

Introduction

Viral movement is an essential characteristic of a virus being well-adapted to the host. It is known that a virus must be able to both replicate and move in its host to cause a systemic infection, however, the exact mechanisms of movement remain unknown. Plant viruses have specialized movement proteins to help move the virus throughout the host. Using a host plant cDNA library, some host factors involved in virus movement have been studied using the yeast two-hybrid system. This system allows for yeast growth in nutrient deficient conditions based on protein-protein interactions. The introduction will attempt to familiarize the reader with plant viruses, cell to cell movement, systemic movement, geminiviruses, and the yeast two-hybrid system.

Plant Viruses

Viruses are obligate intracellular pathogens that use host machinery to create viral progeny. Animal viruses are able to establish a primary infection by attaching to cellular membrane receptors and entering host cells. After replication and release from the initial infected host cell the animal virus moves through the host using mainly either the circulatory, lymphatic, or nervous system (Fields *et al.*, 1996). Plant viruses also need to move throughout their hosts in order to establish a systemic infection. The similar goal is accomplished by a different method however. Plant viruses move through their hosts by two different mechanisms. G. Samuel in 1934 when writing about tobacco mosaic virus (TMV) stated a theory that there was a slow cell to cell movement of virus via the plasmodesmata combined with a rapid distribution through the plant phloem (Carrington *et al.*, 1996). After initial replication in a plant cell the virus moves cell to cell through the plasmodesmata in the cell wall that attaches neighboring cells. The local cell-to-cell

movement cannot establish a systemic infection for the virus, however, for which the virus must be able to enter the plant's long distance transport system. Many viruses can replicate within a host, however, only viruses that are well adapted to their host are able to move systemically and cause plant disease symptoms.

Cell to Cell Movement

After initial replication the virus moves to adjoining cells to further produce viral progeny. Each plasmodesma is ~50 nm in diameter lined with a plasma membrane that combines both adjoining cells, contains a desmotubule extending through linking endomembrane systems of neighboring cells, and protein globules with bridging proteins that form linkages between the plasma membrane and desmotubules (Lucas *et al.*, 1993; Lucas and Gilbertson 1994). Primary plasmodesmata are comprised of a single plasma membrane-lined channel while secondary plasmodesmata are branched and formed across preexisting cell walls (Oparka *et al.*, 1999a). Viruses are able to pass through the plasmodesmata by increasing the size exclusion limit (SEL) from <1 kD for passive diffusion to ~10 fold higher (Carrington *et al.*, 1996, Wolf *et al.*, 1991). The TMV movement protein (MP) has been shown to increase the SEL to allow a 20 kD dextran across the plasmodesmata and can affect the SEL several cells away (Waigmann *et al.*, 1994). The age of the host is a factor as there is a substantial decrease in the plasmodesmal permeability during the transition in a leaf from sink to source as a plant matures (Oparka *et al.*, 1996). Another method of cell to cell movement is through tubules which project unidirectionally into one cell and are derived from plasmodesmata that have lost their desmotubules (Maule 1991). Specialized movement proteins accomplish the movement of a plant virus from cell to cell through the plasmodesmata.

Capsid proteins are also sometimes needed although the method of movement with a capsid protein is often tubule induced (Santa Cruz *et al.*, 1998). The movement proteins are believed to have been acquired by viruses from host functions similar to the *knotted 1* homeobox protein that moves cell to cell by increasing the SEL and transports its own mRNA (Hake 1992). Viral movement proteins can bind ssRNA or ssDNA, increase the SEL, and colocalize with components of the cytoskeleton and ER-derived membranes (Boyko *et al.*, 2002). To move the viral genome within the cell the MP may use the host movement system and associations between the movement proteins and actin, myosin, and microtubules have been found (Boyko *et al.*, 2002). The microtubules and filamentous actin may in fact deliver the viral genome to the plasmodesmata in concert with the movement protein (Lazarowitz and Beachy 1999).

Systemic Movement and Host Interactions

The phloem is a long-distance transport system in plants to deliver nutrients and messages throughout the plant. To enter the phloem a plant virus must pass through specialized plasmodesmata from the epidermis, through the mesophyll, into the bundle sheath and phloem parenchyma, and finally into the companion cell-sieve element complex and back out (Carrington *et al.*, 1996). The entry into and then back out of the phloem is essential for the plant virus to move throughout the plant. The SEL gating can differ from the mesophyll to the companion cell and the TMV movement protein alone can not increase the SEL of bundle sheath and phloem parenchyma plasmodesmata (Carrington *et al.*, 1996, Ding *et al.*, 1992). Immunocytochemical analysis has shown that viruses unable to enter the phloem parenchyma or companion cells are blocked in systemic movement at entry into or out of the sieve element (Schaad and Carrington

1996). Using the heavy metal cadmium in tobacco plants the exit from the sieve element was blocked for the tobacco adapted turnip vein clearing virus suggesting that the entry into and then out of the phloem may be in some cases by different methods (Citovsky *et al.*, 1998). Viral proteins found in the sieve element must have been transported there for the sieve element possesses no protein synthesizing capacity (Santa Cruz *et al.*, 1998). Plant viruses have established many different ways to move within the different hosts they infect. The umbraviruses do not contain a coat protein and in nature are dependent upon luteoviruses for aphid transmission. However, without a luteovirus the umbravirus can still move systemically in *Nicotiana bethamiana* and *N. clevelandii* plants. The umbravirus ORF3 protein can stabilize viral RNA and allows for movement. TMV, which normally needs a capsid protein for rapid systemic movement, was also able to move systemically in the presence of ORF3 (Ryabov *et al.*, 2001). Capsid proteins are important in the sieve element because unprotected viral RNA in the translocation stream inside the sieve element would probably be degraded (Oparka and Turgeon 1996). In order for movement to occur through the phloem the viral movement proteins and capsid proteins probably must interact with host proteins. The virus is able to move by adapting to the way the host plant normally moves essential hormones and proteins throughout the plant. The CmPP16 protein from *Cucurbita maxima* was shown to possess properties similar to viral movement proteins. The messenger RNA was shown to have the ability to move through the phloem whereas the protein was confined to the sieve element. The CmPP16 protein showed functional similarity and limited sequence similarity to the movement protein of red clover necrotic mosaic virus (Xoconostle-Cazares *et al.*, 1999). Alanine-scanning mutations in the movement protein of red clover necrotic mosaic virus

showed that mutants exhibited host-specific defects in the ability to enter the companion cell-sieve element from the bundle sheath and/or phloem parenchyma (Wang *et al.*, 1998). A brome mosaic virus strain, M2, with an expanded host range was studied and the RNA3, which encodes both the movement protein and the coat protein, was believed to have several distinct functions that contributed to the adaptation to new hosts (De Jong and Ahlquist, 1995). A later study examining the movement proteins of M2 showed the difference at four amino acid positions (De Jong *et al.*, 1995).

Geminiviruses

Geminiviruses are single stranded DNA viruses that are divided into three different genera depending upon their transmission vector (van Regenmortel *et al.*, 2000). Members of the genus *Mastrevirus*, have a monopartite genome, infect primarily monocotyledons, and are transmitted by leafhoppers. Members of the second genus, *Curtovirus*, contain a monopartite genome, infect dicotyledons, and are transmitted by either leafhoppers or treehoppers. The third genus, *Begomovirus*, has members with monopartite or bipartite genomes, that infect dicotyledons, and are transmitted by whiteflies (van Regenmortel *et al.*, 2000). The bipartite begomoviruses include both squash leaf curl virus (SLCV) and bean golden mosaic virus (BGMV). Both viruses can establish an infection in *N. benthamiana*, however, BGMV is not well adapted and accumulates 25-fold less viral DNA than tomato golden mosaic virus (TGMV), induces no visible symptoms and requires a functional capsid protein for systemic movement (Petty *et al.*, 1995; Pooma *et al.*, 1996).

The bipartite geminiviruses contain two circular ssDNA components named A and B, each of which is separately encased in a twinned icosahedral capsid. The A

component contains 5 open reading frames (ORFs) of varying functions while the B component contains only 2 ORFs with the ORF being named after their respective DNA component and direction of transcription (Figure 1). The AL1 protein is the only essential protein needed for DNA replication, but the efficiency is enhanced by the actions of a functional AL3 (Hanley-Bowdoin *et al.*, 1990; Sunter *et al.*, 1990). AL1 acts as a site-specific endonuclease to initiate rolling circle DNA replication (Laufset *et al.*, 1995). The AL2 protein controls the transcriptional levels of the AR1 and BR1 genes (Sunter and Bisaro 1992). AR1 is the coat protein which is required for whitefly transmission (Azzam *et al.*, 1994). The other A component ORF AL4 is either non-functional or dispensable in plant infections for TGMV (Pooma *et al.*, 1996) and BGMV (Hoogstraten *et al.*, 1996).

The B component of bipartite geminiviruses encodes two movement proteins, BR1 and BL1. BR1 and BL1 are believed to act in concert to potentiate virus movement in a host plant and although both show host adaptation the compatibility between them is of far greater importance (Schaffer *et al.*, 1995). The BR1 and BL1 proteins are each necessary for both local and systemic movement. The AL1 replication protein and AL2 trans-activator of BR1 and AR1 are also required for successful systemic movement of TGMV in *N. benthamiana* (Jeffrey *et al.*, 1996). Geminiviruses are DNA-based and must replicate in the host nucleus. BR1 is believed to be a shuttle protein that binds viral ssDNA and moves the complex across the nuclear membrane into the cytoplasm where BL1 can join and move through the plasmodesmata to adjacent cells (Lazarowitz and Beachy 1999). The BR1 of SLCV has in fact been immunolocalized to the nucleus of phloem parenchyma cells of systemically infected pumpkin and squash leaves and shown

to have properties of a typical ssDNA binding protein (Pascal *et al.*, 1994). In coat protein mutants of TGMV the ability to accumulate ssDNA in plants has been shown to be BR1 dependent (Jeffrey *et al.*, 1996). Microinjection studies showed that the BR1 protein of bean dwarf mosaic virus redirects ssDNA and dsDNA from the nucleus to the cytoplasm and although the passageway for movement from the nucleus to the cytoplasm has not yet been determined it is believed to be through the nuclear pores used by cellular proteins and RNA (Görlich and Mattaj 1996). The BL1 protein has been shown to alter the SEL of plasmodesmata and induce the transport of fluorescently labeled nucleic acids to adjacent cells (Noueiry *et al.*, 1994 Ding *et al.*, 1995). One model of geminivirus movement states that while the BR1 protein is usually localized to the nucleus the BR1:BL1 complex is redirected to the cell periphery when both are expressed and after the movement through the plasmodesmata to a new cell the BR1 redirects the viral ssDNA to the nucleus (Sanderfoot and Lazarowitz 1995). Another model of movement suggests that BR1 acts intracellularly while BL1 moves intercellularly and any interaction between the two is to impose a size constraint on the viral genome (Rojas *et al.*, 1998).

The ability of the AR1 coat protein to affect movement is also controversial. The AR1 protein is needed for the systemic movement of monopartite geminiviruses (Rojas *et al.*, 2001). There is no clear role of AR1 in the cell-to-cell spread of bipartite geminiviruses, however, AR1 may have a role in systemic spread. The coat protein is essential in virus systemic movement in poorly adapted virus host combinations, such as BGMV in *N. benthamiana* (Pooma *et al.*, 1996). A host specific or virus specific adaption of the BR1 movement protein may determine the need for a functional AR1 coat

protein in systemic spread (Pooma *et al.*, 1996). A second opinion of AR1 function in movement was expressed in a paper on mutations in the BR1 protein that were masked by a functional AR1 coat protein (Qin *et al.*, 1998). The conclusions by the authors stated that AR1 and BR1 may compete in the nucleus for ssDNA to bind which would exclude encapsidated virions from any role or ability to move in the host (Qin *et al.*, 1998).

