Abstract

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Geminiviruses are a group of plant-infecting viruses that include the genera *Begomovirus* and *Curtovirus*. Although members of these two genera are similar in regards to their mechanisms of replication and encapsidation, they exhibit differences in movement as well as in transcriptional activation of late gene promoters. This study evaluated protein-protein interactions of the begomovirus AL2 protein and a curtovirus C2 protein using the yeast two-hybrid system. The AL2 proteins of TGMV, BGMV, and CabLCV were evaluated for their ability to self-interact, as well as to interact with heterologous AL2 proteins, and host proteins. The AL2 proteins tested were all shown to contain the ability to self-interact and to interact heterologously with one another. However, interactions between the AL2 proteins and the host proteins could not be assayed due to lack of expression of certain AL2 'bait' proteins. The BCTV C2 protein was also evaluated for self-interaction and interaction with the begomovirus AL2 proteins. Unlike AL2, C2 was not shown to contain the property of self-interaction and the preponderance of the evidence demonstrated that it also did not interact with AL2. However, the BCTV C2 protein was shown for the first time to possess transcriptional activation activity. In addition, self-interaction of TGMV AL2 was tested using a biochemical assay. However, technical problems prevented the detection of TGMV AL2 self-interaction using an immunocapture approach, although such self-interaction was observed using the yeast two-hybrid system.

Comparative analysis of the begomovirus AL2 protein and the curtovirus C2 protein

by

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List of Abbreviations

TGMV	tomato golden mosaic virus
BGMV	bean golden mosaic virus
CabLCV	cabbage leaf curl virus
BCTV	beet curly top virus
ACMV	African cassava mosaic virus
TMV	tobacco mosaic virus
LexA	LexA DNA-binding domain
B42AD	B42 activation domain
pLexA	plasmid encoding the LexA DNA-binding domain
pB42AD	plasmid encoding the B42 activation domain
ADH1	alcohol dehydrogenase promoter
GAL1	galactose promoter
LEU2	leucine reporter gene
His	histidine
Trp	tryptophan
Leu	leucine
glu	glucose
gal	galactose
TAL2	TGMV AL2 protein
BAL2	BGMV AL2 protein
CAL2	CabLCV AL2 protein
C2	BCTV C2 protein
N-terminal	amino terminal
C-terminal	carboxy terminal
ΔC	carboxy-terminal truncation
ΔN	amino-terminal truncation
W124A	mutation of tryptophan to alanine at position 124
LexA-	LexA carboxy-terminal fusion protein
-LexA	LexA amino-terminal fusion protein
B42AD-	B42AD carboxy-terminal fusion protein
Nb	*Nicotiana benthamiana*
Pos	an autoactivating 'bait' comprised of the activation domain of the yeast transcription factor Gal4p
p53	human protein 53
SV40	simian virus 40
T	large T-antigen
HA	hemagglutinin epitope tag
GST	glutathione *S*-transferase
IgG	immunoglobulin G
Y-PER	yeast protein extraction reagent

List of Abbreviations (cont.)

IP	immunoprecipitation
Rpm	revolutions per minute
ORF	open reading frame
PCR	polymerase chain reaction
DSS	disuccinimidyl suberate

Introduction

Geminiviruses

Viruses are obligate intracellular pathogens that use their host's replication machinery to create viral progeny. Geminiviruses infect a wide range of plant species and are responsible for significant losses in a variety of vegetable, fiber and cereal crops throughout the world (Bennett, 1971). These viruses are characterized by a twinned particle morphology and a circular, single-stranded DNA genome (Hamilton *et al*., 1982, 1983). Geminiviruses typically replicate their DNA by a rolling circle mechanism in the nucleus of the plant cell using double-stranded (ds)DNA intermediates. Viral dsDNA forms are also used as templates for transcription (Bisaro, 1996). Despite these similarities, members of the family *Geminiviridae* are different with regards to genome structure, vector type, and host range (Matthews, 1991). Viruses belonging to the genus *Mastrevirus* have a monopartite genome, infect primarily monocotyledons, and are transmitted by leafhoppers. Members of the genus *Curtovirus* also have a monopartite genome and are transmitted by leafhoppers but infect dicotyledons. An example of a member of this genus is beet curly top virus (BCTV). In contrast, viruses belonging to the genus *Begomovirus* have bipartite genomes, infect dicotyledons, and are transmitted by whiteflies. Examples of begomoviruses are tomato golden mosaic virus (TGMV), bean golden mosaic virus (BGMV), and cabbage leaf curl virus (CabLCV).

Bipartite begomoviruses from the New World have genomes comprised of two DNA components (DNA A and DNA B, figure 1) that generally are both required for infectivity. The A component contains five open reading frames (ORFs) and the B component contains

two ORFs which are named according to their respective DNA component and direction of transcription. In these viruses, proteins necessary for DNA replication, regulation of viral gene expression, and encapsidation are encoded by DNA A, while DNA B encodes proteins necessary for movement in the infected plant (Rogers *et al*., 1986; Sunter *et al*., 1987; Sunter and Bisaro, 1991, 1992; Etessami *et al*., 1988; Noueiry *et al*., 1994). Members of the genus *Curtovirus* differ in their genome organization from the begomoviruses in having a single circular DNA component which contains all the genes required for replication and movement. The nucleotide sequences of three different BCTV strains have been determined (Stanley *et al*., 1986; Stenger, 1994) and sequence comparisons have revealed that the BCTV genome contains seven conserved ORFs, three on the virion-sense DNA strand and four on the complementary-sense DNA strand (figure 2).

It has been hypothesized that the complementary-sense ORFs of the BCTV genome are homologues of their bipartite virus counterparts and so their products should have similar functions. Indeed, mutational analysis has indicated that the BCTV C1 protein, like AL1, is essential for viral DNA replication (Elmer *et al*., 1988; Briddon *et al*., 1989) and that C3, like AL3, is involved in the control of this process (Sunter *et al*., 1990; Stanley *et al*., 1992; table 1). Functional homology of the C3 and AL3 proteins was confirmed in experiments which showed that the BCTV *C3* gene can complement a TGMV *al3* mutant and vice versa (Hormuzdi and Bisaro, 1995). However, evidence suggests that the BCTV *C4* gene, which appears to be a symptom determinant (Stanley and Latham, 1992), plays a more significant role in viral pathogenesis than does *AL4* in the bipartite begomoviruses. In African cassava mosaic virus (ACMV) and TGMV, mutations in the *AL4* gene have been shown to have little

or no effect on virus replication, infectivity, or disease development (Etessami *et al*., 1991; Pooma and Petty, 1996), suggesting that the protein is either non-functional or functionally redundant with an essential begomovirus protein. With the virion-sense ORFs, it has been determined that *V1*, like the *AR1* ORF of the bipartite begomoviruses, encodes the viral coat protein which in BCTV is required for systemic infection (Briddon *et al*., 1989). The coat proteins of both curtoviruses and begomoviruses are necessary for transmission by their respective vectors (Briddon *et al*., 1990; Azzam *et al*., 1994).

While the viral proteins involved in replication and encapsidation of begomoviruses and curtoviruses are quite conserved, those involved in virus movement through the plant are somewhat different. The B component of the begomovirus DNA genome encodes two movement proteins, BR1 and BL1. These proteins are believed to act in concert to direct virus movement in a host plant (Schaffer *et al*., 1995). The BR1 and BL1 proteins are both necessary for local and systemic movement of the virus, and the expression of both is sufficient for the systemic movement of the DNA A component (Jeffrey *et al*., 1996). In contrast, the curtovirus genome does not contain a separate component harboring the movement proteins. The coat protein (V1) and the V3 protein have been shown to be involved in the systemic spread of BCTV, since *v1* and *v3* mutants both produced wild-type levels of replication in protoplasts but were not infectious in plants (Hormuzdi and Bisaro, 1993; Frischmuth *et al*., 1993). This differs from the begomoviruses in that coat protein expression is not required for their movement (Pooma *et al*., 1996).

In addition to differences in viral movement, begomoviruses and curtoviruses are different with regards to their regulation of gene expression. It has been shown that

begomoviruses conform to the general strategy of DNA virus transcription mechanisms in which early gene products activate the expression of viral genes required later in the infection cycle. In TGMV, for example, the transcriptional activator protein, AL2, is required for infectivity because it is necessary for expression of the coat protein, AR1, and one of the movement proteins, BR1 (Sunter and Bisaro, 1992). Consistent with this function, AL2 has been shown to contain a minimal 15 amino acid transcriptional activation domain at its C-terminus (Hartitz *et al*., 1999). In the curtoviruses, a viral protein required for coat protein expression has yet to be identified. Although the BCTV genome contains an ORF positionally analogous to AL2, known as C2, *c2* mutants can systemically infect plants and are not impaired in their ability to express coat protein (Stanley *et al*., 1992; Hormuzdi and Bisaro, 1995). In addition, while several different begomoviruses produce a protein that can *trans*-activate the coat protein gene promoter of a TGMV *al2* mutant in tobacco protoplasts, BCTV is unable to complement this mutant (Sunter *et al*., 1994). Thus, the similar position of the *AL2* and *C2* ORFs on their respective genomes does not reflect a similar role in transcriptional regulation, and these genes are considered to be paralogues.

