An *Arabidopsis thaliana* protein interacts with a movement protein of *Turnip crinkle virus* in yeast cells and *in vitro*

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Plant virus movement proteins bind host components to promote virus movement from initially infected cells to neighbouring cells. In this study, cDNA clones encoding p8 and p9, two small proteins required for the movement of *Turnip crinkle virus*, were used as ‘bait’ in a yeast two-hybrid system to screen an *Arabidopsis thaliana* cDNA library for interactive proteins. One *A. thaliana* clone was identified that encodes a protein, designated Atp8, which interacted with p8 in yeast cells and *in vitro*. The apparent full-length of Atp8 mRNA was sequenced and shown to encode a protein with two possible transmembrane helices, several potential phosphorylation sites and two ‘RGD’ sequences.

Introduction

The process of infection by plant viruses depends upon cell-to-cell movement of virus within a plant host, which is in some cases followed by systemic movement. Virus-encoded movement proteins (MPs) are usually, if not always, required for movement, and many viruses require other viral proteins such as the coat protein (CP) for cell-to-cell and/or systemic movement. The movements of several plant viruses are host dependent and cell-type specific, suggesting that specific host factors play key roles (Waigmann et al., 1998). While several viral MPs have been characterized as RNA- and ssDNA-binding proteins (Citovsky et al., 1990; Pascal et al., 1994), localized to plasmodesmata, and shown to increase the size-exclusion limits of plasmodesmata (Wolf et al., 1991; Waigmann et al., 1994), the molecular mechanisms of movement and interactions with host components remain largely unknown. The MP of *Tobacco mosaic virus* (TMV) associates with the cytoskeleton (Heinlein et al., 1995; McLean et al., 1995), and plant virus MPs, in general, are hypothesized to interact with cellular macromolecules through hydrophobic interactions (Mushegian & Koonin, 1993). Recently, interactions between plant proteins and the MPs of *Cucumber mosaic virus* (CMV; Ham et al., 1999), *Tomato spotted wilt virus* (TSWV; Soellick et al., 2000) and TMV (Dorokhov et al., 1999; Chen et al., 2000) have been described.

*Turnip crinkle virus* (TCV) encodes two small MPs, p8 and p9, which are both required for (Hacker et al., 1992), and can function in trans in (Li et al., 1998) cell-to-cell movement. In an effort to identify host proteins that interact with the TCV-encoded movement proteins, an *Arabidopsis thaliana* cDNA library was screened in a GAL4-based yeast two-hybrid system against TCV p8 and p9 ‘baits’. One *A. thaliana* cDNA clone was identified that encodes a protein, tentatively designated Atp8 (*A. thaliana*/p8), which interacted with p8 in yeast cells and *in vitro*. Atp8 mRNA was cloned and sequenced to show that Atp8 mRNA potentially encodes a protein with two possible transmembrane helices, several potential phosphorylation sites and two ‘RGD’ sequences. We suggest that the characteristics of Atp8 are consistent with those expected of a host protein for participation in plant virus cell-to-cell movement.

Methods

- **Recombinant plasmid ‘baits’ in the yeast two-hybrid system.** DNA fragments of TCV p8, p9 or the TCV CP were amplified from pTCV-3d† (Heaton et al., 1989) by PCR and inserted into the polylinker site of pGBT9 of the GAL4-based MATCHMAKER Two-Hybrid System (Clontech) to generate DNA-binding domain/target protein hybrid plasmids. Briefly, a BglII-tagged upstream primer (5’ GAACCTTagatGGATGATCTGGAACG 3’) and a SalI-tagged downstream primer (5’ CTGACATgctagCTTAGAATGTTG 3’) were used to amplify p8 cDNA fragments, an EcoRI-tagged upstream primer

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(5’ AAAGaattcAAAGTTTCTGCTAGTCA 3’) and a BanHI-tagged downstream primer (5’ CTTCGgaattcTAGATCTTTTCCA 3’) were used to amplify p9 cDNA fragments, and an EcoRI-tagged upstream primer (5’ CGTgaattcGAAAATGATCC 3’) and a BanHI-tagged downstream primer (5’ GAggatccACTATTACCTTCC 3’) were used to amplify CP cDNA fragments, all from pTCV-3d1. Each amplified cDNA fragment was cut with the corresponding restriction enzymes and ligated to pG TB9 to construct the DNA-binding domain/target protein hybrid plasmids pGBT-p8, pGBT-p9 and pGBT-pCP, respectively.