The Yeast Two-Hybrid System

The yeast two-hybrid system can be utilized to detect specific protein-protein interactions (Gyuris *et al.*, 1993). The two-hybrid system can be used to detect interactions between two cloned proteins or for searching a DNA library for a gene encoding a protein that may interact with a known bait protein. The LexA based system is able to select for protein-protein interactions due to the expression of a nutritional reporter gene. This allows for the search of large numbers of yeast containing a constructed bait protein with a fusion protein or library. The bait protein is inserted into a pLexA vector containing a *HIS3* marker (figure 2) while the prey library or fusion protein vector, pB42AD, contains a *TRP1* marker (figure 3). The fusion gene in pB42AD can only be expressed in media that contains galactose as the carbon source. Media using glucose as the carbon source will repress expression of the fusion protein. The interaction between a bait and a prey fusion protein will induce the expression of a reporter gene, *LEU2*, allowing for the growth of yeast that contain interacting proteins on minimal medium lacking leucine (figure 4). While yeast two-hybrid systems are sensitive enough to detect relatively weak protein-protein interactions the detection of an interaction does not necessarily indicate that there is a corresponding interaction between

the proteins in their natural environment (Fields and Sternglanz 1994). Potential host interactions necessary for the cell-to-cell and systemic movement of geminiviruses were studied the using yeast two-hybrid system to test the geminivirus movement proteins against a host *N. benthamiana* cDNA library while keeping in mind that artifacts of the yeast two-hybrid system may show up as positives while not necessarily being biologically relevant. The movement proteins used in the screen were the BGMV BR1 and SLCV BR1 movement proteins and the BGMV AR1 coat protein. Interactions between the movement proteins BGMV BR1, SLCV BR1, BGMV BL1, and the BGMV AR1 coat protein were also evaluated.

Materials and Methods

Polymerase chain reaction

5 ng of plasmid DNA was added to 10 μ L 10x PCR Buffer (500 mM KCl, 100 mM Tris-HCL pH 9.0, 1% Triton® X-100), 10 μ L 25 mM MgCl₂, 8 μ L 5mM dNTPs, 5 μ L of each primer(100ng/ μ l), 50 μ L sterile dH₂O, 1 μ L *Taq* 5,000 u/mL DNA polymerase (Promega). The sample was then topped with 100 μ L mineral oil and placed into a Perkin-Elmer PCR machine. The samples was run through 35 cycles of the following: 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 1 minutes. 10 μ L of the sample was electrophoresed to check for products.

Electrophoresis

Separation by electrophoresis through a 1% agarose (BRL, electrophoresis grade) in TBE (89 mM Tris base, 89 mM boric acid, 2 mM disodium EDTA, pH 8.0) gel containing 1 μ g/mL ethidium bromide. The gel was run for 1 hour at 90 volts.

Topo isomerase-mediated cloning of PCR products

Each reaction (1 μ L PCR product, 1 μ L salt solution (1.2 M NaCl, 0.06 M MgCl₂) 3 μ L sterile water, and 1 μ L topo isomerase-activated pCR2.1 vector) was set up in an Eppendorf tube and the reaction mixed by flicking with finger. After incubating at room temperature for 5 minutes, 2 μ L of the reaction was added to 25 μ L one shot competent *Escherichia coli* Top10F' cells and placed on ice for 5 minutes. The cells were heat shocked in a 42°C water bath for 30 seconds and then placed on ice immediately. 250 μ L SOC media (2% bactotryptone, .5% yeast extract, 1% 1 M NaCL, 1% 2 M glucose, 1% 2 M MgCl₂, .25% KCl) were added; the tube was capped tightly and shaken at 37°C and 200 rpm for 1 hour. Aliquots of 10 μ L and 50 μ L of the transformation mixture, plus 190

μL dH₂O or 150 μL dH₂O on LB plates (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 2% bactoagar, pH 7.5) containing 50 $\mu\text{g}/\text{mL}$ kanamycin. The plates were incubated overnight at 37°C.

Endonuclease digest

Restriction endonuclease digests were performed according to the manufacturer's directions. The appropriate buffers and reagents were added following instructions. Most digests were incubated for approximately 1.5 hours but some required an overnight incubation at the appropriate temperature. Digests were analyzed by electrophoresis on 1% agarose gels in either TBE or TAE (400 mM Tris base, 50 mM sodium acetate, and 10 mM disodium EDTA pH 7.8) buffer.

Photography

Photographs of agarose gels and yeast dilution plates were taken using either the Polaroid camera, film type 667 or a gel doc video system (BIO-RAD).

Competent bacterial cells

A single colony of *E. coli* DH5 α was inoculated into 3 mL of L broth (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) and grown overnight at 37°C with shaking. 25 μL of overnight culture was subcultured into 7.5 mL of broth in a sterile 15 mL plastic centrifuge tube and grown at 37°C with shaking for 4 hours. Bacteria were pelleted by centrifuging for 3 minutes at approximately 5000 rpm. The culture supernatant was decanted and the pellet resuspended in 7.5 mL of cold 50 mM CaCl₂ by gentle vortexing. After incubation on ice for 20 minutes the cells were again centrifuged at 5000 rpm. After pouring off the supernatant the pellet was resuspended in 1 mL of cold 50 mM CaCl₂.

Transformation of *E. coli*

200 μ L of competent cells were added to each Eppendorf tube with the appropriate amount of DNA (0.2 μ L of purified plasmid DNA, or a complete ligation reaction) for each transformation. Cells were incubated on ice for 40 minutes, heat-shocked in a 42°C waterbath for 3 minutes, and transferred back to ice for 5 minutes. 0.8 mL of 2xTY broth (1.6% bactotryptone, 1% yeast extract, 0.5% NaCl) was added and cells were placed in 37°C waterbath for at least 10 minutes. 200 μ L aliquots of each transformation were spread onto L plates containing 50 μ g/mL ampicillin. Plates were allowed to dry and incubated at 37°C overnight.

Small scale preparation of plasmid DNA (miniprep)

A single colony from a transformation plate was picked and placed in 3 mL L broth containing 100 μ g/mL ampicillin. The culture was grown overnight at 37°C with shaking. 1.5 mL of the culture was poured into an Eppendorf tube and centrifuged for 1 minute to pellet the cells. The pellet was resuspended in 100 μ L of TEG (25 mM Tris-HCl, pH 8, 10 mM EDTA, 50 mM glucose). When cells were completely resuspended, 200 μ L of freshly prepared alkaline-SDS (200 mM NaOH, 1 % sodium dodecyl sulfate) was added. The tube was capped and mixed by several rapid inversions and left on ice for 5 minutes. 150 μ L of 3M potassium acetate (pH 4.8) were added, mixed thoroughly, and centrifuged at 4°C for 5 minutes. The supernatant was recovered in a clean eppendorf tube and 0.5 mL phenol:chloroform was added, mixed vigorously to emulsify, and centrifuged at RT for 2 minutes to separate the phases. The aqueous phase was recovered in a clean eppendorf tube 1 mL of ethanol added, mixed by rapid inversion, and left to stand at room temperature for 2 minutes. After centrifuging at room

temperature for 5 minutes the supernatant was removed by decanting. The pellet was dried under a vacuum for 10 minutes. The pellet was resuspended in 50 μ L of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) containing 50 μ g/mL RNase A and incubated at 37°C for 30 minutes. 1 μ L of 10 mg/ml proteinase K was then added and the incubation continued at 37°C for another 30 minutes. 50 μ L of phenol:chloroform was added and mixed vigorously to emulsify. After centrifugation for 3 minutes to separate the phases the aqueous phase was recovered in a clean eppendorf tube. To the phenol phase 50 μ L dH₂O was added, mixed, and centrifuged again. The two aqueous phases were pooled together and 100 μ L of chloroform added, mixed, and centrifuged for 1 minute. The aqueous phase was recovered in a clean eppendorf tube and 25 μ L of 10 M ammonium acetate, 250 μ L of ethanol were added. After mixing gently, the samples were stored at -20°C overnight to precipitate the DNA. 10 minutes of centrifugation at 4°C pelleted the DNA and after completely removing the supernatant and vacuum drying the pellet for 10 minutes the DNA was resuspend in 50 μ L of dH₂O and stored at -20°C.

Gel purification of DNA

A restriction digest was separated by electrophoresis on a 1 % agarose gel in TBE containing ethidium bromide. After cutting out the required bands with a razor blade the gel slices were weighed in an Eppendorf tube to determine the volume. 0.5 gel slice volumes of “TBE modifier”(GeneCleanII kit, Bio101), and 4.5 gel slice volumes of NaI solution were added to the Eppendorf tube. The liquids were mixed gently and incubated in a 50°C waterbath for 5 minutes to dissolve the agarose. If the agarose was not completely dissolved after 5 minutes of incubation, the tube was vortexed gently at room temperature until it was. 5 μ L of thoroughly resuspended “Glass-milk” (GeneClean II kit,

Bio101) was added and mixed hard to disperse the glass evenly. After incubating at room temperature for 5 minutes the glass was pelleted by centrifugation and the supernatant removed. 0.5 mL of “NEW wash”(GeneCleanII kit, Bio101) was added to the glass pellet and mixed vigorously to resuspend. The glass pellet was pelleted again by centrifugation and the supernatant removed. The “NEW wash” was added twice more for a total of three washes and after removing the supernatant from the final wash, the glass pellet was allowed to dry on the bench for 10 minutes (the open tube covered with a KimWipe to prevent airborne particles from dropping in it). The DNA was eluted by adding 10 μ L of dH₂O to the dried pellet. The pellet was mixed very gently to resuspend the glass in the water without splashing up the sides of the tube (glass will dry there and any bound DNA will be lost). The pellet was incubated in 50°C waterbath for 5 minutes, centrifuged for 1 minute to pellet the glass, and the supernatant transferred to a fresh Eppendorf tube. The elution step was repeated to elute all of the bound DNA and the supernatant pooled with the first one. The success of the gel purification was checked by electrophoresis of 5 μ L of the eluted fraction on an agarose gel.

Transformation of yeast

5 mL of YPD were inoculated with a yeast colony (strain EGY48) and incubated at 30°C with shaking at 250 rpm overnight. A sample of the culture was used to make 1:10 and 1:100 dilutions in sterile distilled water (dH₂O). A 10 μ L aliquot of the 1:100 dilution was placed in a hemocytometer and a portion of the overnight culture containing 2.5×10^8 cells was added to 50 mL of YPD and incubated at 30°C with shaking at 250 rpm for four hours. After the four hour incubation period the cells were counted again to ensure that the cell population had doubled. The culture was then transferred to a sterile

50ml falcon tube and spun at 3,000 rpm for 5 minutes. The supernatant was poured off and the pelleted cells were resuspended in 25 mL of sterile dH₂O. The cells were resuspended and spun again for 5 minutes. After the supernatant was again removed from the cell pellet it was resuspended in 1 mL of 100mM lithium acetate. The suspension was transferred to a 1.5 mL Eppendorf tube and centrifuged to pellet the cells. The cells were resuspended in 450 μ L of 100mM lithium acetate and 50 μ L aliquots for each transformation were aliquoted into 1.5 mL Eppendorf tubes, spun down, the supernatant removed. The transformation mix (240 μ L 50% polyethylene glycol 3350, 36 μ L 1.0 M lithium acetate, 25 μ L sheared and denatured (2 mg/mL) salmon sperm DNA, 1 μ L of plasmid, and 49 μ L sterile dH₂O) was directly added to the cell pellet which was then resuspended by vigorous mixing. The cells were then incubated for 30 minutes at 30°C and heat shocked in a 42°C water bath for 25 minutes. After the heat shock, the cells were pelleted by centrifugation and gently resuspended in 1 mL sterile dH₂O. 200 μ L of the transformation mix was plated onto selective minimal media which lacked 0.02% L-Histidine HCl monohydrate, 0.1% L-Leucine, or 0.02% L-Tryptophan, (L-Isoleucine 0.03%, L-Valine 0.15%, L-Adenine hemisulfate salt 0.02%, L-Arginine 0.02%, L-Lysine HCl 0.03%, L-Methionine 0.02%, L-Phenylalamine 0.05%, L-Threonine 0.2%, L-Tyrosine 0.03%, 0.672% Yeast Nitrogen Base, L-Leucine 0.1% , L-Tryptophan 0.02%, L-Uracil 0.02%) containing 2% glucose incubated for two days at 30°

Protein gel electrophoresis and western blotting

Yeast strain EGY48 transformed with appropriate plasmids was grown overnight in minimal selective media at 30°C with shaking at 250 rpm. The cell density of the culture was determined by counting serial dilutions on a hemacytometer and an amount

containing 2.5×10^8 cells was transferred into 50 mL of YPD (2% peptone, 1% yeast extract, 2% glucose) media and grown for 4 hours at 30°C with shaking at 250 rpm. After 4 hours another cell count was performed to confirm that the population had doubled at least twice. The culture was poured into a 50 mL centrifuge tube and centrifuged for 5 minutes at 3000 rpm. The supernatant was decanted and the cell pellet resuspended in the remaining supernatant. The mixture was transferred into an Eppendorf tube and stored at -80°C.