Despite the lack of a role for the C2 protein in coat protein expression, *c2* mutants have displayed a phenotype in one investigation. In this study, a significant number of *Nicotiana benthamiana* plants inoculated with BCTV *c2* mutants began demonstrating signs of recovery three weeks after the disease symptoms first appeared (Hormuzdi and Bisaro, 1995). However, this enhanced recovery phenotype appears to be dependent on the host, as it was not observed in sugar beet (*Beta vulgaris*) inoculated with BCTV *c2* mutants (Stanley *et al*., 1992). In another study, *N. benthamiana* and *Nicotiana tabacum* plants containing

TGMV *AL2* or BCTV *C2* transgenes were inoculated with TGMV, BCTV, or tobacco mosaic virus (TMV). The expression of the transgenes resulted in enhanced susceptibility of the plants to viral infection, suggesting that both AL2 and C2 have a role in host defense suppression (Sunter *et al*., 2001). In addition, it was shown that an *AL2* transgene lacking the C-terminal activation domain elicited the enhanced susceptibility phenotype, indicating that host defense suppression does not require direct transcriptional activation by AL2 (Sunter *et al*., 2001). Therefore, the transcriptional activation function of the begomovirus AL2 protein is independent of host defense suppression.

The Yeast Two-Hybrid System

One of the major focuses of this research was to evaluate protein-protein interactions involving the begomovirus AL2 protein and the curtovirus C2 protein both with themselves, each other, and with host proteins. One of the most commonly used methods to detect *in vivo* protein-protein interactions is the yeast two-hybrid system (Gyuris *et al*., 1993). This system takes advantage of the fact that multiple eukaryotic *trans*-acting transcriptional regulators are composed of physically separable, functionally equivalent domains such as a DNA-binding domain that binds a specific promoter sequence and an activation domain that directs transcription of the gene. Both domains are required for activation of the gene. In the yeast two-hybrid system, DNA fragments encoding proteins to be assayed for interaction are fused separately to a DNA-binding domain and an activation domain. If interaction between the proteins occurs, the domains are brought into close physical proximity and transcriptional activation of a reporter gene will occur. In the MATCHMAKER LexA two-hybrid system (Clontech), the DNA-binding domain is provided by the *Escherichia coli* LexA protein while

the activation domain is an 88-residue acidic peptide (B42) that has been shown to activate transcription in yeast (Ma and Ptashne, 1987). Two different cloning vectors are used to generate fusions of these domains to two separate DNA fragments encoding proteins to be co-expressed in yeast and assayed for interaction. The 'bait' protein is inserted into the pLexA vector containing the DNA-binding domain and a *HIS3* marker (figure 3), while the 'prey' protein is inserted into the pB42AD2 vector containing the activation domain (B42AD) and a *TRP1* marker. The activation domain fusion gene is under control of the yeast *GAL1* promoter, so it is only expressed in medium that contains galactose as the carbon source (figure 4). The interaction between a 'bait' and a 'prey' will induce the expression of a reporter gene, *LEU2*, allowing the growth of yeast that contain interacting proteins on medium lacking leucine (figure 5). A two-hybrid interaction in this system is also dependent on the presence of galactose, which is required to induce expression of the B42AD fusion proteins.

While the yeast two-hybrid system can be an extremely useful tool for evaluating potential protein-protein interactions, for some proteins, this may not be the best approach. Since results from yeast two-hybrid assays depend on the activation of the *LEU2* reporter gene, one of the most important experiments to conduct prior to the interaction assay is one that tests the autoactivation potential of the 'bait' protein. If the 'bait' protein activates the *LEU2* reporter gene in the absence of any 'prey' protein, then it cannot be used in interaction assays. In addition to lacking autoactivation activity, in order to be effective, the 'bait' protein must be, when expressed, stable in yeast, and it must accumulate in the nucleus where it can bind to the promoter of the *LEU2* reporter gene. Even if the 'bait' protein meets all

these criteria, detection of specific interactions with 'prey' proteins is not assured and a number of controls must be performed. Among the most important of these controls are to ensure that growth of yeast is galactose dependent, since expression of the 'prey' protein requires galactose. It is also necessary to ensure that a potential 'prey' protein interacts specifically with the 'bait' of interest. This can be evaluated by testing whether the 'prey' protein will allow growth of yeast when co-expressed with the LexA DNA-binding domain alone, or when co-expressed with LexA fusions to control proteins with which the 'prey' is not expected to interact (so-called irrelevant 'baits'). If a pair of 'bait' and 'prey' proteins passes all of these tests, it is likely that the two proteins interact. However, it is generally considered necessary to confirm their interaction by alternative biochemical approaches to guard against possible artifacts of the yeast two-hybrid system.

Additionally, there are two variations of the LexA yeast two-hybrid system known as the Gal4/VP16 and the Gal4/B42 yeast two-hybrid systems. Both of these systems utilize Gal4 as the DNA-binding domain but the Gal4/VP16 system uses VP16 as the activation domain while the Gal4/B42 system uses B42 as the activation domain. Each variation of yeast two-hybrid system has its own advantages and disadvantages, and the LexA system is best suited for the interactions assayed in this project.

Methods

PCR

Each 50 µl PCR reaction was set up with 5 ng template DNA, 5 µl of 10X thermo buffer (100mM Tris.HCl, pH 9.0, 500mM KCl, 1% Triton X-100), 5 µl 25mM $MgCl_2$, 2 µl 5mM dNTP cocktail (5mM each of dATP, dCTP, dGTP, and dTTP), 250 ng of each primer, 0.25 µl Taq DNA polymerase and dH_2O to 50 µl in a 0.5 ml microfuge tube. The PCR reaction was incubated at 94ºC for one minute, 50ºC for one minute, and 72ºC for one minute for 25 cycles. After the final cycle, the reaction was held at 4ºC.

TOPO® cloning

Each cloning reaction (1 µl PCR product, 1 µl salt solution, 3 µl sterile water, and 1 µl TOPO® vector) was set up in a 1.5 ml microfuge tube using the TOPO® TA Cloning kit (Invitrogen) and the reactions were mixed by flicking. After a five minute incubation at room temperature, 2 µl of the reaction was added to one vial of One Shot® Chemically Competent *E. coli* cells and placed on ice for five minutes. The cells were heat shocked in a 42ºC water bath for 30 seconds and then placed on ice immediately. 250 µl 2xTY medium was added and the tube was capped tightly and shaken horizontally at 37ºC at 250 rpm for one hour. After spreading 10 µl of the transformation mix and 190 µl dH_20 onto LB plates (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 3mM NaOH) containing 50 µg/ml kanomycin and 50 µg/ml x-gal, 50 µl of the transformation mix and 150 µl dH_2O was also spread. The plates were allowed to dry and incubated overnight at 37ºC.

Gel purification of DNA fragments

A restriction digest was run on a 1% TBE (89mM Tris.HCl, 89mM boric acid, 2mM disodium EDTA, pH 8.0) agarose gel containing 1 µg/ml ethidium bromide. After cutting out the required DNA fragments with a razor blade, the gel slices were weighed in a 1.5 ml microfuge tube to determine their volume. The DNA fragments were purified using the GENECLEAN II kit (Bio 101 Inc.). 0.5 gel slice volumes of TBE modifier and 4.5 gel slice volumes of NaI solution were added to the microfuge tube. The tube was mixed gently and incubated in a 50ºC waterbath for five minutes to dissolve the agarose. If the agarose was not completely dissolved after five minutes of incubation, the tube was mixed gently at room temperature until it was. 5 µl of thoroughly resuspended GLASSMILK was added to the tube and mixed hard to disperse the glass evenly. After incubating at room temperature for five minutes the glass was pelleted by centrifugation and the supernatant was removed with a P1000. 0.5 ml of NEW wash was added to the glass pellet and mixed hard to resuspend. The glass was pelleted again by centrifugation and the supernatant was removed. The NEW wash was added twice more for a total of three washes. After removing the supernatant from the final wash, the glass pellet was allowed to dry on the bench for ten minutes (the open tube was covered with a KimWipe to prevent airborne particles from dropping in it). The DNA was eluted by adding 10 µl of dH_2O 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 to prevent any DNA from being lost. The tube was incubated in a 50ºC waterbath for five minutes, centrifuged for one minute to pellet the glass, and the supernatant was transferred to a fresh microfuge tube. The elution step was repeated to elute all of the bound DNA and the

supernatant was pooled with the first one. The success of the gel purification was checked by running 5 µl of the eluted fraction on a 1% agarose gel.

Preparation of competent bacterial cells

A single colony of *Escherichia coli* DH5αF' was inoculated into 3 ml 2xTY broth (1.6% bactotryptone, 1% yeast extract, 0.5% NaCl) and grown overnight at 37°C with shaking at 250 rpm. 25 µl of overnight culture was subcultured into 7.5 ml of 2xTY broth in a sterile 15 ml plastic centrifuge tube and grown at 37°C with shaking for four hours. The bacteria was pelleted by centrifuging for three minutes at 5000 rpm. The culture supernatant was removed and the pellet was resuspended in 7.5 ml of cold 50 mM $CaCl_2$ by gentle mixing. After incubation on ice for 20 minutes, the cells were again centrifuged for three minutes at 5000 rpm. After pouring off the supernatant, the pellet was resuspended in 1 ml of cold 50 mM $CaCl_2$.

Transformation of *E. coli*

200 µl of competent *E. coli* DH5αF'cells was added to each 1.5 ml microfuge tube with the appropriate amount of DNA (0.2 µl of purified plasmid DNA, or a complete ligation reaction) for each transformation. The cells were incubated on ice for 40 minutes, heat-shocked in a 42°C waterbath for three minutes, and transferred back to ice for five minutes. 0.8 ml of 2xTY broth was added and the cells were placed in a 37°C waterbath for at least ten minutes. 200 µl aliquots of each transformation were spread onto LB plates containing 50 µg/ml ampicillin to select for the plasmid. The plates were allowed to dry and incubated at 37ºC overnight.