pGBT-p8 cut with N hel was re-ligated to construct pGBT-p8d1, which had a deletion of amino acid residues 36 and 37. The ends of N hel cut pGBT were ‘polished’ with the Klenow fragment of DNA polymerase I (GibcoBRL) and re-ligated to construct pGBT-p8d2, which has a deletion of the C-terminal half of the 72 residue protein.

■ cDNA library screen, DNA sequencing and analysis. DNA from an A. thaliana cDNA library (Clontech) was inserted into the GAL4 activation domain of pGAD10 (Clontech) to generate activation domain/A. thaliana hybrid plasmids. The activation domain/A. thaliana hybrid plasmids were amplified in E. coli strain DH5α, and plasmid DNA was prepared according to the manufacturer’s protocol.

Recombinant ‘bait’ vectors, the DNA-binding domain/target protein hybrid plasmids pGBT-p8, pGBT-p9 and pGBT-pCP, were used to transform yeast strain HP7c by the mini-transformation protocol described by Gietz et al. (1992). Yeast cells harbouring recombinant ‘bait’ vectors were transformed with the activation domain/A. thaliana hybrid plasmids by the large-scale transformation described by the manufacturer of the cDNA library. Transformants were spread on selective medium and incubated at 30 °C for 4–6 days. Filter assays for β-galactosidase activity were as described by the manufacturers of the MATCHMAKER Two-Hybrid System (Clontech). Yeast colonies were lifted from agar plates with circles of sterile Whatman #1 filter paper. The filters with colonies were frozen in liquid nitrogen and subsequently allowed to thaw at room temperature. The filters were placed, colony-side up, on a second filter presoaked in ‘Z’ buffer/X-Gal solution comprising 100 ml ‘Z’ buffer (20 mM NaH2PO4, 40 mM Na2HPO4, pH 7.0, 10 mM KCl, 1 mM MgSO4), 167 ml X-Gal stock solution (20 mg/ml of 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside in N,N-dimethylformamide), and 0.27 ml β-mercaptoethanol. The filters were incubated at room temperature. After the appearance of a blue colour, generally after 30–60 min, the filters were aligned with the agar plates to identify β-galactosidase-producing colonies. The β-galactosidase-positive colonies were re-streaked onto selection medium (SD, –Trp, –Leu) to allow segregation of cDNA fusion plasmids, and well-isolated colonies were reassayed as described above for β-galactosidase activity.

Leu’ Trp’ transformant strains were generated according to the manufacturer’s protocol. Briefly, Leu’ Trp’ LacZ’ transformants were cultured for 1–2 days in liquid SD synthetic medium that lacked Leu and contained Trp. These conditions maintained the activation domain/A. thaliana hybrid plasmids, but the DNA-binding domain/target protein hybrid plasmid (pGBT-p8) was randomly lost from approximately 10–20% of the transformants. Samples of SD agar plates were plated onto SD agar with Trp and without Leu and incubated at 30 °C for 2–3 days. Colonies were transferred onto each of two SD agar plates, one lacking Leu and Trp and the other lacking only Leu. The Trp auxotrophs (those that grew on + Trp but not on − Trp plates) were assayed for β-galactosidase activity. Colonies that were positive for β-galactosidase activity were discarded as false positives and those that were negative were tested further.

The remaining candidate plasmids were isolated and used to transform yeast strain HP7c in the following combinations: (1) a candidate plasmid alone, (2) a candidate plasmid and pGBT9 (no ‘bait’ insert), (3) a candidate plasmid and pGBT-p8, and (4) a candidate plasmid and pGBT-pCP, which encodes TCV CP, a non-interacting protein. Cotransformants in combinations 2, 3 and 4 were selected on SD synthetic medium that contained His, but lacked Trp and Leu. Transformants in combination 1 were selected on SD medium that lacked Leu, but contained Trp and His. Candidate plasmids in combinations 1, 2, 3 and 4 that resulted in positive β-galactosidase assays were discarded as false positives. Candidate plasmids in combination 3 that resulted in positive β-galactosidase assays were used in additional transformations to further eliminate false-positive clones.

