, restoring Ser-247, Ser-258, Thr-261, Ser-265, and Ser-267, respectively. These mutant proteins were purified and analyzed for their ability to be phosphorylated by the cell walls. The results in Figure 3B show that while restoration of Ser-247 and Ser-267 did not result in P30 phosphorylation (lanes 4 and 8), recreation of each individual Ser-258, Thr-261, and Ser-265 residue restored the phosphorylation (lanes 5–7). Thus, the results of P30

mutational analysis identified Ser-258, Thr-261, and Ser-265 as the sites of phosphorylation.

Effect of the P30 carboxy-terminal serine and threonine residues on RNA and ssDNA binding

P30 contains two single-strand nucleic acid-binding domains, A and B [Fig. 4B; Citovsky et al. 1992a]. Domain B includes the entire carboxy-terminal part of the protein (Fig. 4). Here, we studied the potential role of carboxy-terminal serine and threonine residues on the P30 interaction with RNA and ssDNA. In the sb-2 mutant, removal of all four carboxy-terminal serines (at positions 247, 258, 265, and 267) and the threonine residue (at position 261) resulted in a substantial modulation of P30 single-strand nucleic acid-binding activity (Fig. 5). Although sb-2 binding to RNA was almost unaffected, its ability to bind ssDNA was blocked completely (Fig. 5). The effect of individual carboxy-terminal serine and threonine residues on ssDNA binding was then studied. One by one, these residues were restored in the sb-2 mutant to produce substitution mutants sb-3 through sb-7. Separate restoration of Thr-261 (sb-5 mutant) or Ser-267 residues (sb-7 mutant) was sufficient to regain the wild-type level of P30 binding to ssDNA (Fig. 5). Other carboxy-terminal serine residues had no effect on ssDNA binding. Practically no effect on binding to RNA could

Figure 4. Structural and functional domains of P30. (A) P30 carboxy-terminal serine/threonine clusters. Amino acid sequence between positions 210 and 268 is shown in one-letter code; specific amino acid substitutions are shown above (sb-1 - sb-8) or below (sb-9) the corresponding residue in the wild-type sequence. (B) P30 domains. Domains A (residues 112–185) and B (residues 186–268) are responsible for single-strand nucleic acid-binding activity [Citovsky et al. 1992a]; domain C (residues 65–86) may be required for the correct protein folding [Citovsky et al. 1992a]; domain D contains phosphorylation sites and may be involved in anchoring P30 to the cell wall. Numbers in parentheses indicate specific positions of amino acid residues.

Figure 5. Binding of P30 substitution mutants to RNA and ssDNA. (Hatched bars) Binding to ssDNA; (solid bars) binding to RNA. For experimental conditions, see Materials and methods.

be detected for all of the individual substitution mutants (Fig. 5).

The role of P30 phosphorylation sites (Ser-258, Thr-261, and Ser-265 residues) in single-strand nucleic acid binding was studied further using two substitution mutants of this entire region. In the sb-8 mutant, these amino acids were substituted with uncharged alanine residues, whereas in sb-9 they were substituted with negatively charged aspartate residues (Fig. 4A). Figure 5 shows that neither sb-8 nor sb-9 had any effect on ssDNA or RNA binding, suggesting that P30 phosphorylation may not directly affect its interaction with nucleic acids.

Cell wall-associated protein kinase activity as a function of leaf maturation

In addition to the biochemical characterization of P30 phosphorylation by a plant cell wall-associated protein kinase, the phosphorylation assay was used to begin to characterize the enzyme itself. The presence of the serine/threonine-specific protein kinase capable of phosphorylating P30 was determined in cell walls of different vegetative plant organs. The highest level of P30 phosphorylation was observed with a cell wall-enriched fraction of mature leaves (Fig. 6A, lane 2). Phosphorylation of P30 by a cell wall-enriched fraction of the stem was significantly lower (lane 3), and no phosphorylation was detected using cell wall-enriched fractions of tobacco apical bud (which includes the apical meristem with enclosing very young leaves) (lane 1) or roots (lane 4). Soluble fractions of the apical bud (Fig. 6B, lane 1), leaf (lane 2; see also Fig. 2, lane 2) and root tissues (lane 4) were unable to phosphorylate P30. In contrast, the soluble fraction of the stem (Fig. 6B, lane 3) exhibited a low level of P30 phosphorylation comparable to that observed with the stem cell walls (Fig. 6A, lane 3). These results may reflect physiological differences between leaf and stem tissues. Because P30 phosphorylation occurred predominantly in leaf cell walls, the cell wall-associated protein kinase was characterized further using this tissue.