Small scale purification of plasmid DNA

A single colony from a transformation plate was picked and inoculated into 3 mls of 2xTY broth containing 100 μg/ml ampicillin. The culture was grown overnight at 37ºC with shaking. 1.5 ml of the culture was dispensed into a 1.5 ml microfuge tube and microfuged for one minute to pellet the cells. The pellet was resuspended in 100 μl of TEG (25 mM Tris-HCL, pH 8.0, 10 mM EDTA, 50 mM glucose). When cells were completely resuspended, 200 μl 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 incubated on ice for five minutes. 150 μl of 3M KOAc (pH 4.8) was added and the tube was mixed hard and microfuged at 4ºC for five minutes. The supernatant was recovered into a clean microfuge tube and 0.5 ml phenol:chloroform was added, mixed hard to emulsify, and microfuged at room temperature for two minutes to separate the phases. The aqueous phase was recovered into a clean microfuge tube and 1 ml of EtOH was added, mixed by rapid inversion, and incubated at room temperature for two minutes. After microfuging at room temperature for five minutes, the supernatant was completely removed. The pellet was dried under vacuum for ten minutes. The pellet was resuspended in 50 μl of TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA) containing 50 μg/ml RNase A and incubated at 37°C for 30 minutes. 1 μl of 10 mg/ml proteinase K was then added and the incubation was continued at 37°C for a further 30 minutes. 50 μl of phenol:chloroform was added and mixed to emulsify. After microfuging for three minutes to separate the phases, the aqueous phase was recovered into a clean microfuge tube. 50 μl dH_2O was added to the phenol phase, mixed, and microfuged again. The two aqueous phases were pooled together and 100 μl of chloroform

was added, mixed, and microfuged for one minute. The aqueous phase was recovered in a clean microfuge tube and 25 µl of 10M ammonium acetate and 250 µl of EtOH were added. After gentle mixing the samples were stored at -20°C overnight to precipitate the DNA. The DNA was pelleted by centrifugation at 4°C for ten minutes and after completely removing the supernatant, the pellet was vacuum-dried for 10 minutes. The pellet was resuspended in 50 µl of dH_2O and stored at -20°C.

Restriction digets

Each 10 µl restriction digest was set up with 1 µg of DNA, 1 µl of appropriate NEB buffer, 0.01 units of appropriate restriction enzyme, and dH_20 to 10 µl (1µg/ml BSA was included if specified) in a 1.5 ml microfuge tube. The tube was flicked and quick-spun to mix. The digests were incubated for two hours in a 37ºC waterbath unless otherwise specified. The results were checked by adding 5 µl of 0.05% bromo-phenol blue dye to the 10 µl digests and running them on a 1% TAE (400mM Tris.HCl, 50mM sodium acetate, and 10mM disodium EDTA pH 7.8) agarose gel containing 1µg/ml ethidium bromide.

DNA sequencing

All DNA sequences were performed by Iowa State University using 2.5 µg/µl of purified plasmid DNA and 5 pmol/µl of appropriate primer.

Transformation into EGY48 yeast cells

3 ml of glucose rich medium (20mg/ml peptone, 10mg/ml yeast extract, and 20 mg/ml glucose) was inoculated with a loopful of fresh EGY48 yeast and grown at 30°C with shaking at 250 rpm overnight. The culture was removed from the incubator the following day and a cell count was performed to determine the amount of overnight culture needed to

inoculate $2.5x10^8$ cells/ml into 50 ml of room temperature glucose rich medium. The 50 ml subculture was grown at 30 ^oC with shaking at 250 rpm for approximately four hours. After the four-hour incubation period the cells were counted again to ensure that two doublings had occurred and there were enough cells to successfully conduct the transformation. The 50 ml culture was aseptically poured into a sterile 50ml falcon tube and centrifuged at 3,000 rpm for five minutes to pellet the cells. The supernatant was poured off and the cell pellet was resuspended in 25 ml of sterile dH_2O. The cells were centrifuged as before for five minutes. After the supernatant was again removed from the cell pellet it was resuspended in 1 ml of 100mM LiAc. The suspension was transferred to a 1.5 ml microfuge tube and quick-spun in the microfuge to pellet the cells. The pellet was resuspended to 500 µl with 100mM LiAc and 50 µl aliquots were dispensed into 1.5 ml microfuge tubes. The tubes were quick-spun and the supernatants were removed. To each cell pellet the following was added in order: 240 µl 50% PEG, 36 µl 1M LiAc, 25 µl 2 mg/ml sheared and denatured salmon sperm DNA, and 50 µl of sterile dH2O containing purified plasmid DNA as appropriate. The tubes were mixed until the pellets were resuspended. The cells were then incubated for 30 minutes at 30^{o}C and heat shocked in a 42ºC water bath for 20 minutes. The cells were next pelleted by centrifugation at 10,000 rpm, the supernatants were removed and the pellets were resuspended in 1 ml sterile dH_20. 200 µl of the transformation mix was spread onto minimal glucose medium (20 mg/ml glucose and 6.72 mg/ml yeast nitrogen base) and incubated for 72 hours at 30ºC.

Preparation of transformed EGY48 yeast cells for lysis

Transformed EGY48 yeast cells were grown overnight in glucose minimal medium at 30°C with shaking at 250 rpm. A cell count was performed to determine the amount of overnight culture needed to inoculate 2.5 x 10^8cells/ml into a 50 ml subculture. This amount was dispensed into either 50 ml of room temperature glucose rich medium and grown for approximately four hours (for yeast transformed with LexA fusion plasmid) or into 50 ml of room temperature minimal media containing galactose (20 mg/ml) and raffinose (10 mg/ml) as the carbon sources for approximately ten hours (for yeast transformed with B42AD fusion plasmid) at 30°C with shaking at 250 rpm. Following the four or ten hour incubation, another cell count was performed to make sure that the yeast had undergone two doublings (at least 2x10^7 cells). The 50 ml subculture was aseptically transferred into a sterile 50 ml centrifuge tube and centrifuged for five minutes at 3000 rpm. The supernatant was discarded and the pellet was washed in 25 ml of sterile dH_20. The cells were again pelleted by centrifugation for five minutes at 3000 rpm. The supernatant was poured off and the cells were resuspended by mixing and transferred to microfuge tubes. The resuspended pellets were microfuged briefly at 14,000 rpm and the supernatants were removed.

Lysate preparation from yeast cells grown in glucose rich medium

Each yeast cell pellet was resuspended in an equal volume of 2x Laemmli buffer (Laemmli 1970) and boiled for five minutes in a water bath. The samples were then microfuged five minutes at room temperature at 14,000 rpm to pellet the cells and generate the soluble fractions.

Lysate preparation from yeast cells grown in galactose minimal medium

See Table 3.

SDS PAGE

The protein gel casting stand was assembled and the 10 ml 12% polyacrylamide separating gel (3.3 ml dH_2O, 2.5 ml 1.5M Tris.HCl, pH 8.8, 4 ml 30% acrylamide stock (30:0.8 acrylamide: *bis*- acrylamide), 100 µl 10% sodium dodecyl sulfate (SDS), 100 µl ammonium persulfate(APS), 5 µl TEMED) was added until approximately one inch from the top of the casting stand. 100µl tert-amyl alcohol was added to the unpolymerized gel. After polymerization and removal of the tert-amyl alcohol, the 6 ml 4% polyacrylamide stacking gel (4.33 ml dH_20, 0.75 ml 1M Tris.HCl, pH 6.8, 0.8 ml 30% acrylamide stock, 60 µl 10% SDS, 60 µl 10% APS, 5 µl TEMED) was added to the top of the casting stand. Following polymerization of the stacking gel, 20 µl of cleared yeast cell lysate was added to each gel well. The protein gel was run in 1x protein gel running buffer (0.38M glycine, 50 mM tris base, 0.1% SDS) at 100 volts until the bromophenol blue reached the bottom of the stacking gel. The protein gel was then run at 150 volts until the bromophenol blue reached the bottom of the separating gel. The protein gel was stained in 0.5% Coomassie Blue dye (75mM picric acid, 25% MeOH) for 20 minutes at 50 rpm and rinsed in tap water to assess the relative amount of protein in each cleared lysate fraction.

Transfer of protein to nitrocellulose membrane - western blot

The western blot apparatus (Bio-Rad) was set up as follows: fiber pad, blotting paper, protein gel, nitrocellulose membrane, blotting paper, and fiber pad making sure there were no

air bubbles between the gel and nitrocellulose. The apparatus was closed inside a cassette, soaked in 1x western transfer buffer (1.92 M glycine, 250 mM tris base, 20% MeOH) and placed into a western blot electrode. After immersing in 1x western transfer buffer the western blot was run overnight at approximately 35 volts. The following day the nitrocellulose membrane was removed from the western blot assembly and incubated in 25 ml of 5% milk blocking solution (5% nonfat milk, 0.05% Tween-20, and 50 ml 1x TBS) and rotated at 50 rpm for one hour at room temperature. The blocking solution was poured off and 25 ml of a 1:200 dilution of primary antibody (anti-LexA MAb or anti-HA F-7 MAb in 50 ml 1x TBS with 5% nonfat milk and 0.05% Tween-20) was added and rotated at 50 rpm for one hour at room temperature. After pouring off the primary antibody, the blot was washed with 25 ml 1x TBS for five minutes a total of five times by rotation at 50 rpm. 25 ml of a 1:2000 dilution of secondary antibody (anti-mouse IgG - horseradish peroxidase conjugate) was added and rotated at 50 rpm for one hour at room temperature. After pouring off the secondary antibody and washing again five times with 1x TBS for five minutes each, 5 ml of Supersignal® West Pico stable peroxide solution and 5 ml Supersignal® West Pico luminol enhancer (Pierce) was added and rotated for five minutes at 250 rpm at room temperature. The solution was poured off and in the darkroom, x-ray film was exposed to the nitrocellulose membrane for 30 seconds and five minutes. The film was developed for five minutes, rinsed in tap water, fixed for five minutes, and rinsed again in tap water.