Positive two-hybrid interactions were further verified by cotransforming yeast strain SYP526 (supplied in the MATCHMAKER kit with pGAD interacting candidates and pGBT-p8. The lacZ reporter gene in strain SYP526 is under the control of a different promoter than in strain HP7c. The two promoters share only the GAL4-responsive elements. Therefore, positive two-hybrid interactions observed in both strains are likely to require binding of the GAL4 DNA-binding domain specifically to the GAL4-responsive elements (Bartel et al., 1993). After the elimination of false positives, one candidate activation domain/A. thaliana hybrid plasmid remained that was designated pAt p8-1.

To isolate additional cDNA clones of At p8 mRNA, the cDNA insert of pAt p8-1 was 32P-labelled with the RadPrime DNA Labelling System (Gibco BRL) and used to screen the A. thaliana cDNA library in E. coli by in situ hybridization as described by Maniatis et al. (1982).

Sequences of cDNA inserts were determined with a DNA Sequence Kit, Version 2.0 (USB) and the recommended protocol. A portion of the sequencing was performed by an ‘in-house’ sequencing group (College of Veterinary Medicine, KSU) using an ABI 373A DNA Sequencer, Version 1.2.1. Nucleotide and deduced amino acid sequences were analysed with the PC/Gen software package (IntelliGenetics).

■ Protein expression, purification and in vitro binding assay. The glutathione S-transferase (GST) expression vector pGEX-cs3 (kindly provided by D. Parks, Oregon State University, USA) was used to express p8 and At p8 in E. coli. An upstream primer (5’ GAACCTTA-GATCTGGATGATCTTTCCA 3’) and an EcoRI-tagged downstream primer (5’ GAggatccACTATTACCTTCC 3’) were used to amplify p8 cDNA fragments from pTCV-3d1. Amplified p8 cDNA fragments were digested to completion with BamHI and with EcoRI, and ligated to BamHI/EcoRI-digested pGEX-cs3 to construct pGEX-p8. pGEX-p8 was cut with N hel and ligated to construct mutant pGEXp8d1 with a two amino acid deletion. N hel-digested pGEX-p8 was ‘blunt-ended’ and re-ligated to construct mutant pGEX-p8d2 with a C-terminal deletion. cDNA fragments of At p8-1 were isolated by EcoRI digestion of pAt p8-1, and ligated into EcoRI-digested pGEX-cs3. The plasmids were used to transform E. coli strain DH5α. Protein expression was induced by 0.3 mM IPTG. GST fusion proteins were purified on glutathione-Sepharose 4B (Pharmacia) following the recommended protocol. A full-length At p8 fragment was cloned into the expression vector pPROEX1 (Gibco BRL). His-At p8 was induced in E. coli by addition of 0.6 mM IPTG, and purified on Ni–NTA resin (Gibco BRL). The Protein Biotinylation System (Gibco BRL) and the recommended protocol were used to label purified His-At p8 and GST–At p8 with biotin. Approximately 2.0 µg each of the purified proteins was blotted to a nitrocellulose membrane (Schleicher & Schuell) through a ‘slot-blot’ manifold (Bio-Rad). Blots were probed with approximately 5.0 µg of biotin-labelled His-At p8/GST–At p8 in Tris-buffered saline (TBS; Tris base, 20 mM, pH 7.5, NaCl, 500 mM), washed in Tween–Tris-buffered saline (TTBS; TBS + 0.05% Tween 20), incubated with ExtrAvidin–alkaline phosphatase (Sigma) in TBS, washed in TTBS and assayed in an NBT–BCIP alkaline phosphatase solution.
DNA, RNA and protein blots. Two TCV p8 mutants were constructed by the methods described above. pTCV-3d1, which contains a full-length cdna copy of the TCV genome, and two deletion mutants, pTCV-p8d1 and pTCV-p8d2, were linearized with XhoI, and transcribed in vitro as described by Heaton et al. (1989). Nicotiana benthamiana plants or isolated protoplasts were inoculated with in vitro-synthesized viral RNAs as previously described (Heaton et al., 1989).