P30 phosphorylation was systematically examined in cell wall-enriched fractions of leaves at different developmental stages. The protein kinase activity was assayed

in young apical leaves (3.3 and 5.2 cm long), an intermediate leaf still undergoing expansion growth (9.8 cm long), and a mature fully expanded leaf (13.5 cm long). Because leaf maturation begins at the tip and progresses toward the base (Turgeon 1989; Ding et al. 1992), each of the leaves (with the exception of the youngest 3.2-cm apical leaf) was sampled at three distinct developmental regions: the tip, the midsection, and the base (Fig. 7).

Figure 7 shows that the cell wall-enriched fraction of the youngest leaf (3.2 cm) was unable to phosphorylate P30. This is consistent with the absence of the cell wall-associated protein kinase activity in the tobacco apical bud (Fig. 6A, B, lane 1). The next, more expanded, leaf (5.2 cm) showed a detectable level of protein kinase activity in the cell wall-enriched fraction from its most mature tip part, whereas the less mature tissue from the midsection and base of the same leaf lacked this kinase activity. High levels of P30 phosphorylation were observed using cell wall-enriched fractions of the tip and midsection segments of the intermediate-size leaf (9.8 cm); still, a lower level of P30 phosphorylation was observed with the least mature basal part of the same leaf. All three segments (tip, midsection, and base) of the mature fully expanded leaf (13.5 cm) produced high levels of P30 phosphorylation (Fig. 7). These results suggest that the cell wall-associated protein kinase activity closely follows basipetal leaf development.

Alternatively, correlation between P30 phosphorylation and leaf development could be attributed to high

Figure 6. P30 phosphorylating activity in various tobacco plant organs. Cell wall-enriched (A) or soluble (B) fractions of a wild-type tobacco apical bud (lanes 1), mature leaf (lanes 2), stem (lanes 3), and roots (lanes 4) were prepared and analyzed for their ability to phosphorylate purified P30 as described in Materials and methods. The numbers at left indicate the molecular masses (in kD) of protein standards. Arrowheads indicate the position of P30. (C) Inability of young leaf cell wall-enriched fraction to dephosphorylate P30. (Bar Y) Cell wall-enriched fraction from a young apical leaf incubated with P30; (bar M) cell wall-enriched fraction from a mature leaf incubated with P30; (bar Y + M) cell wall-enriched fraction from a mature leaf incubated with P30 followed by addition of an equal amount of the young apical leaf cell walls and continued incubation for 30 min at 25°C. All other experimental conditions were as described in Materials and methods. P30 phosphorylation was quantified by counting radioactivity in the P30 bands excised from a Coomassie brilliant blue R-250-stained polyacrylamide gel.

Figure 7. Phosphorylation of P30 as function of leaf maturation. Tobacco leaves of the indicated sizes were removed from the plant and their tip (T), midsection (M), and base (B) segments were excised. Tissue samples of similar size (0.07–0.09 g) of these segments were used to prepare cell wall-enriched fractions; these fractions were assayed for their ability to phosphorylate purified P30. Identical results are routinely obtained with similar sized leaves from independent tobacco plants.

levels of a putative phosphatase activity in immature leaves. To test this possibility, P30 phosphorylated by mature leaf cell walls (Fig. 6C, bar M) was incubated further with a cell wall-enriched fraction from a young leaf. Although the latter cell wall preparation was unable to phosphorylate P30 (Fig. 6C, bar Y), its presence did not affect P30 phosphorylation by the mature leaf cell walls (bar M + Y). Thus, the basipetal pattern of P30 phosphorylation by tobacco leaf cell walls likely reflects developmental changes in protein kinase activity rather than in phosphatase activity.

Discussion

In this study we developed an assay for *in vitro* phosphorylation of the TMV movement protein P30 by cell wall-enriched fractions of tobacco leaves. Using this system, the P30 phosphorylation sites were localized to the carboxy-terminal Ser-258, Thr-261, and Ser-265 residues (Fig. 4). Phosphorylation patterns of endogenous P30 (i.e., P30 expressed in transgenic plants) and exogenously added P30 (i.e., P30 produced in *E. coli* and phosphorylated by wild-type plant cell wall-enriched fractions) were similar. Because P30 transgenic plants have been reported to produce a functional movement protein (Deom et al. 1987), the P30 phosphorylation described in this study likely reflects a native biological modification of this protein.