Autoactivation test

10^{-1}, 10^{-2}, and 10^{-3} dilutions of yeast transformed with each 'bait' plasmid to be tested were set up. Eight μl of each dilution was spotted onto plates containing minimal glucose

medium lacking histidine but containing leucine (+ Leu) to select for the plasmid and minimal glucose medium lacking histidine and leucine (- Leu). Both a positive control that demonstrated autoactivation by growth on the - Leu glucose medium and a negative control that had been previously shown to not autoactivate were also included. Samples that showed growth on the - Leu glucose medium demonstrated autoactivation of the leucine reporter gene without a 'bait'/'prey' interaction and were thrown out. Yeast strains containing 'baits' that grew on the + Leu glucose medium but did not grow on - Leu glucose medium contained non-autoactivating proteins and were used in interaction assays.

Interaction test

10^{-1}, 10^{-2}, and 10^{-3} dilutions of yeast transformed with DNA encoding both a 'prey' protein and a 'bait' protein that did not autoactivate were set up. Eight µl of each dilution was plated onto three different mediums: medium lacking both histidine and tryptophan containing glucose as the carbon source that allowed growth of yeast transformed with both 'bait' and 'prey' plasmids (+ Leu glu), a medium lacking histidine, leucine, and tryptophan containing galactose as the carbon sources that allowed expression of B42AD fusion proteins (- Leu gal), and a glucose medium lacking histidine, tryptophan, and leucine containing glucose as a carbon source which demonstrated galactose dependence of 'prey' protein expression (- Leu glu). Positive control strains expressing both 'bait' and 'prey' proteins previously shown to interact and negative control strains expressing 'bait' and 'prey' proteins previously shown to not interact were included. Yeast strains co-expressing 'bait' and 'prey' proteins that grew on the – Leu gal plate but did not grow on the – Leu glu plates contained interacting proteins.

Immunocapture assay

Binding of the antibody to immobilized protein G

The immunocapture assay was performed using the Seize™ X Yeast Immunoprecipitation Kit (Pierce). 0.4 ml of ImmunoPure® Plus Immobilized Protein G was added to a column and placed inside a microfuge tube. The tube was capped and spun for one minute at 14,000 rpm in a microfuge and the flow-through collected in the tube was discarded. The gel was washed twice by adding 0.4 ml of Binding/Wash Buffer 1 (0.14M NaCl, 0.008M Na_2PO_4, 0.002M K_2PO_3, and 0.01M KCl, pH7.4) to the column containing the Immobilized Protein G and mixing it gently end-over-end for two minutes. The tube was spun in the microfuge at 14,000 rpm for one minute and the buffer collected in the tube was poured out. 100μg of purified HA F-7 monoclonal antibody was prepared in an equal volume of Binding/Wash Buffer 1 and added to the column. The tube was capped and shaken at 100 rpm for 15 minutes to allow the antibody to bind the matrix. The tube was then spun for one minute at 14,000 rpm in the microfuge and the flow-through was discarded. The column was washed three times by adding 0.5 ml of Binding/Wash Buffer 1, capping the tube, and inverting it gently end-over-end 10 times. The flow-through was discarded and after the final wash, the column was transferred to a new microfuge tube and 0.4 ml of Binding/Wash Buffer 1 was added.

Cross-linking of the bound antibody

13 mg of DSS was dissolved in 1.0 ml of DMSO. 52 μl of the DSS solution was added to the center of the column containing the bound antibody support. The microfuge

tube was capped and mixed for one hour at room temperature at 100 rpm. The tube was then spun for one minute at 14,000 rpm in the microfuge. The tube was emptied and 500 µl of Quenching/Wash Buffer 2 (25mM Tris.HCl, 0.15M NaCl, pH7.2) was added to the column. The tube was capped and mixed at 100 rpm for 10 minutes. The tube was then spun for one minute at 14,000 rpm in the microfuge. The microfuge tube was emptied and 500 µl of Quenching/Wash Buffer 2 was added again to the column. The tube was capped, mixed end-over-end ten times, and spun for one minute at 14,000 rpm in the microfuge. The column was placed into a new microfuge tube and 190 µl of Immunopure® Elution Buffer was added to remove the excess DSS and uncoupled antibody. The tube was capped and mixed end-over-end ten times. The tube was spun at 14,000 rpm in the microfuge and emptied. After the elution, the column was placed into a new microfuge tube and washed three times by adding 500 µl of Quenching/Wash Buffer 2, capping the tube, inverting the tube ten times, and spinning the tube at 14,000 rpm in the microfuge. After the final wash, the column was placed into a new microfuge tube.

Immunoprecipitation of the antigen

The cleared yeast lysate sample to be run over the column was diluted 1:1 with Binding/Wash Buffer 1 and added to the column. The sample was incubated by mixing at 100 rpm at room temperature for one hour. The tube was spun for one minute at 14,000 rpm in the microfuge. The column was placed into a new microfuge tube and washed four times with 500 µl of Quenching/Wash Buffer 2. The tube was capped, inverted ten times, and spun in the microfuge one minute at 14,000 rpm. The flow-through was discarded. After the final wash, the column was placed into a new microfuge tube.

Elution of the immunoprecipitated antigen

190 µl of ImmunoPure® Elution Buffer was added to the column. The tube was capped, inverted ten times, and spun at 14,000 rpm in the microfuge. The elution was repeated two additional times and the three eluted fractions are assessed for protein accumulation by SDS PAGE and western blot.

Results

I. Analysis of begomovirus AL2 proteins and curtovirus C2 protein using the yeast two-hybrid system

Homologous and heterologous interactions of begomovirus AL2 proteins

Previously, it was shown using the yeast two-hybrid system that TGMV AL2 protein (TAL2) self-interacts (Jordan, J.K. and Petty, I.T.D., unpublished results). To eliminate its autoactivation potential, 13 C-terminal amino acids were deleted from TAL2, and the resulting protein, TAL2ΔC, was expressed as a C-terminal fusion to the LexA DNA-binding domain. This fusion protein will be designated LexA-TAL2ΔC. In contrast, full-length, wild-type TAL2 was expressed as a C-terminal fusion to the B42 activation domain. This fusion protein will be designated B42AD-TAL2.

One of the first questions addressed during this project was if other begomovirus AL2 proteins would also self-interact. The first thing done was the construction of plasmids encoding either the BGMV AL2 protein (BAL2) or CabLCV AL2 protein (CAL2) as C-terminal fusions to the LexA DNA-binding domain. Because BAL2 and CAL2, like TAL2, are transcription activators, it was assumed that fusion of the full-length wild type AL2 protein to LexA would result in an autoactivating 'bait'. To avoid this potential problem, deletion mutants of BAL2 and CAL2 lacking the 13 C-terminal amino acids (BAL2ΔC and CAL2ΔC, respectively) were used. PCR products of the truncated *AL2* ORFs flanked by *Nco*I (5') and *Xho*I (3') restriction sites were generated, cloned, and sequenced (Perera, P.S. and Petty, I.T.D., unpublished results). These plasmids were digested with *Nco*I and *Xho*I and the resulting 367 bp DNA fragments coding for the BAL2ΔC and CAL2ΔC proteins were gel purified and inserted into *Nco*I/*Xho*I cut pLexA vector. The resulting plasmids

encoded LexA-BAL2ΔC and LexA-CAL2ΔC fusion proteins, respectively. Expression of these fusion proteins in yeast strain EGY48 was confirmed by western blot of whole cell extracts (figure 6).

To confirm that LexA-BAL2ΔC and LexA-CAL2ΔC lacked autoactivation activity, their ability to confer leucine prototrophy on EGY48, mediated by the *LEU2* reporter gene, was tested. Serial dilutions of yeast cell suspensions were spotted onto minimal medium lacking histidine (to select for the plasmid) and either containing or lacking leucine (figure 7). Control strains containing the empty pLexA vector (negative control) or pLexA-Pos (which encodes an autoactivating 'bait' comprised of the activation domain of the yeast transcription factor Gal4p fused to LexA; positive control) were also included. The results of this assay confirmed that LexA-BAL2ΔC and LexA-CAL2ΔC lacked the ability to autoactivate the *LEU2* reporter gene and that they could be used as 'baits' in a yeast two-hybrid assay.