Genomic DNA was isolated from 3- to 4-week-old A. thaliana plants as described by Gelvin & Schilperoort (1988). A. thaliana genomic DNA was digested to completion with EcoRI or BamHI, resolved in 0-9% agarose gels, and transferred to nylon membranes (Schleicher & Schuell). DNA blots were probed with radiolabelled Atp8 DNA fragments (Maniatis et al., 1982). A Trizol RNA Kit (GibcoBRL) and its recommended protocol were used to isolate total RNA from plant tissues. An Oligoex mRNA Kit (Qiagen) was used to purify mRNA from total RNA. mRNA or total RNA was resolved in 1% agarose gels, and transferred to nylon membranes (Schleicher & Schuell). RNA blots were hybridized with a radiolabelled cdna probe as previously described (Heaton et al., 1989). Protein samples were prepared as previously described (Heaton et al., 1991). Proteins were resolved in a 10% polyacrylamide–SDS gel and blotted to nitrocellulose membranes. The blots were probed with an anti-TCV CP serum as previously described (Heaton et al., 1989).

Results

pAtp8-1 identified by interaction of its protein product with the product of pGBT-p8 in yeast cells.

One of the TCV movement proteins, p8 (pGBT-p8), was used as ‘bait’ against an A. thaliana ecotype Col-0 cDNA library constructed in the vector pGAD10. Theoretically, 10^7 clones that contained both a bait plasmid and a cdna plasmid were spread on a selective medium in each independent screening, but an average of 150 clones grew on SM medium were spread on a selective medium in each independent screening, but an average of 150 clones grew on SM medium. Of approximately 2200 clones that grew on SM, 36 were initially β-galactosidase positive. After elimination of false positive clones as described in Methods, one positive clone remained, which was designated pAtp8-1.

A C-terminal portion of p8 is required for interaction with Atp8

In an effort to determine if the product of pAtp8 specifically interacts with TCV p8, the ORFs for several proteins, including TCV CP and TCV p9, as well as the MP of Tomato bushy stunt virus (p22; Hearne et al., 1990; Schothof et al., 1995), were tested in yeast cells. Yeast strain HF7c was co-transformed with pAtp8-1 plus pGBT-CP, pGBT-p9 or pGBT-p22. Neither the marker gene His^+ nor lacZ was activated in yeast. These results further substantiate that the product of pAtp8-1 interacts specifically with TCV p8 in yeast cells.

To begin delimitation of the regions of p8 responsible for the interaction with Atp8, two deletion mutants of p8 were generated. In p8d1, two amino acid residues (36 and 37) in the central region of the 72 residue protein were deleted, and in p8d2 the C-terminal half of the protein was deleted by introducing a stop codon after 33 amino acid residues. Mutant p8d1 interacted with pAtp8-1 in yeast cells, but p8d2 did not. Thus, the N-terminal half of p8 is apparently not sufficient for interaction with Atp8.

Atp8 interacts with p8 in vitro

An in vitro protein-binding assay was used to further confirm the interaction between Atp8 and p8. E. coli-expressed and purified GST–p8, GST–p8d1, GST–p8d2, GST, His–Atp8, and additional control proteins including BSA and the GibcoBRL high molecular mass marker proteins (myosin, phosphorylase b, bovine serum albumin, carbonic anhydrase, β-lactoglobulin and lysozyme), were blotted onto a nitrocellulose membrane. The membrane was probed with biotin-labelled His–Atp8 in TBS. As shown in Fig. 1, full-length His–Atp8 showed a strong interaction with GST–p8 and GST–p8d1 but not with itself, GST–p8d2, GST or the other control proteins. These results demonstrate that Atp8 specifically interacts with p8, but fails to interact with the N-terminal half of p8 in vitro. The N-terminal portion of Atp8, which interacted with p8 in the original two-hybrid screen, showed the same interaction with p8 in vitro (data not shown). These in vitro results substantiated the interaction in yeast cells.