The possible role of P30 phosphorylation sites (Ser-258, Thr-261, and Ser-265) and their flanking serine residues (Ser-247 and Ser-267) in single-strand nucleic acid binding was examined using specific amino acid substitutions. The specificity of this interaction (i.e., P30 binding to ssDNA vs. binding to RNA) was substantially

modified by substitution of amino acid residues Thr-261 and Ser-267, one of which (Thr-261) represents a P30 phosphorylation site; this substitution of both residues resulted in inhibition of ssDNA binding with little or no effect on binding to RNA. Our previous results (Citovsky et al., 1992a) show that deletion of the entire carboxy-terminal part of P30 had no effect on P30 interaction with ssDNA or RNA. Thus, the effect of amino acid substitutions at positions 261 and 267 may be the result of P30 conformational changes. This observation predicts an important role of Thr-261 and Ser-267 residues in maintaining the native conformation of the full-length P30 protein.

Previous studies suggested the presence of at least three functional P30 domains (Fig. 4B). The region between amino acid residues 68–86 (domain C) was suggested to be involved in the correct folding of the active protein (Citovsky et al. 1992a), and the regions between amino acid residues 112–185 and 185–268 were shown to contain two independently active single-strand nucleic acid-binding domains, A and B (Citovsky et al. 1992a). Using *in vitro* phosphorylation experiments, a phosphorylated region, designated domain D, was then identified in the carboxyl terminus of P30 (Fig. 4B). Interestingly, the P30 phosphorylation sites do not correspond to any known consensus phosphorylation sites for protein kinases (as searched using the PROSITE computer program).

Does P30 phosphorylation play a biological role in virus interaction with host plants? To address this question, one should consider the effects of P30 on the physiology of the host plant. The two known activities of P30, single-strand nucleic acid binding and increase in plasmodesmal permeability, could alter normal cellular functions. For example, active P30 may form complexes with cellular RNAs and interfere with host cell metabolism. Also, P30-induced increase in plasmodesmal permeability may alter intercellular communication, an important biological process. Thus, inactivation or attenuation of P30 activity may be critical for survival of the host plant. We propose that phosphorylation functions to deactivate P30 by sequestering it to plant cell walls. The following observations support this model. (1) Young apical leaves of P30 transgenic plants lack the protein kinase activity responsible for P30 phosphorylation; and in these leaves, P30 is found predominantly in the soluble fraction (Deom et al. 1990; V. Citovsky, unpubl.). (2) Mature leaves with the highest levels of P30 phosphorylation efficiently accumulate P30 in their cell walls (Deom et al. 1990; V. Citovsky, unpubl.). (3) Young apical leaves, potentially unable to sequester P30 by phosphorylation, are more susceptible to virus infection (for review, see Culver et al. 1991). (4) Deletion of the carboxy-terminal 33 amino acids (which include the P30 phosphorylation sites) results in a slight but consistent increase in the size of TMV-induced necrotic lesions on the infected leaves (Gafny et al. 1992). This increase in TMV virulence may be due in part to the inability of the host plant to sequester the truncated P30.

The present study of P30 phosphorylation in tobacco

describes a serine/threonine-specific protein kinase activity tightly associated with the plant cell wall. While many protein kinases have been found in plants [Ranjeva and Boudet 1987; Lawton et al. 1989; Feiler and Jacobs 1990; Alderson et al. 1991; Ferreira et al. 1991; Harper et al. 1991; Lin et al. 1991; Stein et al. 1991; Suen and Choi 1991; Trewavas and Gilroy 1991], none have been described as a cell wall-associated protein kinase. Here, the cell wall-associated protein kinase activity appears to be developmentally regulated, present predominantly in mature or near-mature leaves.

As the leaf matures, its cells elongate; to maintain symplastic continuity, mature cells develop a new type of intercellular connection, the secondary plasmodesmata. In contrast to primary plasmodesmata, which form during cell division, secondary plasmodesmata are more complex morphologically [Ding et al. 1992] and are formed by insertion into existing cell walls (for review see Robards and Lucas 1990). In tobacco leaves, secondary plasmodesmata are found to develop basipetally; furthermore, in P30 transgenic plants, secondary plasmodesmata specifically accumulate P30 [Ding et al. 1992]. Because plasmodesmata are often found in isolated cell wall fractions from plant tissue [Taiz and Jones 1973; Mozner and Kloth 1991], the correlation between the development of secondary plasmodesmata and the protein kinase activity suggests an intriguing possibility that the cell wall-associated protein kinase may represent a functional component of secondary plasmodesmata. Alternatively, this protein kinase may be involved in a signal transduction pathway that leads to secondary plasmodesmata formation. Thus, the cell wall-associated protein kinase is potentially a molecular marker for the development of secondary plasmodesmata.