In order to test BAL2 and CAL2 for self-interaction, 'prey' plasmids were also made that encoded either BAL2ΔC or CAL2ΔC as C-terminal fusions to the B42 activation domain. To do this, the same DNA fragments encoding BAL2ΔC and CAL2ΔC used in the construction of the 'bait' plasmids were inserted into the pB42AD2 'prey' vector which had been digested with *Nco*I and *Xho*I (Yu, M. and Petty, I.T.D., unpublished results). The resulting plasmids encoded B42AD-BAL2ΔC and B42AD-CAL2ΔC fusion proteins, respectively. These 'prey' plasmids were then co-transformed with the appropriate pLexA-BAL2ΔC and pLexA-CAL2ΔC 'bait' plasmids into EGY48 yeast cells and self-interaction tests were performed (figure 8). Serial dilutions of yeast cell suspensions were spotted onto

minimal medium (1) lacking histidine and tryptophan (to select for both plasmids), (2) lacking histidine, tryptophan, and leucine with glucose as the carbon source, and (3) lacking histidine, tryptophan, and leucine with galactose as the carbon source. Growth on medium lacking leucine is dependent on expression of the two-hybrid reporter gene, *LEU2*. A two-hybrid interaction in this system is also dependent on the presence of galactose, which is required to induce expression of the B42AD fusion proteins. Control strains containing the pLexA and pB42AD vectors (negative control) and vectors encoding fusions of LexA to a fragment of human p53 (LexA-p53) and of B42AD to the simian virus 40 (SV40) large T-antigen (B42AD-T) were also included. Human p53 and SV40 T-antigen have previously been shown to interact, and were used as a positive control. The results of the tests indicated that yeast containing LexA-BAL2ΔC could grow in the absence of exogenous leucine in the presence of a vector encoding B42AD-BAL2ΔC, but only when galactose was used as the carbon source. No growth was observed when the pB42AD empty vector was expressed in the presence of the pLexA empty vector. Similar results were obtained for LexA-CAL2ΔC paired with B42AD-CAL2ΔC. Thus, like TAL2, BAL2 and CAL2 also self-interact in the yeast two-hybrid system.

Since all three begomovirus AL2 proteins tested showed homologous interaction, it was of interest to determine whether interaction would also occur between heterologous AL2 proteins. To do this, the EGY48 yeast strain was transformed with the six heterologous combinations of TGMV, BGMV, and CabLCV AL2 'bait' and 'prey' plasmids. Interaction tests were performed as before (figure 9). The results of the tests indicated that yeast containing any of the three begomovirus AL2 'bait' plasmids could grow in the absence of

exogenous leucine in the presence of any of the three begomovirus AL2 'prey' plasmids, but only when galactose was used as the carbon source. No growth was observed when the pB42AD empty vector was expressed in the presence of the pLexA empty vector. The results of the tests showed that all the heterologous combinations of AL2 'bait' and 'prey' proteins interacted in the yeast two-hybrid system.

Comparative analysis of a curtovirus C2 protein with begomovirus AL2 proteins

Geminiviruses of the genus *Curtovirus* encode a protein, designated C2, which is thought to represent a paralogue of the begomovirus AL2 protein. The BCTV C2 protein is similar to AL2 in its capacity to suppress plant defenses (Sunter *et a*l., 2001), but differs from AL2 in lacking the ability to *trans*-activate TGMV coat protein gene expression (Sunter *et al*., 1994). Consequently, another major focus of this project was to compare the characteristics of the BCTV C2 protein with those of begomovirus AL2 proteins. First, a plasmid was made encoding the full-length BCTV C2 protein (C2) as a C-terminal fusion to the LexA DNA-binding domain. A PCR product of the full-length *C2* ORF flanked by *Nco*I (5') and *Xho*I (3') restriction sites was generated, cloned, and sequenced (Leming, C.L. and Petty, I.T.D., unpublished results). This plasmid was digested with *Nco*I and *Xho*I and the 535 bp DNA fragment coding for full-length C2 protein was gel purified and inserted into *Nco*I/*Xho*I cut pLexA vector. The resulting plasmid encoded a LexA-C2 fusion protein. To test whether this C2 'bait' protein had autoactivation activity, pLexA-C2 was transformed into yeast and an autoactivation test was performed by spotting serial dilutions of yeast cell suspensions onto minimal medium lacking histidine (to select for the plasmid) and either containing or lacking leucine (figure 10). As before, control strains containing the empty

pLexA vector (negative control) or pLexA-Pos (positive control) were also included. Similar to the strain containing the pLexA-Pos autoactivation control 'bait', yeast expressing LexA-C2 were able to grow on medium lacking leucine. The results of this assay showed that the full-length C2 'bait' protein autoactivated the *LEU2* reporter gene in yeast strain EGY48.

Because the full-length C2 protein autoactivated, deletion mutants of C2 lacking 22 C-terminal or 22 C-terminal and 33 N-terminal amino acids (C2ΔC and C2ΔNΔC, respectively) were also evaluated as potential 'baits'. An alignment of the BCTV C2 protein with the TGMV AL2 protein (figure 11) shows that both contain a C-terminal acidic-rich region, which in C2 consists of 22 amino acids while in AL2 consists of 13 amino acids. Since deletion of the 13 amino acid C-terminal sequence from begomovirus AL2 proteins allows them to be fused to the C-terminus of LexA without creating an autoactivator, the equivalent region was deleted in C2ΔC. The alignment of the C2 and AL2 proteins also revealed that C2 contains an N-terminal 33 amino acid extension in comparison to AL2. Whether this N-terminal extension is actually present on C2 protein expressed in BCTV-infected plants is not clear (Stanley *et al*., 1992; Hormuzdi and Bisaro, 1995). Thus, removal of the 33 N-terminal amino acids would eliminate a possibly unessential portion of the *C2* sequence while still allowing expression of a functional C2 protein.

PCR products of the truncated *C2* ORFs flanked by *Nco*I (5') and *Xho*I (3') restriction sites were generated, cloned, and sequenced. These plasmids were digested with *Nco*I and *Xho*I and the resulting DNA fragments coding for the truncated C2 proteins were gel purified and inserted into the *Nco*I/*Xho*I cut pLexA vector resulting in plasmids encoding LexA-C2ΔC and LexA-C2ΔNΔC fusion proteins, respectively. These plasmids were transformed

into yeast and autoactivation tests were set up as before, with pLexA-Pos as the positive control and the empty pLexA vector as the negative control (figure 10). The results of these tests showed that neither LexA-C2ΔC, nor LexA-C2ΔNΔC 'bait' protein autoactivated. A western blot of whole cell extracts confirmed that these fusion proteins were being expressed in yeast strain EGY48 (figure 12).

The next characteristic examined was if BCTV C2 protein self-interacted. To do this, DNA fragments encoding full-length C2, C2ΔC, or C2ΔNΔC were inserted into the pB42AD2 vector resulting in plasmids encoding B42AD-C2, B42AD-C2ΔC and B42AD-C2ΔNΔC fusion proteins, respectively. Expression of these fusion proteins in yeast strain EGY48 was confirmed by western blot of whole cell extracts (figure 13). Plasmids encoding either LexA-C2ΔC or LexA-C2ΔNΔC were transformed into yeast together with a 'prey' plasmid encoding either B42AD-C2, B42AD-C2ΔC, or B42AD-C2ΔNΔC. Serial dilutions of each of the resulting strains were spotted onto minimal medium (1) lacking histidine and tryptophan (to select for both plasmids), (2) lacking histidine, tryptophan, and leucine with glucose as the carbon source, and (3) lacking histidine, tryptophan, and leucine with galactose as the carbon source (figure 14). A yeast strain expressing LexA-p53 and pB42AD-T was included as a positive control while a strain expressing B42AD-C2ΔC and LexA alone was included as a negative control. No galactose dependent growth on medium lacking leucine was observed for the C2ΔC 'bait' co-expressed with the C2ΔC 'prey' or for the C2ΔNΔC 'bait' co-expressed with the C2ΔNΔC 'prey'. In addition, no self-interaction was observed for any of the other combinations of C2 'bait' and 'prey' proteins (data not

shown). Thus, unlike the begomovirus AL2 proteins tested, the curtovirus C2 protein does not appear to exhibit self-interaction in the yeast two-hybrid system.

Since the paralogous begomovirus AL2 and BCTV C2 proteins have both been shown to be involved in the suppression of host defenses, it was of interest to perform tests to determine if BCTV C2 might interact with begomovirus AL2. Yeast were transformed with all combinations of begomovirus AL2 and BCTV C2 'bait' and 'prey' plasmids. These strains were tested for galactose-dependent, leucine prototrophy as before (figures 15 and 16). Also included was a positive control strain expressing LexA-p53 and B42AD-T, and negative control strains expressing either LexA-C2ΔC plus B42AD, or LexA plus B42AD-C2ΔC. In these tests, growth on medium lacking leucine was observed for strains expressing LexA-C2ΔC together with B42AD-CAL2ΔC. The same results were observed for strains expressing LexA-C2ΔC with either B42AD-TAL2 or B42AD-BAL2ΔC (data not shown). This growth phenotype appeared specific for the AL2 'preys' because growth was not observed for yeast strains expressing LexA-C2ΔC with B42AD, or with B42AD-C2. However, no growth was seen for strains expressing LexA-C2ΔNΔC with any of the AL2 'prey' proteins (data not shown). Also, while the LexA-C2ΔC 'bait' appeared to interact specifically with AL2 'preys', the B42AD-C2ΔC 'prey' did not interact with either the LexA-BAL2ΔC or LexA-CAL2ΔC 'baits'. In fact, none of the AL2 'baits' conferred leucine prototrophy when co-expressed with either the B42AD-C2ΔC or B42AD-C2ΔNΔC 'preys' (data not shown; table 2).

Evaluation of interactions between AL2 and C2 proteins and proteins encoded by candidate host cDNAs

N. benthamiana is a permissive host of TGMV, BGMV, CabLCV and BCTV (Qin and Petty, 2001; Hormuzdi and Bisaro, 1994). Previously, four candidate *N. benthamiana* cDNA clones were identified in yeast two-hybrid screens using TAL2 'baits' (Jordan and Petty, unpublished results). It was of interest to determine whether the same host factors interact with other begomovirus AL2 proteins, or with BCTV C2 protein. However, before this question could be addressed, it was necessary to confirm the specificity of interaction between proteins encoded by individual host cDNAs and TAL2 'baits'.