Atp8 genes show restriction fragment length polymorphisms in three differentially susceptible A. thaliana ecotypes

Blots of genomic DNA from three differentially TCV-susceptible A. thaliana ecotypes, Dijon-0, Col-0 and No-0,
were probed with an Atp8 cDNA fragment to ascertain gene distribution. Col-0 is the most susceptible to TCV in that it shows more severe symptoms than the other two ecotypes (data not shown), while Di-0 is relatively resistant to TCV infection (Oh et al., 1995). The blot analysis showed that Atp8 genes are most likely present as single-copy genes in all three genomes. Polymorphisms in the lengths of restriction fragments were observed after complete digestion with BamHI, EcoRI or MunI (GibcoBRL) (Fig. 2a). RNA blot analysis showed that Atp8 mRNAs were present at approximately equal levels in all three ecotypes (Fig. 2b).

**Phenotypes of p8 mutants**

The two p8 mutations described above were introduced into the TCV genome resulting in TCV-p8d1 and TCV-p8d2. Wild-type and mutant viral RNAs were transcribed in vitro, and used to inoculate *N. benthamiana* plants. No symptoms were observed in plants inoculated with either TCV-p8d1 or TCV-p8d2. Total RNAs and proteins were isolated from inoculated and upper leaves and used in RNA and protein blots. As shown in Fig. 3(a, b), no viral RNA or CP was detected in leaves inoculated with TCV-p8d1 or TCV-p8d2.

![Fig. 2. Blot analyses of *A. thaliana* genomic DNA and mRNA. (a) Genomic DNA isolated from *A. thaliana* ecotypes Dijon, No-0 or Col-0 was digested with BamHI, EcoRI or MunI as labelled, resolved in a 0–9% agarose gel, blotted to a nylon membrane, and hybridized with 32P-labelled, Atp8-specific DNA. (b) Total RNAs isolated from 3-week-old plants of *A. thaliana* ecotype Dijon, No-0 or Col-0 were resolved in a 1% agarose gel, blotted to a nylon membrane, and hybridized with 32P-labelled, Atp8-specific DNA.](image)

Isolated *N. benthamiana* protoplasts were also inoculated with *in vitro*-synthesized viral RNAs. Twenty-four hours after inoculation, protoplasts were collected, and total proteins and RNAs were isolated and used in RNA and protein blot analyses. As shown in Fig. 3(c, d), both mutant viral RNAs and CPs accumulated to wild-type levels, demonstrating that both p8 mutants replicated and accumulated to wild-type levels. The lack of symptoms and accumulation in inoculated plants was, therefore, due to inefficient cell-to-cell movement *in planta*.

**Atp8 is potentially a membrane protein with two ‘RGD’ sequences**

The partial Atp8 cDNA fragment identified in the yeast two-hybrid system was approximately 0.5 kb long and did not represent the full-length (2–3 kb) of Atp8 mRNA, as determined by RNA gel analysis. To obtain the full-length sequence of Atp8 mRNA, the *A. thaliana* cDNA library was screened with 32P-labelled partial Atp8 cDNA fragments. Five clones were identified by hybridization with partial Atp8 cDNA fragments and their inserts were sequenced. The sequences overlapped and represented the 2–2.2 kb full-length sequence of Atp8 mRNA. The deduced amino acid sequences of Atp8 is listed in Fig. 4. The sequence of Atp8 mRNA contains a non-coding 5′ sequence of 131 nucleotides and a 3′ non-coding region of 306 nucleotides upstream of a poly(A) tail. One long ORF was identified that potentially encodes a 67 kDa protein. The original Atp8 clone isolated from yeast cells encoded amino acid residues 66–236 of Atp8. The nucleotide sequence of Atp8 mRNA showed significant identity to two *A. thaliana* cDNA sequences (accession nos
An Arabidopsis protein binds TCV MP

Fig. 4. Deduced amino acid sequence of Atp8. Atp8 mRNA potentially encodes a protein with two RGD cell-attachment sequences (bold) and two transmembrane helices (bold italics). Residues encoded in the original clone that interacted with p8 in yeast cells are italicized.

N96677 and H76652) of unknown function, and the deduced amino acid sequence of Atp8 aligned with a single A. thaliana protein (AC084165) of unknown function. Atp8 contains two possible transmembrane helices, several potential phosphorylation sites and two ‘RGD’ sequences (Fig. 4).