Materials and methods

Preparation of plant cell wall-enriched fractions

Wild-type tobacco plants (*N. tabacum* cv. Turk) or transgenic tobacco expressing P30, produced as described previously [Citovsky et al. 1992b], were used as sources of plant tissue. Frozen plant tissue (2 grams) was ground to a fine powder and homogenized in 2 ml of buffer H (0.1M (HEPES) at pH 7.4, 10 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF) plus 2% Triton X-100. Following centrifugation (1000g for 5 min at 4°C), the pellet was homogenized in 10 volumes of buffer H plus 2% Triton X-100 and centrifuged again. This procedure was repeated twice followed by six washes (1000g for 5 min at 4°C) in buffer H plus 2% Triton X-100 and two washes in buffer H. The resulting white insoluble material was suspended in one volume buffer H, aliquoted, quick-frozen in liquid nitrogen, and stored at -70°C.

Preparation of P30 produced in *E. coli*

Deletion mutants of P30 were described previously [Citovsky et al. 1990, 1992a]. Substitution mutants of P30 were constructed using oligonucleotide-directed mutagenesis [McClary et al. 1989] and verified by dideoxynucleotide sequencing [Kraft et al. 1988]. The amino acid substitutions made in all substitution mutants are described in the text and in Figure 4A. P30 and its deletion and substitution derivatives were produced in *E. coli*,

purified to near homogeneity as described previously [Citovsky et al. 1990, 1992a], and verified by Western blot analysis [Lehto and Dawson 1990].

Phosphorylation assay

Soluble (80–100 µg of protein) or cell wall-enriched fractions (10–20 µg of protein in 25 µl of buffer H) were mixed with 5 µl of 10× kinase buffer (0.2 M HEPES at pH 7.4, 50 mM MgCl₂, 0.5 M KCl) and 20 µl of buffer H alone or containing 2 µg of P30 or its mutant derivatives. The reaction was started by the addition of 0.5 µl of [γ -³²P]ATP (equal to 5 µCi, 3000 Ci/mmol), continued for 15 min at 25°C, and stopped with 50 µl of extraction buffer [75 mM Tris-HCl (pH 6.8), 9 M urea, 4.3% SDS, 7.5% β -mercaptoethanol [Lehto and Dawson 1990]]. The samples were boiled for 5 min and centrifuged (12,000g for 5 min at 25°C), and the extracted proteins were resolved on 12.5% SDS-polyacrylamide gels [Laemmli 1970]. Following gel electrophoresis, the proteins were either electrotransferred onto Immobilon-P membranes or stained with Coomassie brilliant blue R-250. Radiolabeled proteins were visualized by autoradiography of the Immobilon-P membranes or dried gels.

Phosphoamino acid analysis

Radiolabeled bands of P30 were excised from the Immobilon-P membranes, and the protein was hydrolyzed in 6 N HCl for 1 hr at 100°C [Kamps 1991]. Eluted hydrolysis products were dried in vacuo, resuspended in 1 ml of double-distilled water, redried, resuspended in 4–5 µl of water, and mixed with 2 µl of unlabeled phosphoamino acid standards [1 mg/ml in first dimension thin-layer electrophoresis (TLE) buffer [Hunter and Sefton 1980]]. Phosphoamino acids were separated and identified by thin-layer chromatography (TLC) [Cooper et al. 1983].

Gel mobility shift assay

Radioactively labeled 75-mer DNA oligonucleotide [nucleotides 600–674 of the *virE2* locus of *Agrobacterium* pTiC58 plasmid [Hirooka et al. 1987] end-labeled by phosphorylation with T4 polynucleotide kinase [Ausubel et al. 1987]] and 182-nucleotide-long synthetic RNA [produced from the *petD*-d24 template, as described previously [Citovsky et al. 1990]] were used as probes for the binding of P30. Indicated amounts of protein were incubated for 15 min at room temperature with 0.1 µg of the probe; samples were then loaded onto a 4% native polyacrylamide gel and electrophoresed as described previously [Citovsky et al. 1990, 1992a]. Following electrophoresis, the gels were dried and autoradiographed. For quantification of binding, radioactive protein-probe complexes were excised from dry polyacrylamide gels and their radioactivity was determined by counting Cerenkov radiation.

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