Two *N. benthamiana* proteins encoded by cDNAs named Nb#26 and Nb#39 had been isolated using LexA-TAL2ΔC as a 'bait'. To evaluate the specificity of the interactions, Nb#26 or Nb#39 proteins were co-expressed in yeast with either LexA-TAL2ΔC, LexA alone, or LexA with a C-terminal fusion to human lamin (LexA-lam). It was observed that all three 'baits', LexA-TAL2ΔC, LexA, and LexA-lam, conferred leucine prototrophy on yeast strain EGY48 when co-expressed with either Nb#26 or Nb#39. Therefore, the proteins encoded by these host cDNAs appeared not to interact specifically with TAL2, and they were not characterized further.

Two other host cDNA clones were identified by the yeast two-hybrid screen using a 'bait' composed of a TAL2 point mutant containing a substitution of alanine for tryptophan at amino acid residue 124 (W124A) fused to the N-terminus of LexA (Jordan, J.K. and Petty, I.T.D., unpublished results). The fusion protein encoded by this 'bait' plasmid was designated TAL2(W124A)-LexA. Nucleotide sequencing showed that cDNA clones Nb#62

and Nb#51 were from the same host gene and represented full-length and 5'-truncated copies of the mRNA, respectively (Jordan, J.K. and Petty, I.T.D., unpublished results). The first test performed was an interaction test to determine if TAL2(W124A)-LexA, which was previously shown to be a non-autoactivating 'bait'(Jordan, J.K. and Petty, I.T.D., unpublished results), interacted specifically with Nb#51 and Nb#62 (figure 17). To evaluate the specificity of the interactions, proteins encoded by cDNAs Nb#51or Nb#62 were co-expressed in yeast with either TAL2(W124A)-LexA or LexA alone. It was observed that TAL2(W124A)-LexA conferred leucine prototrophy on yeast strain EGY48 when co-expressed with either Nb#51 or Nb#62. These results confirmed the ability of Nb#51 and Nb#62 to interact specifically with TAL2 fused to the N-terminus of LexA. However, it was not known whether TAL2 fused to the C-terminus of LexA would also be able to interact. To determine this, yeast strains co-expressing LexA-TAL2ΔC and either Nb#51 or Nb#62 were tested for the ability to grow on medium lacking leucine (figure 17). Positive controls used in these tests were yeast strains co-expressing TAL2(W124A)-LexA with either Nb#51 or Nb#62 and negative controls were strains co-expressing LexA alone with Nb#51 or Nb #62. Unlike TAL2(W124A)-LexA, co-expressed LexA-TAL2ΔC did not allow growth with either the Nb#51 or Nb#62 encoded host proteins.

Because Nb#51 and Nb#62 could interact with TAL2 only when it was fused to the N-terminus of LexA, the available 'baits' for BAL2, CAL2, and C2, which were all LexA C-terminal fusions, could not be evaluated for possible interaction with these proteins. Therefore, it was necessary to construct plasmids that encoded LexA N-terminal fusions of BAL2, CAL2 and C2. To do this, PCR products of the *BAL2* and *CAL2* ORFs, containing

the W124A mutation without the stop codon and flanked by *Bam*HI (5') and *Xho*I (3') restriction sites, were generated, cloned, and sequenced (Du, X. and Petty, I.T.D., unpublished results). In addition, similar PCR products encoding C2ΔC and C2ΔNΔC were generated, cloned, and sequenced. After digestion with *Bam*HI and *XhoI*, gel purified DNA fragments encoding BAL2(W124A), CAL2(W124A), C2ΔC, or C2ΔNΔC proteins were inserted into *Bam*HI/*Xho*I cut pNLexA vector. The resulting plasmids encoded BAL2(W124A)-LexA, CAL2(W124A)-LexA, C2ΔC-LexA, and C2ΔNΔC-LexA fusion proteins, respectively. These plasmids were transformed into yeast and expression of the fusion proteins was tested by western blot (figure 18). Whereas TAL2(W124A)-LexA protein readily could be detected with the anti-LexA antibody, no protein detection was observed for BAL2(W124A)-LexA, CAL2(W124A)-LexA, C2ΔC-LexA, or C2ΔNΔC-LexA. Nucleotide sequencing confirmed that the geminivirus *AL2* or *C2* ORFs were correctly cloned in-frame with LexA, so the lack of protein accumulation must be due to other factors. The failure to obtain 'bait' plasmids from which LexA N-terminal fusions to other begomovirus AL2 proteins or BCTV C2 protein could be expressed precluded evaluation of their possible interaction with host proteins encoded by cDNAs Nb#51 and Nb#62.

II. Biochemical approach - Immunocapture assay to test TGMV AL2 self-interaction

Overview of immnocapture assay

The second major focus of this thesis was to biochemically test the TGMV AL2 self-interaction previously shown by yeast two-hybrid analysis. To do this, an immunocapture approach was used. In this approach, an antibody immobilized to a column matrix binds protein 'x' expressed in soluble fractions of yeast cell lysates. Protein 'x' captured by the

specific antibody can be eluted from the column and the success of the 'capture' confirmed by western blot. To test for interaction between protein 'x' and another protein, 'y', a cleared lysate containing both proteins can be passed over the protein 'x' antibody-specific column. Protein 'x' should bind to the column, and, if interaction occurs between proteins 'x' and 'y', protein 'y' should also be retained on the column through binding to protein 'x'. The interaction is detected by western blot of the eluted fractions; if interaction occurred, proteins 'x' and 'y' would both be detected in the eluted fractions using specific antibodies. In the immunocapture assays used in this project, protein 'x' is the HA-tagged B42AD fusion protein and protein 'y' is the LexA fusion protein.

There are five yeast strains that were used in the immunocapture assay to test self-interaction of TAL2. LexA-p53 co-expressed with B42AD-T was used as one of the positive control strains since these proteins were previously shown to interact physically in biochemical assays. An alternative positive control strain used in the assay contained pBait and pTarget which encode proteins shown to interact via the yeast two-hybrid system and assumed to also physically interact biochemically (OriGene). The negative control strains for the assay that should not physically interact in biochemical assays included LexA-TAL2ΔC co-expressed with B42AD alone and B42AD-TAL2 co-expressed with LexA alone. The fifth strain used in the assay was the test strain containing pLexA-TAL2ΔC and pB42AD-TAL2. All the strains were required to be grown in galactose minimal medium to induce expression of the B42AD fusion proteins. However, as data will show, protein extraction from yeast cells grown in galactose was more difficult than for those grown in

glucose, making it difficult to obtain high levels of soluble protein for both the B42AD and LexA fusion proteins.

(i) Efforts to obtain 'bait' and 'prey' fusion proteins in soluble fractions of yeast cell lysates

(1) Lysate preparation using Y-PER™ or IP buffer of yeast cells grown in galactose minimal medium

The first methods used to obtain 'bait' and 'prey' fusion proteins in supernatant fractions of cleared yeast cell lysates involved the use of Y-PER™ or IP buffer. Y-PER™ was used because it was the reagent recommended by the manufacturer of the immnunocapture assay to extract soluble protein from the yeast cells, and IP buffer was used because previous studies had shown that it was effective in the extraction of soluble protein from yeast cells (Miyoshi *et al*., 2002). Lysates of the pLexA-p53 plus pB42AD-T positive control strain, the two negative control strains, and the test strain were made by adding either four times the volume of Y-PER ™ or four times the volume of IP buffer and an equal volume of glass beads to the yeast cell pellets and mixing vigorously at room temperature for 20 minutes (methods A and B, table 3). Soluble fractions were obtained by centrifugation at 14,000 rpm for five minutes at room temperature. The supernatant fractions prepared by method A were analyzed by western blot (figure 19) and showed positive results for B42AD fusion protein detection for the strains containing pLexA plus pB42AD-TAL2 and pLexA-TAL2ΔC plus pB42AD-TAL2 using anti-HA primary antibody. However, negative results for B42AD fusion protein detection were observed for the supernatant fractions for the strains containing pLexA-p53 and pB42AD-T and pLexA-TAL2ΔC and pB42AD, and negative results were also observed for LexA fusion protein detection in the supernatant

fractions of all four strains when analyzed using anti-LexA primary antibody. Similar results were obtained for method B (data not shown). The results indicated that none of the LexA fusion proteins and only one of the B42AD fusion proteins (B42AD-TAL2) was present in the soluble protein fractions at levels high enough to be detected by western blot following the use of either of these two lysis procedures.

(2) Attempts to improve LexA fusion protein levels in Y-PER™-generated cleared lysates of yeast cells grown in galactose minimal medium

Because none of the LexA fusion proteins was present at levels high enough to be detected by western blot using the first lysis procedures, Y-PER™ lysis was carried out for three different time periods to see if this would aid in protein detection. Since the strain expressing LexA-TAL2ΔC and B42AD-TAL2 was shown to accumulate B42AD-TAL2 protein in the soluble fraction when cells were lysed with Y-PER™ for 20 minutes, this strain was used to see if LexA fusion protein could also be detected if lysis was allowed to occur for longer time periods. Lysates of the strain expressing LexA-TAL2ΔC and B42AD-TAL2 were made by adding four times the volume of Y-PER™ and an equal volume of glass beads to the cell pellet and mixing vigorously for 20, 40, and 60 minutes at room temperature (method A, table 3). Soluble fractions were generated by centrifugation at 14,000 rpm for five minutes at room temperature and assessed for protein accumulation by western blot (figure 20). Positive results were obtained for B42AD-TAL2 protein detection in the soluble fractions when analyzed using anti-HA primary antibody, while negative results were observed for LexA-TAL2ΔC detection in the soluble fractions using anti-LexA primary antibody. The results of the western blot analysis showed that while detection of B42AD-TAL2 occurred in all three of the soluble fractions generated by method A, detection of the

LexA fusion protein did not improve even when lysis was allowed to occur for longer time periods.