The casting apparatus was assembled and a 12% polyacrylamide separating gel (3.3 mL dH₂O, 2.5 mL 1.5 M Tris HCl, pH 8.8, 4 mL 30% acrylamide stock (30 acrylamide:0.8 bis-acrylamide), 100 µL 10% SDS, 100 µL ammonium persulfate, 5 µL N,N,N',M'-tetramethylethylenediamine) was added three-quarters of the way to the top. 100 µL tertiary-amyl alcohol was added to the unpolymerized gel. After polymerization and removal of tertiary-amyl alcohol a 4% polyacrylamide stacking gel (4.33 mL dH₂O, .75 mL 1 M Tris HCl, pH 6.8, .8 mL 30% acrylamide stock, 60 µL 10% SDS, 60 µL 10% APS, 5 µL TEMED) was added. Resuspended yeast cell pellets were removed from the -80°C freezer and 0.5 mL Laemli 2x reagent (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) was added. The samples were mixed vigorously until thawed then boiled for 5 minutes in a water bath. After the water bath the samples were then mixed vigorously for 30 seconds and centrifuged for 5 minutes. 20 µL of the protein supernatant was loaded onto the gel. Protein gel running buffer (1.92 M glycine, 250 mM tris base, 0.1% SDS) was poured into the top and bottom of the apparatus until the top of the gel was covered. The gel was electrophoresed at 100 volts until the bromophenol blue reached the bottom of the gel. The western blot apparatus was assembled and

immersed in 1x western transfer buffer (1.92 M glycine, 250 mM tris base, 20% MeOH). The western blot was electrophoresed overnight at 33 volts. The following day the nitrocellulose was removed from the western blot assembly and placed in a sterile tray. 50 mL of 5% milk blocking solution, 2.5 g dried milk, 125 μ L 10% Tween, and 50 mL TBS (2 M NaCl and 0.5 M HCl) was added and rotated for 1 hour. The milk blocking solution was poured off and 50 ml primary antibody(1:200 dilution of anti-monoclonal antibody (Santa Cruz Biotechnology) or 1:200 dilution of anti-HA epitope tag monoclonal tag antibody F-7 (Santa Cruz Biotechnology) inTBS with 5% milk, 0.05 % Tween-20) was added and rotated for 1 hour. The blot was then washed with TBS 5 times for 5 minutes each. A 1:2000 dilution of anti-mouse IgG horseradish peroxidase conjugate in blocking solution was added and rotated for 1 hour. After washing again 5 times with TBS, 7.5 mL of supersignal chemiluminescent substrate stable peroxide solution and 7.5 mL supersignal chemiluminescent substrate luminol enhancer (Pierce) were combined and added to the blot. This was rotated for 5 minutes. In the dark room the nitrocellulose was exposed to film for 30 seconds and again for 5 minutes (Kodak). The film was removed and placed in developer for 5 minutes. After removing the film from developer and washing in H₂O the film was placed in fixer for 5 minutes and washed again in H₂O.

Transformatin with cDNA library

Yeast strain EGY48 containing a pLexA derivative (“bait” plasmid) was grown in SD-H broth with 2% glucose at 30°C overnight. Cells were made competent as described previously and aliquots were transformed with 2 μ g pooled library plasmid DNA (*N. benthamiana* cDNA library in pB42AD “prey” vector). The number of transformation

aliquots was determined empirically such that the total number of yeast transformants obtained was 7.2×10^6 . individual transformation mixtures were pooled and serial dilutions were plated to determine the total number of transformants. The remainder of the pooled transformation mixtures was spread onto SD-HT medium with 2% glucose (30 15-cm plates) and incubated at 30°C for 48 hours.

Yeast Colony Scraping

Yeast colonies cotransformed with 'bait' and cDNA library 'prey' plasmids were recovered from the plates and pooled. 5mL of sterile dH₂O was placed onto plate that was to be scraped and the colonies on the plate are resuspended using a sterile spreader. The suspension was removed from the plate using a glass 10 mL pipette and stored in a 50 mL Falcon tube. Another 5mL of sterile dH₂O was placed on the same plate. After repeating the scraping until the plate was completely clear of colonies the 5 mL suspension was removed from the plate and used to resuspend the colonies on the next plate and collected in the Falcon tube. This procedure was repeated until the colonies were collected from all 30 plates. The cells were then pelleted by centrifuging the Falcon tube at 3,000 rpm for 5 minutes. The cells were then resuspended in 25 mL dH₂O and pelleted. The cell pellet was resuspended in a 65% glycerol mixture (25 mM Tris-HCl, .1 M MgSO₄, pH 8). Five aliquots of 500 µL were removed from the cell suspension 65% glycerol mixture and placed in Eppendorf tubes. These aliquots and the remainder of the cell suspension were stored at -80°C.

Yeast two-hybrid screening

A 0.5 mL aliquot of the pooled yeasted transformations in 65% glycerol mixture was added to 4.5 mL of SD-HT broth containing 2% galactose and 1% raffinose (gal/raf)

and incubated for 4 hours at 30°C with shaking at 250 rpm. Serial dilutions series of the culture were then plated on SD-HT gal/raf and incubated at 30°C for 48 hours. The amount of culture that contained 10 million yeast colonies was. A duplicate culture of pooled yeast transformants in SD-HT gal/raf was set up and after 4 hours of incubation the appropriate amount was added to dH₂O to make a total volume of 3 mL. This was then plated 200 µL at a time on 15 SD-HLT gal/raf plates. A dilution series from the 4 hour mixture was also plated on SD-HT plates to confirm the plating efficiency and all plates were incubated at 30°C. Growth of yeast on the SD-HLT gal/raf plates began to be visible around the third day after plating. Colonies were picked with a sterile toothpick and added to 100 µL of 15% glycerol for storage at -80°C. Colonies that grew from day 3 until day 7 were picked.

Retrieving Plasmid DNA from a Single Yeast Colony

Yeast colonies suspended in 15% glycerol were streaked onto SD-T glucose plates and incubated at 30°C for 48 hours. Single yeast colonies were picked and dispersed into 20 µL of lysis buffer (Stratagene). The mixture was frozen by immersing the Eppendorf tube in a dry ice-methanol bath for 30 seconds, thawed in a 37°C water bath and then heated at 95°C for 5 minutes. The lysate was cleaved by centrifugation at 14,000 x g for 30 seconds and the supernatant transferred to a fresh Eppendorf tube. *E. Coli* XL1-Blue supercompetent cells (Stratagene) were thawed on ice. Transformations were performed by aliquoting 75 µL of the cells into a prechilled 15-mL Falcon 2059 polypropylene tube. 1.3 µL of β-mercaptoethanol were added to each Falcon 2059 polypropylene tube, yielding a final concentration of 25 mM β-mercaptoethanol/tube. The cells were swirled gently and incubated on ice for 10 minutes with gentle swirling

every 2 minutes. After adding 2 μL of the yeast lysate to the reaction tubes and swirling gently the transformation mixtures were incubated on ice for 30 minutes. The transformation mixtures were then incubated in a 42°C water bath for 40 seconds and placed back on ice for 2 minutes. 0.4 mL of SOC media was added to each transformation mixture and incubated at 37°C for 1 hour with shaking at 225-250 rpm. The entire volume of the transformation reaction was plated onto LB medium containing 50 $\mu\text{g}/\text{mL}$ ampicillin and incubated at 37°C overnight.

Cracking Gel

A single *E. coli* colony used to inoculate 3 ml 2xTY broth containing 100 $\mu\text{g}/\text{mL}$ ampicillin and incubated overnight at 37°C with shaking at 250 rpm. A 5 μL aliquot of the overnight culture was mixed with 25 μL of cracking buffer (50 mM NaOH, 0.5% SDS, 5 mM EDTA) and incubated in a 65°C water bath for 40 minutes. 5 μL of PDB dye mix (250 mM EDTA, pH 8, 50% glycerol, 0.1% SDS, 0.1% bromophenol blue) were then added to each tube and a 13 μL aliquot was electrophoresed on a 1% agarose gel in TBE buffer at 100V until the bromophenol blue ran off the bottom of the gel. The gel was stained for 20 minutes with gentle agitation in 250 mL of dH₂O containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide.

RESULTS

Construction and testing of 'bait' protein fusions to the LexA DNA-binding domain (LexA-DB)

Previous work by others showed that BL1 proteins from BGMV, TGMV, or Cabbage leaf curl virus (CabLCV) can be expressed efficiently in yeast as C-terminal fusions to the DNA-binding domain of the LexA protein (J.K. Jordon and I.T.D. Petty, unpublished results). However, when the expression of BR1 proteins from the same viruses was attempted under similar conditions, only BGMV BR1 accumulated efficiently (J.K. Jordon, A.E. Currie and I.T.D. Petty, unpublished results). For brevity, this protein will be referred to as BBR1 (BGMV BR1). Orthologous viral proteins, such as BR1 homologues from different bipartite geminiviruses, might reasonably be expected to interact with the same host protein(s) to carry out their function(s) during infection of a common host plant. If this is the case, biologically relevant interactions should be detected more efficiently with the yeast two-hybrid system when multiple orthologous 'bait' proteins are available to corroborate the results. To use this approach for screening with the yeast two-hybrid system, a second BR1 protein in addition to BBR1 was needed that could be expressed efficiently in yeast. Accordingly, the BR1 proteins from two additional geminiviruses, *Squash leaf curl virus* (SLCV) and Texas pepper virus (TPV, also called *Serrano golden mosaic virus*), were tested for their ability to accumulate efficiently when expressed in yeast as C-terminal fusions to LexA-DB. These proteins will be referred to as SBR1 (SLCV BR1) and PBR1 (TPV BR1), respectively.

The strategy for cloning and expression of the SBR1 and PBR1 proteins was similar. In each case, PCR was used to isolate the corresponding ORF from a cloned copy of the viral DNA B component. The plasmids used containing cloned DNA B were pSLCV10-

E1D from extended host range virus isolate SLCV-E (D.C. Stenger, unpublished results), and pTPV-MB1D from the mosaic-inducing isolate TPV-M (Stenger *et al.*, 1990). Both plasmids were kindly provided by Dr. D.C. Stenger (USDA-ARS, Lincoln, Nebraska). Flanking restriction sites for *Bam*HI and *Not*I (SBR1), or *Bgl*II and *Not*I (PBR1) were incorporated into the PCR primers (Table 1). For PBR1, a *Bgl*II site was used rather than *Bam*HI because this ORF contained a naturally occurring *Bam*HI site that would have complicated subsequent cloning steps. The PCR products were cloned initially into the intermediate plasmid vector pCR2.1-topo and their nucleotide sequence was determined. For PBR1, a clone without PCR-induced mutations, pCR-PBR1.3, was readily identified and the insert was excised by digestion with *Bgl*II and *Not*I, and then ligated between the compatible *Bam*HI and *Not*I restriction sites of the yeast two-hybrid system 'bait' vector, pLexA (Figure 3). The resulting plasmid was named pLexA-PBR1. For SBR1, none of the four clones initially tested was free from PCR-induced mutations, so a clone with the complete wild-type sequence was assembled in an additional step from pCR-SBR1.3, which had a wild-type 5'-proximal sequence, and pCR-SBR1.1, which had a wild-type 3'-proximal sequence. Single *Nco*I restriction sites are present within the SBR1 ORF and the pCR2.1-topo vector, so exchange of *Nco*I fragments between pCR-SBR1.3 and pCR-SBR1.1 was used to generate the intermediate plasmid pCR-SBR13.25 with the completely wild-type sequence of SBR1. The SBR1 ORF was released from pCR-SBR13.25 by digestion with *Bam*HI and *Not*I and ligated between the same sites of the yeast two-hybrid 'bait' vector, pLexA, to create the plasmid pLexA-SBR1 (Figure 3).

The plasmids pLexA-PBR1 and pLexA-SBR1 were individually used to transform yeast strain EGY48 to histidine prototrophy. Whole-cell protein extracts were prepared

from yeast transformants and analyzed by western blotting. Accumulation of the LexA-PBR1 and LexA-SBR1 fusion proteins was evaluated by detection with a monoclonal antibody directed against LexA-DB. While LexA-SBR1 and control proteins LexA-DB and LexA-BBR1 could be detected readily, LexA-PBR1 behaved similarly to LexA-BR1 fusion proteins from TGMV and CabLCV and was not detected (Figure 5).

Although the results of western blot analysis showed that LexA-SBR1 could be expressed efficiently in yeast, this is not the only requirement for an effective 'bait' protein in the yeast two-hybrid assay. It was also important to determine whether LexA-SBR1 had any intrinsic ability to activate transcription of the two-hybrid reporter gene, *LEU2*, in the absence of a specific interaction partner. When it occurs this phenomenon is known as autoactivation. To test for autoactivation, serial dilutions of yeast cell suspensions were spotted onto minimal medium lacking histidine, to select for the 'bait' plasmid which expresses a *HIS3* marker, or lacking both histidine and leucine, on which yeast will only grow if the yeast two-hybrid reporter gene *LEU2* is also expressed. To mimic as closely as possible the conditions of an actual yeast two-hybrid screen, the carbon source used in the selective plates was a mixture of galactose and raffinose. Any yeast containing a 'bait' plasmid should grow when spotted onto minimal medium lacking histidine, and such growth ensures the viability of cells spotted onto the test plate lacking both histidine and leucine. Control strains expressing either LexA-DB alone (pLexA; negative control) or a fusion of LexA-DB with the activation domain of the yeast transcription factor Gal4p (pLexA-Pos; positive control) were also spotted onto both plates. After incubation for five days at 30°C, conditions typical for yeast two-hybrid screening, the extent of growth on both kinds of selective medium was recorded.