Discussion

We used a yeast two-hybrid system to identify an A. thaliana protein, tentatively designated ‘Atp8’, that interacts with p8, one of two small MPs encoded by TCV. The interaction in yeast was verified in vitro. The amino acid sequence of Atp8 is 91% identical to an A. thaliana protein (accession no. AC084165) of unknown function, and has two possible transmembrane helices, several potential phosphorylation sites and two ‘RGD’ sequences.

Atp8 mRNAs are expressed to approximately equal levels in the differentially TCV-susceptible A. thaliana ecotypes Dijon-0, Col-0 and No-0 (Fig. 2B). Atp8 genes show restriction fragment length polymorphisms among the three ecotypes, which had nucleotide sequence differences among Atp8 genes (Fig. 2A). Further studies are needed determine whether the nucleotide differences result in amino acid substitutions that affect TCV resistance and susceptibility. Our preliminary mutagenesis study of the Atp8–p8 interaction is inconclusive. While a mutation of p8 that eliminated the Atp8 interaction also eliminated cell-to-cell movement in a plant host, a second p8 mutation that did not affect the interaction with Atp8 also eliminated cell-to-cell movement.

While speculation from the presence of amino acid sequence motifs is no substitute for empirical data, we believe the presence of two ‘RGD’ cell-attachment sequences in Atp8 deserves attention. RGD-containing proteins and their receptors function in numerous biological phenomena. RGD sequences are recognized by integrins (Campbell et al., 2000), which, in turn, linked to the cytoskeleton (Ruoslathi & Pierschbacher, 1987). RGD-integrin interactions are a major recognition system for adhesion to cells of a large number of adhesive extracellular proteins (Ruoslathi, 1996), and integrin interactions with the actin-based cytoskeleton play important roles in cell signalling processes, including calcium mobilization, protein phosphorylation and alterations in cytoplasmic pH (Hynes, 1992). Integrin-like proteins that display physiological and RGD-binding properties similar to the animal integrins have been described in a broad range of plant species including A. thaliana (Laval et al., 1999; Nagpal & Quatrano, 1999; Canut et al., 1998; Faik et al., 1998), Rubus fruticosus (Faik et al., 1998), maize (Labboure et al., 1999) and Valliseria gigantea (Ryu et al., 1997).

In addition to normal physiological functions, the RGD cell-attachment signal plays a critical role in several animal virus–host cell interactions (Baxt & Becker, 1990; Bergelson et al., 1993; Roivainen et al., 1994; Shafren et al., 1997; Wickham et al., 1993). The cell-surface receptors for the viral RGD motifs are integrins (Roivainen et al., 1994; Wickham et al., 1993), and the cytoskeletal network plays a critical role in receptor-mediated internalization of virus (Kizhatil & Albritton, 1997). Recruitment of the actin cytoskeleton is thought to be an important feature of RGD–integrin-mediated virus internalization.

Recent advances in the elucidation of the mechanisms of TMV movement include findings that the TMV MP associates with the plant cytoskeleton through unknown interactions (Heinlein et al., 1995), and that an extracellular enzyme involved in cell-wall extension (Nari et al., 1991), pectin methylesterase (PME), specifically binds the TMV MP (Chen et al., 2000). These findings are consistent with a model of TMV MP function in which an extracellular or cell surface ‘RGD’
protein (like PME or Atp8) binds the MP, and an integrin-like protein that interacts with the cytoskeleton in turn, recognizes the ‘RGD’ protein. The MP–RGD–integrin complex could function in the intracellular movement and targeting of TMV by the same mechanisms as function for several animal viruses. We should note that the subcellular localization of Atp8 has not yet been determined so we do not know if it is an extracellular, cell-surface or intracellular protein. We also add that, while the RGD sequences are very rare among proteins in general, several PME accessions (AAF63815, T07593, P24791) contain RGD sequences.

While much remains to be done to demonstrate whether the ‘RGD’ motifs of Atp8 and other proteins play a role, we suggest that the idea that TCV, like many animal viruses and perhaps several plant viruses, has recruited RGD–integrin–cytoskeleton trafficking interactions to facilitate virus movement within plant hosts is indeed a tantalizing one.

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