(3) Attempts to improve LexA fusion protein accumulation in cleared lysates generated using an alternative Y-PER™ lysis procedure of yeast cells grown in galactose minimal medium

Since the level of the LexA fusion protein in the soluble fractions generated using lysis method B was still not high enough to be detected by western blot, the Y-PER™ lysis procedure was altered again in that protease inhibitors were added and the lysis and clearing procedures were conducted at 4ºC to ensure that the proteins were not being degraded. Yeast cell lysates of the positive control strain containing pLexA-p53 and pB42AD-T, the two negative control strains, and the test strain were made by adding four times the volume of Y-PER™, an equal volume of glass beads, and protease inhibitors (1mM PMSF, 1µg/ml leupeptin, and 1µg/ml pepstatin A) to the cell pellets. Lysis was achieved by mixing hard at 4ºC for 20 minutes and the soluble fractions were obtained by centrifugation at 14,000 rpm for 20 minutes at 4ºC (method C, table 3). A western blot of the soluble fractions was performed and the results were again negative for LexA fusion protein detection for all four strains when analyzed using anti-LexA primary antibody. Thus, the addition of protease inhibitors and performing the cell lysis and clearing procedures at 4ºC did not aid in the detection of the LexA fusion proteins in the cleared lysates.

(4) Attempts to demonstrate LexA fusion protein accumulation in cleared lysates generated using Y-PER™ and 2x Laemmli buffer of yeast cells grown in glucose rich medium

To determine if the LexA fusion protein accumulation problem was involved with the growth of the yeast cells in galactose minimal medium or the use of Y-PER™ to extract the

protein, the pLexA-p53 and pB42AD-T positive control strain, negative control strains, and test strain were grown in glucose rich medium instead of minimal galactose medium. Lysates were made using Y-PER™ according to method C, or by adding an equal volume of 2x Laemmli buffer to each cell pellet and lysing the cells by boiling for five minutes (method D, table 3). In each case, lysis was followed by centrifugation at 14,000 rpm for five minutes at room temperature to obtain the soluble fractions. The soluble fractions were analyzed by western blot (figure 21). LexA fusion proteins were detected in the soluble fractions produced by both methods, although method D appeared to result in more efficient extraction. However, the results suggested that the protein accumulation problem was most likely being caused by the growth of the yeast cells in galactose minimal medium and not by the use of Y-PER™ to extract the protein, since protein was detected in cleared lysates generated using either Y-PER™ or 2x Laemmli buffer.

(5) Attempts to improve B42AD and LexA fusion protein accumulation in cleared lysates by growth of yeast cells in galactose rich medium

Because the levels of the LexA fusion proteins in the cleared lysates were significantly improved when the yeast cells were grown in glucose rich medium, an alternative growth protocol was developed in which the cells were grown in rich medium containing galactose instead of using galactose minimal medium. The cells were grown overnight in raffinose rich medium followed by overnight growth in rich medium containing both raffinose and galactose. Lysates were made using 2x Laemmli buffer since this had been shown previously to produce detectable levels of LexA fusion proteins in cleared lysates for yeast cells grown in rich glucose medium. The lysates were generated by boiling the cell pellets in 2x Laemmli buffer and the supernatant fractions were made by

centrifugation at 14,000 rpm for five minutes at room temperature (method D, table 3). The supernatant fractions were analyzed by western blot (figure 22) and showed positive results for B42AD fusion protein detection for the strains containing pLexA plus pB42AD-TAL2 and pLexA-TAL2ΔC plus pB42AD-TAL2 using anti-HA primary antibody. However, negative results were again observed for protein detection in the supernatant fractions for the strains containing pLexA-p53 plus pB42AD-T and pLexA-TAL2ΔC plus pB42AD, when analyzed using anti-HA antibody. In addition, supernatant fractions for all four strains were negative for LexA fusion protein detection when analyzed using anti-LexA primary antibody. These results showed that growth of the yeast cells in galactose rich medium did not increase the levels of protein accumulation for either the B42AD or LexA fusion proteins.

(6) Attempts to improve B42AD fusion protein accumulation in cleared lysates generated by alternative cell-breaking procedures of yeast cells grown in galactose minimal medium

Because some of the B42AD fusion proteins were still not being accumulated at levels high enough to be detected by western blot, even when grown in galactose rich medium, alternative methods to break the cells grown in galactose minimal medium were tried. Three new lysis approaches were taken for yeast cells expressing the following 'prey' proteins: B42AD-T, Target, B42AD, and B42AD-TAL2. The first method involved freezing the cell pellet three times in liquid nitrogen, thawing the pellet in a 37ºC waterbath for one minute after each freezing, and adding four times the volume of Y-PER™ and an equal volume of glass beads to the pellet. Lysis was achieved by mixing vigorously at 4ºC for 20 minutes followed by centrifugation at 14,000 rpm for 20 minutes at 4ºC to generate the soluble fractions (method E, table 3). The second method involved freezing the cell pellet

three times in liquid nitrogen, thawing in a 37ºC waterbath for one minute following each freezing, and adding four times the volume of IP buffer and an equal volume of glass beads (method F, table 3). Lysis of these cell pellets and generation of the soluble fractions was done as for method F. The third method involved converting the yeast cells to spheroplasts using lyticase. Lysing of the spheroplasts followed by adding four times the volume of Y-PER™ and an equal volume of glass beads and mixing hard at 4ºC for 20 minutes. The soluble fractions were generated by centrifugation at 4ºC for 20 minutes at 14,000 rpm (method G, table 3). The soluble fractions were analyzed by western blot for B42AD fusion protein detection using anti-HA primary antibody (figure 23). The results were positive for B42AD fusion protein detection in soluble fractions of strains expressing Target and B42AD-TAL2 that were made by method E, and for the soluble fraction made by method F of the strain expressing Target. For method G, the only soluble fraction to show protein accumulation was the fraction expressing Target, and the amount of protein detected was significantly less than that seen with the soluble fractions produced by methods E and F (figure 23). Since Target showed a higher level of protein accumulation than B42AD-T, it was used for all subsequent experiments, together with Bait, as the positive control. In summary, results for soluble B42AD fusion protein accumulation were best for method E, followed by method F, and poor for method G. However, none of the methods produced soluble protein accumulation for all of the B42AD fusion proteins.

Since accumulation of some of the B42AD and LexA fusion proteins was still not being detected by western blot, new primary and secondary antibody dilutions were prepared to see if this would improve the detection of protein accumulation. Yeast cell lysates of the

pBait and pTarget positive control strain, the two negative control strains, and the test strain were made using the two methods previously shown to give the best results for soluble protein accumulation, methods C and E. Western blots of the supernatant fractions were performed and the results were positive for soluble B42AD fusion protein detection for all the strains when analyzed using anti-HA primary antibody (figure 24). In addition, the results were positive for LexA fusion protein detection for all the strains except LexA-TAL2ΔC co-expressed with B42AD-TAL2 when analyzed using anti-LexA primary antibody. The results of the western blot also showed that it was not necessary to freeze-thaw the cell pellet prior to lysis with Y-PER™, as the amount of fusion protein detected in the supernatant fractions made by method C was roughly equal to that in the supernatant fractions generated by method E.

Since the soluble fractions of cleared lysates of the strain expressing LexA-TAL2ΔC plus B42AD-TAL2 did not contain LexA fusion protein detectable by western blot using anti-LexA primary antibody, plasmids encoding LexA-TAL2ΔC and B42AD-TAL2 were co-transformed into yeast a second time and new lysates and soluble fractions were made using lysis methods C and E. A western blot of the soluble fractions was conducted and the results were again negative for LexA-TAL2ΔC detection when analyzed using anti-LexA primary antibody. Since LexA- TAL2ΔC was not detected in cells co-expressing TGMV AL2 'bait' and 'prey', different combinations of TGMV, BGMV, and CabLCV AL2 'bait' and 'prey' plasmids were tried instead. Combinations containing both homologous and heterologous AL2 'bait' and 'prey' proteins previously shown to interact in the yeast two-hybrid system were transformed into yeast and lysates were made using method C. Western blots of the

soluble fractions were performed but the results were all negative for LexA fusion protein detection when analyzed using anti-LexA primary antibody. The results indicated that LexA-AL2 fusion protein accumulation was never high enough to be detected by western blot when it was co-expressed with a B42AD-AL2 fusion protein.

(7) Lysates of separately expressed 'bait' and 'prey' proteins generated using an alternative Y-PER™ procedure

Since the accumulation level of LexA-TAL2ΔC was not high enough to be detected by western blot when it was co-expressed with B42AD-TAL2, each of the proteins (Bait, LexA, LexA-TAL2ΔC, Target, B42AD and B42AD-TAL2) was expressed separately and individual yeast cell lysates were made. The lysates were prepared by method C and the soluble fractions were analyzed for protein detection by western blot using anti-HA and anti-LexA primary antibodies (figure 25). The results of the western blot were positive for protein detection for all the supernatant fractions except for the supernatant fraction from the strain expressing B42AD. Reasons why the strain containing pB42AD did not appear to express the protein in this experiment include that it may not have been transformed into EGY48 correctly or that its DNA may have become mutated at some point prior to transformation. Nevertheless, expression of the LexA and B42AD fusion proteins separately allowed for the best soluble protein accumulation and these soluble protein fractions were used for *in vitro* binding and immunocapture assays.