While yeast bearing pLexA-Pos grew equally on medium lacking histidine or lacking both histidine and leucine, other strains grew well only on medium lacking histidine alone (Figure 6). This result showed that the autoactivation potential of LexA-SBR1 was similar to that of LexA-BBR1, and in each case was only slightly above the background level of LexA-DB alone. Both the BBR1 and SBR1 baits were determined to be suitable for use in the yeast two-hybrid screening procedure.

Although the BR1 and BL1 proteins of bipartite geminiviruses are essential for cell to cell movement and sufficient for systemic movement under some conditions, genetic studies have also identified a role for the coat protein (AR1) specifically in systemic virus movement (reviewed in the Introduction). The evolutionary and likely structural relationship between the AR1 and BR1 proteins suggested that they may exhibit some functional redundancy with respect to systemic movement, so the AR1 protein was also selected as a potential ‘bait’ for yeast two-hybrid screening. Because the coat proteins of bipartite geminiviruses from the New World are all highly conserved in their amino acid sequence, only BGMV AR1 was selected for initial attempts at expression in yeast. For brevity, this protein will be referred to as BAR1 (BGMV AR1). The construction of a ‘bait’ plasmid expressing LexA-BAR1 followed a similar strategy to that used previously for LexA-SBR1. Briefly, PCR was used to isolate the BAR1 ORF from pGA1.2A, a plasmid containing the complete DNA A component of a Guatemalan isolate of BGMV (Fontes *et al.*, 1994). Flanking restriction sites for *Bam*HI and *Not*I were incorporated into the PCR primer (Table 1). The PCR product was cloned initially into the intermediate vector pCR2.1-topo and pCR-BAR1.3, a clone without PCR-induced mutations, was identified by nucleotide sequencing. The BAR1 ORF was then released

from pCR-BAR1.3 by digestion with *Bam*HI and *Not*I and ligated between the same sites of the yeast two-hybrid ‘bait’ vector, pLexA. Independent plasmid clones resulting from this ligation were used individually to transform yeast strain EGY48 to histidine prototrophy. Whole-cell protein extracts were prepared from these yeast transformants as well as control strains and subjected to analysis by western blotting and detection with anti-LexA monoclonal antibody. Expression of the LexA-BAR1 fusion proteins was readily detected in the western blot assay (Figure 5). As for the BR1 ‘bait’ proteins described above, it was also necessary to evaluate the autoactivation potential of yeast strains expressing LexA-BAR1. Serial dilutions of these yeast strains, as well as positive (pLexA-Pos) and negative (pLexA) control strains, were spotted onto minimal medium lacking histidine or lacking both histidine and leucine. Again, all strains grew well on medium lacking histidine alone, but only the strain bearing pLexA-Pos grew well on medium lacking both histidine and leucine (Figure 7). From these results it was concluded that, like LexA-BBR1 and LexA-SBR1, the LexA-BAR1 fusion protein was suitable for use as a ‘bait’ in the yeast two-hybrid screening procedure.

Construction and testing of ‘prey’ protein fusions to the B42 activation domain

As well as using bipartite geminivirus AR1 and BR1 proteins to screen for potential interacting host proteins, the yeast two-hybrid system could also be used to evaluate potential interactions among these viral proteins and with the BL1 protein, all of which are involved in virus movement through the plant. However, for these tests each protein must be expressed not only as a LexA-DB ‘bait’ fusion but also as a fusion to the B42 activation domain B42-AD in the yeast two-hybrid system ‘prey’ vector, pB42AD2. Previously, B42-AD fusions had been made with BBL1 (BGMV BL1) and BBR1 (J.K.

Jordon and I.T.D. Petty, unpublished results), so only B42-AD fusions to BAR1 and SBR1 had to be constructed for this study. In each case the cloning strategy was similar. The BAR1 or SBR1 ORFs were released from the intermediate plasmids used for the 'bait' plasmid cloning (pCR-BAR1.3 and pCR-SBR13.25, respectively) by digestion with *Bam*HI and *Not*I, and then ligated between the same sites of pB42AD2. The resulting plasmids were used to transform yeast strain EGY48 to tryptophan prototrophy. Because expression of B42-AD gene fusions in the 'prey' vectors pB42AD and pB42AD2 is under control of the galactose-inducible *GALI* promoter, these yeast strains were grown in liquid medium containing galactose as a carbon source prior to the preparation of whole-cell protein extracts. The resulting proteins were analyzed by western blotting and detected with a monoclonal antibody directed against the hemagglutinin (HA) epitope tag encoded by pB42AD and pB42AD2 (Figure 8).

Testing for yeast two-hybrid interactions among viral movement proteins and the coat protein

Based on genetic data obtained from infectivity studies in plants it is thought that the BR1 and BL1 proteins interact during virus movement. If this hypothesis is correct, then such an interaction potentially may be detectable using the yeast two-hybrid system. To test this possibility, various combinations of viral protein fusions to LexA-DB and B42-AD were introduced into yeast and their ability to complement growth on minimal medium lacking leucine was determined (Table 2). To screen for potential interacting movement proteins, bait yeast strains were constructed for BAR1, BBL1, BBR1 and SBR1. In each case the appropriate plasmid expressing a LexA-DB fusion protein was used to transform yeast strain EGY48 to histidine prototrophy. Prey yeast strains of

BAR1, BBL1, BBR1 and SBR1 were also constructed. The plasmids expressing each B42-AD fusion protein were then used to transform yeast strain EGY48 to tryptophan prototrophy. As controls, each viral protein in the 'bait' configuration (LexA-DB fusion) was also paired with the 'prey' vector expressing B42-AD alone, and each viral protein in the 'prey' configuration (B42-AD fusion) was paired with 'bait' vectors expressing either LexA-DB alone, or a fusion of LexA-DB with human lamin C (LexA-Lam), an irrelevant bait. None of the viral proteins in the 'prey' configuration interacted with LexA-Lam, but BBR1 and BAR1 preys appeared to interact weakly with LexA-DB alone.

Using the yeast two-hybrid assay, self-association was detected for both the BR1 proteins and AR1, but not BL1. Although the BBR1 bait gave the appearance of autoactivation in this assay, interestingly growth of yeast containing pLexA-BBR1 in combination with the pB42AD vector on minimal medium lacking leucine was dependent on the presence of galactose. This suggests that a weak interaction may be occurring between the LexA-BBR1 fusion protein and B42-AD, or its associated NLS or epitope tag. After accounting for the extent of yeast growth in the presence of B42-AD alone, it appeared that LexA-BBR1 was able to interact not only with B42AD-BBR1, but also with B42AD-BBL1. In contrast to LexA-BBR1, the otherwise equivalent fusion of LexA-DB to the SLCV BR1 protein (LexA-SBR1) showed no detectable interaction with B42-AD alone (Figures 9, 10, 11), which allowed its potential interactions to be detected with greater sensitivity. Thus, LexA-SBR1 appeared to interact with B42-AD fusions of SBR1, BBR1, and BBL1, but not BAR1. Although potential interactions were detected between LexA-DB fusions of BR1 proteins (BBR1, SBR1) and the BGMV BL1 protein (BBL1) fused to B42-AD, when the fusion protein configurations were reversed the

expression of LexA-BBL1 did not allow growth of yeast when paired with any viral protein.

Yeast two-hybrid screening of a *Nicotiana benthamiana* cDNA library

As well as the evaluation of potential interactions between pairs of characterized proteins, such as the viral proteins described in the previous section, the yeast two-hybrid assay also can be used to identify previously unknown interaction partners for specific bait by using it to screen a clone library. For viral proteins it is of interest to determine the identity of host factors with which they may interact during infection. Because the plant species *N. benthamiana* supports systemic infection by numerous geminiviruses, including BGMV and SLCV, it was chosen as a source of mRNA from which a cDNA library was constructed. Polyadenylated mRNA purified from aerial plant parts was used to generate cDNA. This was then directionally-cloned into the pB42AD 'prey' vector to generate a cDNA library comprised of approximately 3.6×10^5 primary transformants in *E. coli* (Y.-M. Hou and I.T.D. Petty, unpublished results). Plasmid DNA isolated directly from the pooled primary transformants was used for all the yeast two-hybrid screening in this study. To screen for potential interacting host factors, bait yeast strains expressing BAR1, BBR1 or SBR1 were used. Each bait strain was then separately transformed with aliquots of the *N. benthamiana* cDNA library in pB42AD and selected for growth on medium lacking both histidine and tryptophan. Transformation with library plasmid DNA prepared from pooled bacterial clones involves sampling of a redundant population of molecules because each colony contributes many individual, identical plasmid molecules to the pool. Accordingly, to ensure that >99% of all cDNA clones present in the library will be included in the sample (i.e. the yeast transformants) the sample must

contain >4.6-fold more colonies than the original library (Kubo and Kondo, 1991). For the cDNA library used in this study, with 3.6×10^5 primary *E. coli* transformants, $>2 \times 10^6$ yeast transformants were generated for each bait strain. To perform the actual yeast two-hybrid screening, yeast colonies doubly transformed with 'bait' and cDNA library 'prey' plasmids were pooled. To ensure that >99% of cDNA library clones present among the pooled yeast transformants would be included in each screen, aliquots containing $>10^7$ colony forming units were used. These aliquots were incubated briefly in medium containing galactose to induce the expression of cDNAs, which are under control of a yeast *GALI* promoter in the pB42AD vector, and then plated on minimal medium containing galactose as a carbon source, but lacking histidine and tryptophan to select for the 'bait' and 'prey' plasmids, and lacking leucine to select for expression of the two-hybrid reporter gene, *LEU2*. Yeast colonies that grew up over a period of 3–7 days were picked and stored in glycerol at -80°C until further analysis was performed. Screening to validate candidate clones took place in several steps. Initially, the recovered yeast colonies were resuspended and spotted onto selective plates containing either glucose or galactose as a carbon source. The growth of yeast clones that represent a genuine two-hybrid interaction should be galactose-dependent on medium lacking leucine because they require galactose-dependent expression of the prey fusion protein to activate *LEU2*. Plasmids were rescued from yeast strains that passed the galactose-dependence test and used to transform *E. coli*. The library prey plasmid was isolated from each and used to transform yeast together with plasmids that expressed either the original bait, the LexA-DB alone, or an irrelevant bait such as a LexA-DB fusion to human lamin C (LexA-lam) or the *Drosophila* homeodomain protein Bicoid (LexA-Bcd; Finley and Brent, 1991). If

bait-specific, galactose-dependent growth on medium lacking leucine was observed, then the prey was considered to be a candidate clone and the cDNA sequence was determined. For comparison, cDNA sequences were also determined for some clones that conferred growth when paired with an irrelevant 'bait' as well as with the original specific 'bait' used for the screen.

Yeast two-hybrid screening results for the BBR1 protein

From a screen of 1.24×10^9 colony forming units from the pooled cDNA library transformants, seven cDNA clones, representing four different genes, were isolated that conferred galactose-dependent growth of yeast in the presence of the LexA-BBR1 fusion protein. A cDNA clone representing a single gene was recovered from two separate yeast colonies, but the fusion junctions were identical between the plasmids, so they likely represent repeated isolation of a plasmid from a single primary transformant. Based on analysis with the BLASTX program (Altschul *et al.*, 1990.) this cDNA clone, Nb#4.31/9.10, probably encodes the 60S ribosomal protein L44 of *N. benthamiana*. The Nb#4.31/9.10 prey allowed yeast growth when coexpressed with LexA-BBR1 and with LexA-SBR1, but not with the LexA-BAR1. This result suggests that the protein encoded by this cDNAs interact only with the homologous BR1 proteins and not with the BGMV coat protein. A cDNA clone, Nb#41.1/46.9 that likely encodes a cytoplasmic NAD⁺-dependent malate dehydrogenase, was also isolated twice from yeast by virtue of its interaction with LexA -BBR1 and failure to interact with LexA-DB alone. Although Nb#41.1/46.9 did not support yeast growth when coexpressed with LexA-SBR1 or LexA-BAR1, it was able to support growth with the irrelevant bait LexA-lam in a very limited fashion.

When analyzed using the BLASTX program, the translated nucleotide sequence of one of these clones, Nb#40.4, did not match closely with any known or predicted proteins in the GENBANK nonredundant database. However, inspection of the sequence revealed an ORF that could potentially encode 98 amino acid residues fused in-frame with the C-terminus of B42-AD. In addition to LexA-BBR1, both the LexA-SBR1 and LexA-BAR1 baits supported yeast growth on medium lacking leucine in the presence of cDNA clone Nb#40.4. These results suggested that the protein encoded by Nb#40.4 can interact both with BR1 proteins (BBR1 and SBR1) and also with the BGMV coat protein (BAR1). Further testing showed that the Nb#40.4 prey could also interact with the LexA-DB vector alone as well as a possible interaction with the LexA-Lam irrelevant bait. Two of the cDNA clones probably encode the δ subunit of the chloroplast ATP synthase. These clones, Nb#4.32 and Nb#8.10, differed in the sequence of the fusion junction between B42-AD and the cDNA and so probably represent independent cDNA clones. Like the cDNA encoded by Nb#40.4, the clones Nb#4.32 and Nb#8.10 allowed yeast growth when coexpressed with LexA-SBR1 or LexA-BAR1 baits, as well as with LexA-BBR1. Interestingly, Nb#4.32 was also able to induce growth with the LexA-Lam bait while Nb#8.10 did not induce irrelevant growth. The final cDNA clone recovered, Nb#43.9, encodes a likely chloroplast RNA binding protein precursor with strong similarity to RNP1 proteins from bean and Arabidopsis. The Nb#43.9 prey allowed growth with the LexA-BBR1 bait, but none of the other viral baits. However, growth was also induced by the irrelevant LexA-Lam protein.

Yeast two-hybrid screening results for the BAR1 protein

From a screen of 2.5×10^9 colony forming units from the pooled cDNA library transformants, five cDNA clones, representing five different genes, were isolated that conferred galactose-dependent growth of yeast in the presence of the LexA-BAR1 fusion protein. After BLASTX analysis of their nucleotide sequences one, Nb#10.2 was found that had no known function. It is a putative protein in *N. benthamiana* and allowed yeast growth when coexpressed with LexA-BAR1 or LexA-BBR1, but not with LexA-SBR1. This result suggested that cDNA Nb#10.2 encoded a protein that interacts with the BGMV proteins BR1 and AR1, but not with SBR1, despite the latter's close sequence similarity to BBR1 (Figure 12). Analysis using BLASTX of nucleotide sequences from the other two cDNA clones recovered was able to identify a likely orthologue with a defined function for each. Nb#1.2 probably encodes an RNA helicase while Nb#24.3 encodes a putative U5 snRNP protein. Nb#22.9 likely encodes a chloroplast 30S ribosomal protein S17 precursor and allowed the growth of yeast when coexpressed with the LexA-BAR1, LexA-BBR1, and the LexA-SBR1 fusion proteins. A rubisco small subunit precursor, Nb#30.10, allowed yeast growth when coexpressed with the LexA-BAR1 fusion protein and the LexA-SBR1 protein, but not the LexA-BBR1 protein. This would indicate possible interaction with the BGMV coat protein and SLCV BR1 movement protein, but not the BGMV BR1 protein. A test with the irrelevant LexA-Bcd bait showed that Nb#22.9 and Nb#30.10 induced growth while the other preys were inconclusive as growth was not found on the control plate lacking only histidine and

tryptophan. All of the other cDNA clones that were sequenced encoded proteins that interacted with the irrelevant bait Lex-lam, but none interacted with the LexA-DB alone.

Yeast two-hybrid screening results for the SBR1 protein

From a screen of 2.13×10^9 colony forming units from the pooled cDNA library transformants, five cDNA clones, representing five different genes, were isolated that conferred galactose-dependent growth of yeast in the presence of the LexA-SBR1 fusion protein. Analysis using BLASTX of nucleotide sequences of the five cDNA clones recovered identified a likely orthologue with defined function in each case. The B42-AD plasmids Nb#s1.2 and Nb#9.7 were recovered from two separate yeast colonies, but the sequenced fusion junctions was identical indicating the likely repeated isolation of a plasmid from a single primary transformant. Based on BLASTX analysis, the cDNA Nb#s1.2/9.7 probably encodes a pectate lyase B protein of *N. benthamiana*. Analysis by BLASTX revealed that Nb#19.3/19.5 probably encodes a rubisco small subunit precursor. Both the Nb#s1.2/9.7 and Nb#19.3/19.5 cDNAs allowed yeast growth when coexpressed with LexA-SBR1, but not with the LexA-BBR1 or LexA-BAR1. The results suggest that Nb#s1.2/9.7 and Nb#19.3/19.5 interact only with the SLCV BR1 protein and not with the BGMV BR1 or BGMV AR1 proteins. The plasmids Nb#1.7 and Nb#15.1 also had fusion junctions that were identical and they encode a putative acetyl transferase according to BLASTX analysis. This cDNA allowed yeast growth when coexpressed with LexA-SBR1, LexA-BBR1, and LexA-BAR1 while growth with the irrelevant LexA-Lam was slight and should be tested again with another irrelevant bait. A cDNA clone, Nb#3.2/8.3/30.3, that likely encodes a chloroplast 30S ribosomal protein S1 precursor

was isolated from three different yeast colonies. This cDNA was able to interact with LexA-SBR1 as well as LexA-BBR1 and LexA-BAR1. However, Nb#3.2/8.3/30.3 was also found to support growth with the irrelevant bait LexA-lam. The fifth cDNA recovered Nb#40.5 probably encodes a putative chloroplast RNA binding protein precursor. This protein induced yeast growth when coexpressed with LexA-SBR1, LexA-BBR1, LexA-BAR1, but also possibly induced both the LexA bait and LexA-lam irrelevant control baits into growth.

DISCUSSION

Technical aspects of yeast two-hybrid screening

After completing the SBR1, BBR1, and BAR1 screens in the yeast two-hybrid system a review of the system and its potential flaws can be made. One of the most troublesome aspects of the screen is that there are potentially biologically-relevant interactions that were not discovered. There are inherent limitations in the screen due to the cDNA “prey” library comprised of approximately 3.6×10^5 primary transformants in *E. coli*. The cDNA library could possibly be missing other potential interactors represented by rare mRNAs due to the small size of the library. However, the effort required to screen a more representative library would be 10-fold greater, which would have made conducting three screens impractical in this instance. Compounding the problem of the small library size are other factors that could limit the detection of biologically relevant interactions. In the yeast two-hybrid system, there are several scenarios in which potential preys may not interact with relevant baits. There are cloning issues in the construction of the prey library as there is only a 1:3 chance that any given cDNA will line up in-frame to form an expressed fusion protein. This would limit the library of 3.6×10^5 primary transformants to an actual number of approximately 1.2×10^5 expressed cDNAs, and so potentially eliminate two-thirds of the possible interacting preys. Another concern is that if a relevant prey represses transcription the *LEU2* gene will not be expressed and yeast growth will not occur. In addition to direct repression, steric hindrance of promoter access could potentially keep interacting preys from activating the reporter gene. Potentially interacting proteins with strong sub-cellular localization signals which direct the prey fusion protein to somewhere other than the nucleus may not

come into contact with the bait fusion protein. The types of proteins that could be omitted due to these signals could include cell wall/membrane proteins that may be involved in trafficking the BR1-viral DNA complex into the cytoplasm or into a neighboring cell. Although the preceding limitations may affect the detection of interacting preys, the likelihood of missing detectable interactors in the library that were expressed is low due to the >99% probability that all independent cDNA clones were included in each screen. This is further given credibility because in the screening process in a number of instances (Table 3) a specific cDNA was isolated in two different yeast colonies.

False-positives are a concern because although they allow the yeast colony to grow in media lacking leucine there is not a specific interaction between the bait and prey that is causing the *LEU2* activation. The false-positive could be caused by a “sticky” prey that allows any fusion protein to activate, or a prey that contains an enzyme that performs the Leu2p enzymatic function and allows for leucine formation without an actual bait:prey interaction. To eliminate these false-positives, clones that allowed growth with glucose or growth without bait specificity (i.e. grow with LexA-DB as well as specific bait) were discarded. Clones that allowed growth with irrelevant baits such as lamin or bicoid were also eliminated. Some of the latter class of false-positives were characterized by sequencing, these false-positives interact with a specific bait and irrelevant bait, but not LexA-DB alone (Table 4).

Whether lamin and bicoid are really irrelevant baits is an important question as well. Lamins are the major structural proteins of the nuclear lamina, a structure that lines the surface of the inner nuclear membrane in higher eukaryotic cells. The nuclear lamina is

composed of a meshwork of 10 nm filaments that are thought to provide a skeletal support for the nuclear envelope and to mediate the attachment of the nuclear envelope to interphase chromatin. The lamina would then be necessary for both structural support and the division of the cell. Additional functions of the nuclear lamina may include the proper organization and anchoring of nuclear pore complexes. During mitosis the lamins also play a crucial role in the disassembly and reassembly of the nuclear envelope (Lenz-Bohme *et al.*, 1997). A major role of BR1 in virus movement is believed to be in the movement from the nucleus into the cytoplasm. The host proteins that were found to interact with BR1 baits, therefore, may have some relation to nuclear movement. It then seems very possible that some of these host proteins could also interact with the lamin baits. The bicoid homeodomain protein controls embryonic gene expression by transcriptional activation and translational repression in fruit flies. This protein has been shown to be a nucleic acid-binding unit that contains an arginine-rich motif similar to the RNA-binding domain of the HIV-1 protein REV, needed for both RNA and DNA recognition (Niessing *et al.*, 2000). It is not known whether the preys that were isolated from the cDNA library screen with the protein baits should have any interaction with bicoid although there are few homeodomain proteins in plants. While the function of the AR1 coat protein is believed to only be in virion formation and in systemic movement, and BR1 is believed to function primarily in shuttling out of the nucleus, the chance remains that there may be other unknown functions. Viruses are so genetically compact that there is a possibility that these viral proteins may provide other functions which could make the “irrelevant” bait of bicoid also possibly relevant. The isolation of some of the false-positives may have been enhanced due to the high abundance of the encoded

proteins and therefore mRNAs. Both the rubisco protein and 30S ribosomal protein are found in large concentrations in the plant and their mRNAs could have been represented many times in the cDNA library.

Candidate interacting cDNA clones

There were two patterns of interaction between candidate clones that reacted with specific baits, but not with irrelevant baits. The first pattern was that the prey interacted with all three specific baits. It is to be expected that biologically relevant *N. benthamiana* proteins might interact with (at least) both BR1s because they are similar in both function and amino acid sequence. However the surprise was that these preys showed an interaction with AR1 too. Evolutionarily, the AR1 and BR1 proteins are paralogues and have both some limited sequence similarity and functional similarity. In monopartite geminiviruses the coat protein equivalent to AR1 is essential for the movement of the virus. Both AR1 and BR1 accumulate within the nucleus and are able to bind ssDNA. The AR1 protein also has an imprecisely defined role in the systemic movement of bipartite geminiviruses. The similarities between the AR1 and BR1 proteins may extend to secondary and tertiary structure as well which would make a host protein that interacts with both much more likely. The preys that were isolated could either have a role in both systemic and local virus movement, have a role in only one type of virus movements and just happen to interact with the other bait, or be a total artifact of the yeast two-hybrid system. There could be artifacts as the system allows for the interaction of proteins that under normal cellular conditions may never be near each other. This interaction could cause activation of the reporter gene, but not be biologically relevant. It is interesting, however, that only a single protein, Nb#4.31, was isolated that interacted with both of the

BR1 proteins and not the AR1 protein. The Nb#40.4 “unknown” protein was isolated with the BGMV BR1 bait. This protein did not match closely any others during the BLAST search (Altschul *et al.*, 1990). The protein did, however, react with the other geminivirus baits meaning that it could be a relevant protein to viral movement with a function in the plant that is not known. Nb#4.32/8.10 was revealed by BLAST search as an ATPase δ subunit found in the chloroplast. ATP is synthesized by a proton-motive force created in a particle made up of a F_0 membrane bound proton conducting unit and a F_1 ATP synthesizing unit. The delta subunit of the ATPase is a 14 kD particle that extends from the stalk of F_0 into the F_1 ATP synthesizing site (Stryer, 1999). While the virus is not known to interact with the ATPase in movement, it is possible that an interaction takes place for energetic reasons or for movement as ATP is circulated throughout the plant from source to sink cells. Nb#1.2 encodes a putative RNA helicase. This prey cDNA allowed a larger level of yeast growth with BGMV BR1 than with SLCV BR1 bait, which may reflect the fact that the BGMV BR1 is expressed at a higher level than the SLCV BR1 bait as shown in the western blots. This putative RNA helicase could be a nuclear protein based on the sub-cellular localization of related proteins identified in BLAST searches. The Rep initiator of geminivirus rolling circle replication has been shown to contain a structure that is related to a large group of proteins that bind RNA in a structure-based evolutionary link (Campos-Olivas *et al.*, 2002) and it is possible that the movement proteins could also bind RNA for either movement or as an artifact of primordial replication and movement. Nb#s1.2/9.7 is a putative acetyl transferase which could be used in the addition of acetyl groups for signaling or the elongation of fatty acids. Even though it is any role that acetyl transferase could play in

the movement of the virus it is interesting that there may be an interaction between the viral movement proteins and a host protein used for the modification of signals. The final protein that was able to induce growth in all of the relevant baits was Nb#40.5 which is a putative chloroplast RNA binding protein. This protein is expected to be found in chloroplasts which the virus probably does not interact with, but it is related to a potato silencing element binding factor (SEBF) which shows a dual function in which it is a chloroplast RNA binding protein and also a nuclear DNA binding protein involved in transcription regulation (Boyle and Brisson, 2001). This protein could also serve dual roles as a nuclear DNA binding protein that could be manipulated by BR1.

The second pattern of prey interactions observed was an interaction with the original bait (used for screen), and (generally) nothing else. It seems odd that a relevant BR1 interaction would not be conserved between both BGMV BR1 and SLCV BR1 baits. This would likely indicate that the host protein is not biologically relevant, however, it may also be a protein that interacts with only a certain species of the geminivirus that has adapted to it. There were some preys that were isolated that interacted with one of the BR1 proteins and the AR1 protein, but not the other BR1. These are potentially not biologically relevant and could be artifacts of the yeast two-hybrid system. The BR1 proteins show far more similarity to each other than to the AR1 protein, so a host protein that interacts with one of the BR1 baits and the AR1 bait, but not the other BR1 bait is unlikely to be relevant. Nb#4.31/9.10 encodes 60S ribosomal protein L44 that allowed yeast growth of both the BGMV BR1 bait and SLCV BR1 bait. While the virus has no known interactions with ribosomes it is unlikely that a relevant host protein would interact with BGMV BR1 and not SLCV BR1 so this may be an artifact of the yeast two-

hybrid system or perhaps the BR1 movement proteins are interacting with the ribosomal proteins before the ribosome is formed. Nb#43.9/43.10 encodes a putative ribonucleoprotein precursor that also allowed growth only with the BGMV BR1 bait. This protein is expected to bind RNA in the chloroplast which may not be needed by the virus, but as described earlier there could be an RNA binding for reasons not established. Nb#10.2 encodes a protein of unknown function. This protein allowed yeast growth when paired with either BGMV BR1 or BGMV AR1 baits. However, it did not allow yeast growth when paired with the SLCV BR1 bait, which is related more closely to BGMV BR1 either BR1 protein is to AR1. Nb#24.3 encodes a putative U5 snRNP protein. U5 is a member of the small nuclear RNAs that cause the splicing of messages in the nucleus. The U5 snRNP is involved with the ends of exons by binding to the 3' ends of the first exon and the 5' end of the second to position them for splicing (Stryer, 1999). This again is an RNA binding protein that has no known movement functions as of yet. Nb#s1.2/9.7 encodes a putative pectate lyase according to BLAST. The pectate lyase function of degrading the middle lamella portion of a plant cell wall is one that was originally found in pathogens. The interaction is an appealing one even though it was observed only for the SLCV BR1 bait. Nb#19.3/19.5 is a rubisco small subunit precursor. Rubisco is the most abundant protein in a plant making up over 16% of the total chloroplast protein. This protein can be used in the chloroplast to either condense a CO₂ molecule with ribulose 1,5-bisphosphate to form a 6-carbon molecule or catalyze the addition of O₂ to ribulose 1,5-bisphosphate to form phosphoglycolate and 3-phosphoglycerate in photorespiration. This interaction was also observed only with the SLCV BR1 bait and could be an artifact. The rubisco could have bound due to the large

amounts of message possible or there could be a reason of importance to the virus. The large amount may increase the likelihood of binding by chance, however, the virus may also have adapted to take advantage of the large amounts of the protein that is available. A possibility for all interacting preys is that they have more than one function within the cell. The prey could interact with viral movement in a way that is yet unknown and have a second function than the one that has been listed above.

Possible further analysis of candidate clones

The first step to take in analyzing the candidate clones further should be continued testing. For the preys that interact with all specific baits (BAR1, BBR1, SBR1), additional “irrelevant” baits should be tested. These irrelevant baits could further support a specific interaction that is leading to the leucine independent growth. Another way to test the candidate clones for relevance is to try coat protein baits from other, monopartite geminivirus genera, e.g. Curtovirus beet curly top virus (BCTV) or tomato pseudo curly top virus (TPCTV). Although the monopartite viruses contain no BR1 or BL1 movement proteins, BCTV and TPCTV coat proteins can substitute for TGMV AR1 in systemic movement (Pooma, 1997; J.L. Jeffery and I.T.D. Petty, unpublished results). Any host protein that interacts with the BGMV AR1 should also interact with the other coat proteins if it is biologically relevant for coat protein-dependent systemic movement. The yeast two-hybrid system needs a second test to confirm physical interaction by alternative methodology because of the chance of artifacts. One possible approach system is a two-step process using an immunoaffinity chromatography and then a western blot. The column would bind any protein that contained a hemagglutinin epitope tag. These proteins and any interaction partners could then be eluted and the binding partner

detected by western blot with the monoclonal antibody that binds to LexA-DB. Any protein that would appear in the western blot would then have to have an interaction between both the bait and the prey in order to bind and then appear in the western blot. Another possibility is looking for a confirmation method *in planta* which would actually be preferable since this would indicate an interaction in the host. The green fluorescent protein (GFP) has been used in plants to show the interactions between MP and actin filaments. A method could be developed to fluoresce the movement protein bait only in the presence of a host prey.

The potential BBR1 interactor Nb#40.4 has no strong BLAST hit, but a poly-A tail indicates that it corresponds to the 3'-end of an mRNA. To try and determine if this cDNA clone represents an mRNA which encodes an actual protein in the host plant that has not yet been identified or is some kind of artifact, the first step would be to try and identify the corresponding plant mRNA in a Northern blot. This would utilize the Nb#40.4 as a probe against the plant RNA for a message encoding the corresponding plant protein. If the northern blot was positive there could be an attempt to try to isolate additional cDNA clones with more 5'-proximal bases. From such clones more nucleotide sequence data could be determined and BLAST searches could be repeated to try to identify related genes. Gene-silencing (or T-DNA knockout in Arabidopsis) is the next logical step after confirming a physical interaction by an alternative method. However, for this approach the host protein must not be an essential protein for the survival of the plant such as the chloroplast or ribosomal proteins found. A plant that can survive without the protein that is believed to be relevant can be grown without this protein in the T-DNA knockouts or silenced using gene-silencing to eliminate the mRNA by host

degradation. If the protein is relevant for efficient movement of the virus then suppression of host gene expression should mimic the effect of BR1 mutations resulting in either slower and weaker movement compared to the wild type host, or no movement at all of the virus in the suppressed host. An advantage in this method is that the T⁻-DNA knockouts are in Arabidopsis and a library of plants with genes that have been knocked-out is already under construction with many completed.

Viral protein interactions

The results of yeast two-hybrid analysis of protein:protein interactions between the viral movement proteins, and between movement proteins and the coat protein are equivocal. To confirm any interactions irrelevant baits and irrelevant preys would be very informative to define interactions. There is a definite need to confirm any initial interactions with alternative assays just as the earlier preys need to be confirmed. The viral interactions between movement proteins and/or the coat protein need to be proven through direct physical interaction to support viral genetic and co-localization assays in plants. Another area of concern is the lack of accumulation shown in the western blot of the AR1 prey. Results from the western blot make it impossible to gain any insight into the interactions between the movement proteins and the AR1 prey since it has failed to show any accumulation during western blotting. The BGMV BR1 protein also had very limited accumulation in the western blot attempts. The AR1 bait seemed to interact with all of the preys resulting in at least limited growth, including the AR1 prey which has yet to show accumulation. There should be an interaction between the AR1 bait and AR1 prey as a self-association is needed in order to make virions. There is also apparently a

non-reciprocal interaction between BR1 and BL1 which may be due to the partitioning of BL1 protein at the plasma membrane (Pascal *et al.*, 1993). In fact the BL1 bait did not interact with any prey, consistent with the possibility that it may be confined to the cytoplasm. Although SLCV BL1 may be expected to self-associate no interaction between the BL1 bait and BL1 prey has yet to be established in the yeast two-hybrid experiments (Ward *et al.*, 1997). The nuclear exclusion could possibly be overcome by the NLS present in a B42AD fusion protein, however, screening the cDNA library with an NLS-tagged TGMV BL1 bait did not yield any positive interactors (W.D. Graham and I.T.D. Petty, unpublished results). The SBR1 bait seemed to interact with BBL1 in yeast, the biological relevance of which can be tested by construction of a chimeric virus. It would be predicted that the combination of SBR1 and BBL1 proteins would support movement of the chimeric virus. The interactions between the viral movement proteins and the coat protein that are believed to be relevant for viral movement in the host should be continued to be studied in the yeast two-hybrid systems, as well as more biologically relevant systems, to attempt to test the widely held notion that these proteins must interact in order for successful viral movement.

Table 1. Oligonucleotides used in this study.

Description	Oligo No.	Sequence	Restriction Site
BGMV <i>ARI</i> ORF 5' PCR primer	175	5'-TGGATCCTAATGCCTAAGCGTGAT GCGCCGTGGCG	<i>Bam</i> HI
BGMV <i>ARI</i> ORF 3' PCR primer	176	5'-TGCGGCCGCTTAATTGGTTATCGA ATCATAGAAAT	<i>Not</i> I
TPV <i>BRI</i> ORF 5' PCR primer	171	5'-TAGATCTTAATGTATCAGTCAAGT TATAGACGTGT	<i>Bgl</i> II
TPV <i>BRI</i> ORF 3' PCR primer	172	5'-TGCGGCCGCTCAACCAACATAGTC TAGGTCGTATG	<i>Not</i> I
SLCV <i>BRI</i> ORF 5' PCR primer	173	5'-TGGATCCTAATGTATTTCGACGAGC AATAGACGGGG	<i>Bam</i> HI
SLCV <i>BRI</i> ORF 3' PCR primer	174	5'-TGCGGCCGCTCATCCCAAATAGTC CAGTTCAAACG	<i>Not</i> I
pB42AD 5' sequencing primer	184	5'-CCAGCCTCTTGCTGAGTGGAGATG	–

Table 2. Summary of the geminivirus baits and their interactions with the geminivirus preys.

preys	test baits						Bcd
	BBR1	BAR1	SBR1	BBL1	LexA-DB	Lam	
BBR1	+	+	+	-	+/-	-	N.D.
BAR1	+/-	+	+/-	-	-	-	N.D.
SBR1	+/-	+	+	-	-	-	N.D.
BBL1	+/-, +	+	+	-	-	-	N.D.
B42AD	+/-	-	-	-	-	-	N.D.

Notes

‘prey’ proteins indicated were fusions to B42-AD

‘bait’ proteins indicated were fusions to LexA -DB

“irrelevant’ baits were human lamin C (Lam) and Drosophila bicoid homeodomain (Bcd)

N.D. = not done

Table 3. Summary of yeast two-hybrid interactions between viral and *N. benthamiana* encoded proteins.

A. Candidate clones^a

Function	cDNA	Interactions		
		BGMV AR1	BGMV BR1	SLCV BR1
Isolated with BGMV BR1				
unknown	40.4	+	+	+
chloroplast ATP synthase δ subunit	4.32, 8.10	+	+	+
60S ribosomal protein L44	4.31/9.10	-	+	+
chloroplast RNA binding protein RNP1 precursor	43.9/43.10	-	+	-
Isolated with BGMV AR1				
RNA helicase	1.2	+	+	+/-
unknown (putative protein)	10.2	+	+	-
Putative U5 snRNP protein	24.3	+	+/-	-
Isolated with SLCV BR1				
pectate lyase B	s1.2/9.7	-	-	+/-
Putative acetyl transferase	1.7/15.1	+	+	+
rubisco small subunit precursor	19.3/19.5	-	-	+/-
chloroplast RNA binding protein precursor	40.5	+	+	+

B. Likely false-positive clones^b

Function	cDNA	Interactions		
		BGMV AR1	BGMV BR1	SLCV BR1
Isolated with BGMV BR1				
malate dehydrogenase	41.1/46.9	-	+	-
Isolated with BGMV AR1				
chloroplast 30S ribosomal protein S17 precursor	22.9	+	+	+
rubisco small subunit precursor	30.10	+	-	+/-
Isolated with SLCV BR1				
chloroplast 30S ribosomal protein S1 precursor	3.2/8.3/30.3	+	+	+

a interact with one or more viral proteins, but not LexA-DB or irrelevant baits.

b interact with one or more viral proteins and also with one or more irrelevant baits.

Table 4. Summary of preys isolated from *N. benthamiana* screen when introduced to all the different baits.

A. BGMV BR1 isolated preys

Screening Bait:	preys	test baits					
		BBR1	BAR1	SBR1	LexA	Lam	Bcd
BGMV BR1 (BBR1)	4.31	+	-	+	-	-	N.D.
	4.32	+	+	+	-	+	N.D.
	8.10	+	+	-	-	-	N.D.
	9.10	+	-	-	-	-	N.D.
	40.4	+	+	+	-	+/-	N.D.
	41.1	+/- +	-	-	-	+, -	N.D.
	43.9	+	-	-	-	+	N.D.
	43.10	+/-, +	-	-	-	-	N.D.
	46.9	+/-, +	-	-	-	+, -	N.D.

B. BGMV AR1 isolated preys

Screening Bait:	preys	test baits					
		BBR1	BAR1	SBR1	LexA	Lam	Bcd
BGMV AR1 (BAR1)	1.2	+	+	+/-	-	+	N.D.
	10.2	+	+	-	-	+	N.D.
	22.9	+	+, +/-	+	-		+
	24.3	+/-	+, +/-	-	-	+	N.D.
	30.10	-	+, +/-	+/-	-		+

C. SLCV BR1 isolated preys

Screening Bait:	preys	test baits					
		BBR1	BAR1	SBR1	LexA	Lam	Bcd
SLCV BR1 (SBR1)	1.2	-	-	+/-, +	-	-	+/-, -
	1.7	+	+	+	-	+/-	-
	3.2	+	N.D.	+	N.D.	N.D.	+/-
	8.3	+	+	+	N.D.	N.D.	N.D.
	9.7	-	-	+/-	N.D.	N.D.	-
	15.1	+	N.D.	+	N.D.	N.D.	-
	19.3	-	-	+/-	-	-	-
	19.5	-	-	+/-	N.D.	N.D.	-
	30.3	+	N.D.	+	N.D.	N.D.	+
	40.5	+	+	+	+/-	+/-	-

Notes

'bait' proteins were as described for Table 2

'prey' proteins were fusions to B42-AD encoded by the *N. benthamiana* cDNAs indicated

N.D. = not done

Table 5. Summary of plasmids used in this study.

Plasmid Name	Alternative Name	Description	Reference
Viral DNAs			
pGA1.2A	–	complete BGMV DNA A, 1.2 copies in pZf19U	Fontes <i>et al.</i> , 1994
pSLCV10-E1D	–	complete SLCV-E DNA B, <i>EcoRI</i> dimer in pGEM7Zf+	D.C. Stenger, unpublished
pTPV-MB1D	–	complete TPV-M DNA B, <i>HindIII</i> dimer in pGEM7Zf+	Stenger <i>et al.</i> , 1990
pCR-BAR1	pCR-BAR1.34	BGMV <i>ARI</i> ORF in pCR2.1	This study
pCR-PBR1	pCR-PBR1.6	TPV <i>BR1</i> ORF in pCR2.1	This study
pCR-SBR1	pCR-SBR13.25	SLCV <i>BR1</i> ORF in pCR2.1	This study
'bait' plasmids			
pLexA	pEG202	LexA-DB fusion vector	Gyuris <i>et al.</i> , 1993
pLexA-Pos	pSH17-4	LexA fusion to yeast Gal4p activation domain	Golemis <i>et al.</i> , 1994
pLexA-Lam	–	LexA fusion to human lamin C fragment	Clontech, unpublished
pRFHM1	DmBcdΔC	LexA fusion to <i>Drosophila</i> Bicoid fragment	Finley and Brent, 1994
pLexA-53	–	LexA fusion to human p53 fragment	Clontech, unpublished
pLexA-BAR1	pLexA-BAR1.34	BGMV <i>ARI</i> ORF in pLexA	This study
pLexA-BBR1	pLexA-BBR1.1	BGMV <i>BR1</i> ORF in pLexA	A.E. Currie, unpublished
pLexA-BBL1	pLexA-BBL1.7	BGMV <i>BLL1</i> ORF in pLexA	J.K. Jordon, unpublished
pLexA-PBR1	pLexA-PBR1.6	TPV-M <i>BR1</i> ORF in pLexA	This study
pLexA-SBR1	pLexA-SBR13.25	SLCV-E <i>BR1</i> ORF in pLexA	This study
'prey' plasmids			
pB42AD	pJG4-5	B42-AD fusion vector	Gyuris <i>et al.</i> , 1993
pB42AD2	–	modified pB42AD with pLexA polylinker	I.T.D. Petty, unpublished
pB42AD-T	–	B42-AD fusion to SV40 T-antigen fragment	Clontech, unpublished
pB42AD-BAR1	pB42AD-BAR1.5	BGMV <i>ARI</i> ORF in pB42AD2	This study
pB42AD-BBR1	pB42AD-BBR1.1.1	BGMV <i>BR1</i> ORF in pB42AD2	J.K. Jordon, unpublished
pB42AD-BBL1	pB42AD-BBL1.7.1 pB42AD-BBL1.7.2	BGMV <i>BLL1</i> ORF in pB42AD2	J.K. Jordon, unpublished
pB42AD-SBR1	pB42AD-SBR13.2	SLCV <i>BR1</i> ORF in pB42AD2	This study

Table 6. Sequence data from the candidate clones isolated during the yeast two-hybrid screens.

I. BGMV BR1

40.4

CGCCTCTTGCTGAGTGGAGATGCCTCCTACCCTTATGATGTGCCAGATTATGC
CTCTCCCGAATTCGGCACGAGGCCAGACGAGAAAGGAGCTTGTTGGACGCG
AGAGACCCTTCTCACAAAGCACGATAAAGGAAGCAAGCATTCTCATCGACCGG
AGAGATCTCTAAGTCGAGAGTACTCATCTGGAAGACGCGAGAGGTCCATGGA
CAAGGAAGATTTATCTCACAGGCAGATAACACGTGGAAGGAAAAGACATGA
TTTGAGGTCCCCAGTTATGATCATTATCCCATAGGAGTTCAATGGACAGAA
GCAGACATTCCCGCAGCGCCAAAAAAGTAGCAGAGACAGGCATGACATGAG
ATCCCCAGTCTTGATCATTATCTGATGAACTTCAACGCACAGAGAAGAA
CGTCATCGAGCACGTAATGCACGATCTCATGGAAATTACAGGTGGTCACCAA
GCAGGGATGATTCTTAAAATGGAGTAAAAGATTAATCAAAGATGCAAGGGAT
ATAATAGCTGTAATTATCAGGTCTTTTTATTTACTTGTATAAGCTTTGTTACTT
TACATGATTTTCTGATAAGTACTATTGTAATCTTCTTTNAAAAAAAAAANN
NNNNNAAAAANANNNAAAAACN

(4.32 similar to 8.10)

NCCTCTTGCTGAGTGGAGATGCCTCCTACCCTTATGATGNGCCAGATTATGCC
TCTCCCGAATTCGGCACGAGGAACCTAGAACAAACCGTCGCCGACCTCGAA
AAAATCGAAAAAATCTTCGACGACGAAGCAGTGTACAACTTCTTCGTGAGCC
CTATCGTCGGCGAAGAGAAGAAACCGCAACTCGTGGACGAGATCGTTTCATC
CTCGAGCATCCAGCCGCACGTGGCGAATTTCTGAACATTTTAGTAGACATG
AAGCGAGTGGAGCTAATCAAAGAAATTGTAAGAGAGNTCGAGAAAGTATAC
AATACGATTACGGACACGGAACCTTGCTGTGGTCACTTCTGTTGTGAAATTGGA
ATCGCAGCATTTGGCGCAGATAGCGAAAGGAGTGCAGCGATTGACAGGTTCG
AAAAATGTGAGGATTAACCGGCTATTGATGAATCGCTAGNANCTGGATTTA
CAATAAGGTACGGAAATTCAGGATCAAAGNTGATTGATATGAGTGTGAAGAA
ACANCTTGGGGATATTGCTGCTCAACTTNAANTTGGGGATATTCAATTAGCC
TGCATAANNGGTCTGNTTTAAACTATATTTTTCTTCAGCGATTCATTTNTTGT
ANTATNANTTTGGTTCAAAGGCAAATGNAAAGATACTNGCTTGGCTCTAAAT
CTTTGGATGAAANTTCAATTCANTTGGATGNACNCCTCTCNCCNNNCCCCTCCC
CTNTCNCNTCTCTCCNCTCNACCCTCCTCCCNANCCAANCANCCCCCGGAAC
TTTTCANCNTNCCCCNNGGNGGAAGNCCCCNTTNGGNTGGNNGTNNCTCTTG
CNACANTACAANAAGGGANNGNCCAATGGNNCNTCCTTNNCANNNTANACCC
NTTTTCNTTTTTTTNTNTAANANTATNC

(4.31 similar to 9.10)

CAGCCTCTTGCTGAGTGGAGATGCCTCCTACCCTTATGATGTGCCAGATTATG
CCTCTCCCGAATTCGGCACGAGGGACAGATCTAATCCTCTGCCCGGACAAT
GGTGAATGTACCTAAGACAAAGAAGACCTACTGCAAATCCAAGGAATGCAA

Table 6 continued

AAAGCACACCTTGCATAAGGTCACACAATACAAGAAAGGAAAAGATAGTTT
AGCTGCCAGGGAAAGCGTCGTTATGATCGTAAGCAGTCAGGTTATGGTGG
CAGACAAAGCCTGTCTTCCACAAAAAGGCTAAAAC TACAAGAAGATTGTCT
TAAGGTTGCAATGCCAGGGGTGCAAGCATGTCTCTCAGCACCCAATCAAGAG
GTGCAAGCATTTTGAGATTGGTGGGGACAAGAAAGGAAAGGGAACCTCTCTT
TTCTAAATTGCCTGTGATGCAAAATTTGGTAGTTTTCTGTTATACATTTTTAGA
TATCTTTTTTTGNTGGTGTAGTTCAAGAGCTTTTGTTTTGTTAGATTCAAGTTA
ATACTATGCTGTGTTAATTACATTTTGATCAGGAGATCAAAGCTGTACTTTTC
AAGGTTCTATTTAGTATGGGTCTTGGTCCCTAAAAAAAAAAAAAAAAAAAA
CTCGAAAAGCNTTGGACTTCTTCTCCCGAAGTTTGGTCAAGTCTCCAATCNAG
NTGGNCGGGTTTNTACTNGNCNGAAATTACCNAAGATGGAAAGGGCN
AATNGTGGNNAANCCTGTGNCCCTTAANTAACCGAATTTCTTAGGATTAN
GATTTTTTATTAANAGTNTAAAAAANGGGTTCCAAATTAAGGGNCCNTAGT
TTAAACAAATTTTTNTTTGNAACNTTTCNNGGGNGNGGTTTTAGN

(43.9 similar to 43.10)

CGCCTCTTGCTGAGTGGAGATGCCTCCTACCCTTATGATGTGCCAGATTATGC
CTCTCCCGAATTCGGCACGAGGGACTGGCAGCCACAGTGGCTCAAGAGGAGG
CGGCGGCTGTGACGGTGGAGGAGGAAGAAACCGAGGCGGTGGCGGTTGAAG
AGGAAGTGGTGGAGGAACAGGGAGGAGGGTTCTGAAGAGAGTAGTAGTG
AAGGTGAAGCTGTAAATACTAAGCTTTATTTTTGGGAATTTGCCGTATCTTTGT
GATAGTGCACAACCTTGCTGGAATTGTTCAAGACTATGCTACTCCTGAGCTTGT
TGAGGTTCTTTACGATAGGGAAACAGGTAAGCAGAGGATTTGCATTTGTG
ACAATGAGTTCTCTTGAAGATTGCAAAACAGTCATCAAAAATCTAGATAGAA
GAGAGTATGGTGGAGAACCTTAAGGGTGAATTTCTCAGACAAGCCTAAACC
AAAAGAGCCCCTTTATCCAGAAACAGAACACAAACTCTTTGTTGGTAATTTG
GCCTGGTCAGTCACATCTGAAAGTTTGGCACAAGCATTCAAGAATATGGAA
CTGTGGTTGGAGCTAGGGTACTATATGATGGGGAGACTGGGAGATCTCGTGG
CTATGGTTTTGTAAAGCTATGAGAATAGAGAACAATTGGAGAATGCCCTTCAA
AATCTTAATGGAGTGGACTGGATGGAAGGGCAGGCCATTAGCTTAGCACAG
GGGAAGGAACATTAGGATGGACAAGATCTTGNTNTAGTGNAAGAGTGGAAA
ATGCCATCAATGGAGATAATGTTTNTCNTGTTAAGAGGCTAAGCTTAACTTGG
CTAAGGNGTGNCATTGAATACTNTCCATGGGGGCTCTNNTTGTNTTACTCCTC
TAATTGGGANATGCN

II. BGMV AR1

1.2

CCTCTCCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCGGCACGAGGG
CTGATATAATTGCTGAAATATCCGATAGTGTGCATACCCGCATTTAAGGCTGCT
GCGGAGGAGCTTCTAAATACTTCCGACCTGTCACCAGCAGAGTTGCTTGCGA
AAGCTCTTGCCAAGGCTGCTGGGTATTCTGAGATCAAGACTCGGTCTGCTTCTT
ACTTCTATGGAGA ACTGTGTTACTCTGCTTCTTGAGTGTGGGAGGCCCATATT

Table 6 continued

CTCACCTTCCTTTGTCTATAGTGTCTTGAGGAGGTTCCCTGCCTGAGGAGAAGG
TTGAATCAATCAACGGTCTTACTTTGACGGCCGATGGAAAGGGGGCAGTTTTT
GATGTATCTGCTGAGGACTTGGACCAATTTCTTGCAGGCCAAAAAACTGCAC
ATGGCGTAAATTTAGAGGTAGTGGAAGCGTTGCCTCAGTTGCAAGAGAGAGA
GAAGCCAAGAGGTGGAAGATTTGGAGGTGGTGGNCNTGGNTGGTTTTCTCCT
AACANAAGAGGCGGAGGTGGCNTTTTCTGGAGGGAATAGGGGGAAGANGTA
ATGGTAACTTTGGNCNCNNATNGTAAAGAGTGAATGCTGGNCNATTTTTTTG
ACACTGACAATAACAAGAATTTGGTCCAGTTCNTTCCAAATTTTNGATAGAA
ACNGATTTCAATAAAAATTCCTNCTNNGNGTTAATTAATTANANGGNNGGA
CATTTTAAGCTATTGAATGCCANTNGTTTCNCGGGATTTNGTTTGNATTNC
TCCAAGNAAGAGTTTTNAC

10.2

CCTCTCCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCGGCACGAGGG
AGA
GAG
AG
AGGACAATGGCTAAGTTCTGCTACTAGACATGTTAGAATATATGCNGCTTAT
ATTGATCCTGAAACTTTTGTCTTTGNCCANACACAAATGGATAAACTTACT
TATCCTTGACCCTACTGATGAATTTGTTTGGACTGATGATACTTGCACCAAGG
TCTACTCTTACTTCCAAGAACTTGTGATCNTTATGAGGGGAGCTCCATTGACG
GAATACACGCTTCGCCTGATCGGTTTCAGACATAGAGCACTACATAAGAAAGC
TTCTCTATACTGGAGAAATCCAATACAACATGAATGCAAAAAGTTCTTAACTTC
AGTATGGGGAAACCAAGAGTTGGGNTCNATTATAATGGCCAAATGGGAGAT
GTAACCCAGTAAATTCGGGNACAATGTTGTCTATNTTGANACAATGAGTNGC
AGAACTAGTTTTTAAGAATGTAAATGAAAATTCCTCCTTCTAGNAAAA
AAAAAAAAAAAAAAAAAACTCGAGAGCTTTGCTTCNCNCAGGGTNGGCAGTC
CCAANAGGTGCGGCTTTCCTCCTTCCAGAATTTCNAAAGAGGAAAGGCANTC
GNGGGGAACTTNTNCCCTCTAAAAGNGATTCTTNGTTTTTTTTTTTTAAAAGT
NAAAAAAAAAGGNTCCATTAAGCCCN

24.3

CCTCTCCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCGGCACGAGGGG
AGAACCAAACCCTTCCTTGAGCTACTTTTCCCTTTGCTATCCTAGCGACCCGA
GAAAGATGTCGTACTTGTTCCTCACCTTCACTCGGGATGGGCGGTGGATCA
GGCCATTCTCGCTGAAGAGGAACGCCTCGTCATTATCCGCTTCGGCCATGACT
GGGACGAACTTGCATGCAGATGGACGAGGTGCTGGCTTCACTTGCAGATAC
ATTAAAGAATTTTGTGTCATATACCTGGTGGACATCACAGAGGTCCCTGATT
TTAACACAATGTATGAGTTGTACGATCCATCCACTATCATGTTCTTCTTCAGG
AACAAGCACATCATGATTGATCTTGGCACAGGGAAACAATAACAAGATCAAC
TGGGCTCTCAAAGATAACAAGAGTTCATTGATATTGTTGAGACAGTGTACC
GTGGCGCGAGAAAGGGTCCGTGGTCTAGTTATTGCACCTAAAGGATTACTCT
ACCAAGTATCGTTACTGAGTGTCTGTGATATTGTAAGTAAATAAAGANAC
TTTATGATTCAACTGAATCAGCTGTCAAGACCCAGAAGTGTACGAATAACTTT
CCATTTAGTTGCAAGTGGAAAGACCTCTAGTCTTGTTCACCATGTTATGATA

Table 6 continued

AATTATATTGACTGTATTGAAANAANCCNCTCCTTATCNTNTNCTTCTTTC
NATNACAAAAAANNNCCGANAGTTTTGNNCTTTTTCCCGN
GNTTGGCAANCCCCANANGGTG

III. SLCV BR1

(s1.2 similar to 9.7)

CCTCTACCCTTATGATGTGCCAGATTATGCCTCTCCCGAAATTCGGCACGAGG
ATGATGAACAGTTTCAAACAATTGATGCTCGTGGTGTGAATGTACAAATTG
CATCCGGAGCTTGTATTACTATCCAATTTGTCACAAATATCATCATTACGGC
CTTCATATTCATGATTGTAAGCCCACTGGTAATGCCATGGTCCGAACTCACC
ATCACACTATGGTTGGAGGACAATGGCTGATGGTGATGGCATTTCATTTTCG
GTTTCGAGCCATATTTGGATTGACCACAACCTCTCTTTCTAATTGTGCCGATGGC
CTTATTGATGCTATCATGGGTTCAACTGCAATTACCATTTCAAACAATTACTT
TACACATCACAATGAGGTGATTTTATTGGGGCATAGCGACTCCTATATCAGA
GATAAGGTTATGCAGGTGACTATTGCCTTCAACCATTTTGGTGAGGGTCTCAT
CCAGAGAATGCCAAGATGTAGGCATGGTTATTTCCATGTGGGGAACAATGAC
TATACACACTGGGAGATGTATGCAATTGGTGGTAGTGCTGACCTACTATTAAC
AGCCAGGGCAACAGATACCTTGCCCCATTGAACCCCTTTGCCAAAAAGGGAN
AAAAANAGTGGAACCATGGGAAGGACATGGAANCACTGGNACTGGAAANC
AGAGGANATCTTATGCTGAATGGAGCATACTTACTGGCGCTGGACNNCCNCT
CCNCAGTTTTGCAANAACCCAGCTTACTTCTAAACCTCTCTATGGNGGANTA
TTACNN

(1.7 similar to 15.1)

CCCCTCCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCGGCACGAGGGT
TAAGAGGCAATCAAAGTTTCTATCTTGAAGGCTGGCTTTTGGGAATCCATCA
GATCCGGGTTTGTAAAGAATAACACAGTACAGGTTATAGAATCACCATCCAA
TGAAGAAGAAGAGGAAGAAGAGCCTTTGCCTGAGGAATTTGTTCTAATTGAA
AAGACTCAACCAGATGGAACAGTTGAACAGATTATATTCTCTTCTGGCGGAG
ATGTTGATGTGTATGATCTCCAAGCTTTATGTGATAAGGTTGGGTGGCCTCGA
AGGCCACTGTCTAAGCTAGCTGCTGCTCTTAAAAATAGCTATATAGTTGCAAC
TTTGCATTCCAGGAAGTTCTCTTCAGGAGAAGAGGGGAATGGAGAAAAGAAG
CTGATAGGCATGGCTCGTGCAACATCAGATCATGCTTTCATGCAACAATTTG
GGATGTTCTTGTGATCCTTCATATCAGGGACAAGGACTTGGAAAAGCTCTTA
TTGAGAACTGATAAGGACCCTTCTCAAAGAGACATTGGAAATATTTTCATT
GTTTCGCGGGATAGTCAAGTTGTGGAATTTCTATAAGATCTAGGTTTTGAACCTG
GACCAAGGGTATTAAGGGNATGTTTTGGTATCCAATGTATTAAACTGTATA
GTTGGACACAGTTTCGCCTCTTGCTCTGTACAAATTCCTCACGGGGGGGGGG
GAATGGGTTGATCTCGACAACCTGCAGCANTCTCGGAACCTTATAACNTTTTGC
C

Table 6 continued
(19.3 similar to 19.5)

CCTCCTCCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCGGCACGAGGG
TTTAAAGGAAAAGGGAGAAAGAGAAAATCTTTCTGTCTTAAGAGTAATTAGCA
ATGGCTTCCTCAGTTCCTTCCTCAGCAGCAGTTGCCACCCGCAGCAATGTTGC
TCAAGCTAACATGGTTGCACCTTTCACAGGTCTTAAGTCTGCTGCCTCATTCC
CTGTTTCAAGAAAGCAAAACCTTGACATCACTTCCATTGCCAGCAACGGCGG
AAGAGTGCAATGCATGCAGGTGTGGCCACCAATTAACATGAAGAAGTATGAG
ACTCTCTCATACTTCCCGATTTGAGCCAGGAGCAATTGCTCTCCGAAATTGA
GTACCTTTTGAAAAATGGATGGGTTCCCTTGCTTGGAATTCGAGACTGAGAAA
GGATTTGTCTACCGTGAACACCACAAGTCACCAGGATACTATGATGGCAGAT
ACTGGACCATGTGGAAGCTACCTATGTTCCGGATGCACTGATGCCACCCAAGT
GTTGGCTTGANGGGGAAAAGCGAAAAAGGAATACCCCCAGGCCTGGGGGCC
GTNATCATTGGGATTTGACAACGTGNGTGNAGGGGCAGGGCATTNAGTTTCA
TTGCCCTCCAAGNCTGGNNGNTACNGGGNTTCTTATTAGGACACCTANCCCT
NTTGNCTNTCNTTNGGGNAGATTGTTGNAAAGGTNNCTTANNTNTTTTTTCC
TTCCAAAAAATNTTATGTTCTTCTTTTATCGGGGNGTTTGGNTCNCCAGTT
TTGAACNTAAATTTGAAAAAAAAAAAA

40.5

CTTCCNANCTTATGACCNCCAGATTATGCCTCTCCCAATTCGGCACGAGGAGT
AGCAGTACAAGAAAAAGAAGAAAAACAGAGNAAAATCCAAAGAATTAATC
AAAGAAGAAAGCTTTTTGTGTTAACTTACCATGGTCTTTGACTGTTCCCTGAT
ATCAAGACTCTCTTTGCTGAATCTGGAATTGTTGAGGATGTTGAGATTATAAA
GACCAAAGATGGGAAGAATAGAGGCTTTGCCTTTGTGACTATGTCTTCTGGG
GAAGAAGCTCAGGCTGCAATTGACAAATTGGATTCTTATACATTATTAGGAA
GGATCATCAGGGTAGAATTCGCGAAGCGATTTAAGAAACCTGCCGGAGCTCC
TCCTCCTCCAACCTCCTCCTCGAGGGGAAACACGGCATAAACTTTATGTTTCTA
ATCTTGCATGGAAAGTGAGGTCTACCCATCTGAGGGACTTCTTCTCTACAAAC
CACAATCCGGTCTCAGTTAGGGTTGTCTTTGACAACGCTTCAGGAAGAGCTGC
TGGGTATGGTTTTGTGTCAATTTGACACCAAAGAAGAAGCAGAGGCTGCACT
GTCTGCTTTCGATGGAAAGGAACTGATGGGCCGACCAATTCGCTTGAATTC
AGTGAAAGGGATGCAGATGGTTCTGAAACAATGAAGACCGATTCAGGAAAG
TCCTGAAGAGCCATAAATCATTGACCTCTCGACCCCTTTGATGNCTTCTAT
GGGGACNTCAGGCCTCTTAATCTGCCCTTTGCCATAATGATGATGGTACATGA
A