(ii) Attempts to capture 'prey' fusion proteins on columns containing immobilized anti-HA monoclonal antibody

(1) Generation of the anti-HA monoclonal antibody column

In order to construct the anti-HA monoclonal antibody column, the Seize™ X Yeast Immnopreciptation Kit (Pierce) was used. First, protein G was bound to the column matrix. Then the column was washed and 100 µg of anti-HA monoclonal antibody was bound to the immobilized protein G. Anti-HA antibody was bound to the column rather than anti-LexA antibody because the binding capabilities of immobilized protein G for mouse IgG_{2a} antibodies (anti-HA antibody) are much greater than for mouse IgG_1 antibodies (anti-LexA antibody). Following several washes of the column, the anti-HA antibody was cross-linked to the immobilized protein G using disuccinimidyl suberate (DSS). Finally, the column was washed several more times and the supernatant fractions of cleared yeast cell lysates expressing the proteins of interest were incubated with the column matrix material and eluted.

To determine if the antibody was successfully bound to the column, 10 µl of the 190 µl unbound antibody wash fraction after the anti-HA antibody was incubated with the column, and 10 µl of the 190 µl uncoupled antibody flow-through fraction after the DSS cross-linking were analyzed by western blot. Serial dilutions of anti-HA antibody ranging from 1.4 µg to 1.4 pg were used as controls, which revealed that the limit of detection of the assay lay between 1.4 µg of antibody (detected) and 0.14 µg of antibody (not detected). No antibody was detected in either the unbound antibody wash fraction or the uncoupled antibody flow-through fraction. These results indicated that there was less than 1.4 µg of anti-HA antibody in each 10 µl fraction. Therefore, less than 26.6 µg of anti-HA antibody

was in each 190 µl fraction, indicating that the majority of the antibody bound to the column matrix. A protein concentration assay using serial dilutions of bovine serum albumin ranging from 0.25 µg/ml to 2 µg/ml was performed on both fractions to determine a better estimate of how much antibody was present in the two fractions. The results of the protein concentration assay showed that there were undetectable levels of protein in the unbound antibody wash fraction and in the uncoupled antibody flow-through fraction. These results indicated that the majority of the 100 µg of the anti-HA antibody added actually was bound to the column.

Immunocapture assay using soluble fractions of cleared yeast cell lysates containing B42AD fusion proteins

As a control experiment to determine if the immunocapture assay would work, soluble fractions containing B42AD fusion protein generated by lysis methods A and B for the strain expressing LexA and B42AD-TAL2, or the strain expressing LexA-TAL2ΔC and B42AD-TAL2, were incubated with the anti-HA antibody column matrix by rotating at 100 rpm for two hours at room temperature. After incubation and washing, elution was performed and the eluted fractions were analyzed by western blot. The results of the western blot were negative for B42AD fusion protein detection when analyzed using anti-HA primary antibody. The results of this control experiment indicated that some parameter of the immunocapture assay would have to be altered to produce any significant results.

Immunocapture assay using yeast cell cleared lysates made from strains expressing LexA or B42AD fusion proteins separately

Since expression of the LexA-TAL2ΔC and B42AD-TAL2 fusion proteins separately allowed for both of the fusion proteins to accumulate in the soluble fractions at levels high enough to be detected by western blot, it was decided that separate soluble fractions would be

combined and allowed to interact *in vitro* prior to being incubated with the anti-HA antibody column matrix. Bait, LexA, and Target were expressed separately and soluble protein fractions were made for each. A soluble fraction made from the strain containing Bait, or a soluble fraction from the strain containing LexA, were combined individually with a soluble fraction from the strain containing Target in a microfuge tube for 30 minutes at room temperature to allow *in vitro* interaction to occur. The relative amounts of each soluble fraction used were adjusted based on the amount of protein detected on a western blot for each fraction; four times the amount of soluble fraction expressing Bait or LexA was added to soluble fraction expressing Target. The combined soluble fractions were then incubated with the HA antibody column matrix by rotating at 100 rpm for two hours at room temperature. After incubation and washing, elution of the proteins was performed and the eluted fractions were analyzed by western blot (figure 26). The results of the western blot were negative for LexA fusion protein in the eluted fractions, and only a very small amount of B42AD fusion protein was observed in the first eluted fractions. To confirm the negative results for the presence of LexA fusion protein in the eluted fractions, the fractions were concentrated by vacuum drying and resuspended in 2x Laemmli buffer. The samples were then boiled for five minutes. The concentrated samples were assessed by western blot but the results remained negative for the presence of LexA fusion protein.

To determine whether the HA-tagged proteins were either not binding to the column, or binding too tightly to be eluted, the flow-through fractions after incubation of the supernatant fractions with the column material and the column material itself were tested by boiling samples in 2x Laemmli buffer. The fractions were analyzed by western blot using

anti-HA and anti-LexA primary antibodies and the results indicated that the majority of the soluble protein did not bind to the column but came off in the flow-through fraction after the incubation was completed (figure 27). Also, a very small amount of B42AD fusion protein was trapped in the column matrix, but no LexA fusion protein was detected. These results indicated that the principal problem with the immunocapture assay involved the failure of HA-tagged proteins to bind to the anti-HA antibody on the column, and that the majority of such protein was washed off the column and did not remain bound.

In an attempt to improve the binding conditions, the combined soluble fractions and the anti-HA antibody column matrix were incubated overnight either at room temperature, or at 4ºC. Again, the eluted fractions were tested for the presence of the fusion protein by western blot and again the results were negative with either anti-HA or anti-LexA primary antibodies. The soluble fractions incubated with the column matrix, the flow-through fractions after incubation, and the column matrix itself were also analyzed for the presence of the fusion protein by western blot (figure 28). The results indicated that roughly equal amounts of each protein were present in the initial soluble fractions and in the flow-through fractions, and no protein was trapped in the column matrix itself. Therefore, it was concluded that nearly all of the HA-tagged soluble protein was being washed off the anti-HA antibody column instead of binding to it.

Discussion

I. Evaluation of begomovirus AL2 proteins using yeast two-hybrid assays

Self-interaction of AL2 proteins may be due to a conserved sequence motif

A sequence alignment of the TAL2, BAL2, and CAL2 proteins reveals that the proteins share a 60% identity in amino acid sequence (figure 29), indicating that there are multiple conserved sequence motifs within the proteins. Data from the yeast two-hybrid assays showed that all three begomovirus AL2 proteins self-interact. Therefore, self-interaction of AL2 may be due to the presence of one such conserved sequence motif that would allow the protein to form dimers, or higher order oligomers. Furthermore, oligomerization might regulate the activity of the AL2 protein. One possibility is that AL2 oligomers could have increased biological activity while monomers would have low, or no activity. An example of a protein that illustrates this phenomenon is the yeast transcriptional activator protein Gal4p that can only bind DNA as a dimer (Carey *et al*., 1989). Prior studies have shown that one function of AL2 is to activate coat protein gene expression (Sunter and Bisaro, 1992). Once the coat protein is expressed, encapsidation of the viral DNA begins. Since coat protein expression too early in the infection cycle would not allow for the maximum production of viral particles, it would make sense for an AL2 oligomer to be active and a monomer to be inactive. This way, the coat protein would not be expressed at high levels until many AL2 proteins have been translated to allow for the formation of oligomers.

Unfortunately, it cannot be determined what type of oligomer AL2 would form using a yeast two-hybrid assay. In order to show this, gel filtration chromatography could be conducted. If the gel-filtration column is calibrated with proteins of similar shape to AL2

and known molecular weights, the molecular weight of AL2 passed through the column could be determined. The molecular weight of the eluted AL2 protein could then be compared to the molecular weight of AL2 as a monomer and the aggregation of AL2 can be determined. In addition, density gradient ultra-centrifugation could be performed using purified AL2 along with a standard of known molecular weight proteins. Following centrifugation, calibration curves can be generated and comparison of AL2 with the standards can determine the likely aggregation state of AL2 in solution.

While it would make sense for the oligomer form of AL2 to be the active form and the monomer to be the inactive form, the reverse could also be true; AL2 monomers could have increased biological activity while oligomers have less. This type of arrangement could allow for regulation of AL2 protein activity. As too many proteins are produced and begin to oligomerize, the activity of the protein would be reduced. In order to determine which of the scenarios is true, AL2 could be mutated in a way that would eliminate its oligomerization potential while still maintaining its other biological functions. Then, an *in vitro* transcription assay could be conducted to determine the effect mutant AL2 would have on transcription of coat protein mRNA versus wild-type AL2. If mutant AL2 resulted in a decreased level of transcription of coat protein mRNA compared to wild-type AL2, it would indicate that the active form of AL2 is an oligomer while the inactive form is a monomer. However, if mutant AL2 resulted in an increase in transcription compared to wild-type AL2, then the results would suggest that the monomeric form of AL2 would be the active form.

Although sequencing data have revealed that the sequences of TAL2, BAL2 and CAL2 share a 60% identity in amino acid sequence, there is still a 40% difference due to the

divergence of the proteins over time (figure 29). Despite this amount of sequence difference, data have also shown that AL2 proteins of several New World begomoviruses can *trans*-activate the coat protein gene promoter of a TGMV *al2* mutant (Sunter *et al*., 1994). It has also been shown that BAL2, CAL2, and TAL2 are functionally equivalent and can be substituted among the three viruses (Gillette *et al*., 1998; Hung and Petty, 2001; Qin and Petty, 2001). In addition, the AL2 homologue of the Old World begomovirus ACMV complements coat protein expression of a TGMV *al2* mutant even though it shows an even greater sequence divergence from TAL2 than do BAL2 and CAL2 (Sunter *et al*., 1994). If AL2 is under selection pressure to retain its self-interaction property for *in vivo* biological function of the protein, it would be predicted that the highly divergent AL2 homologue of ACMV should share this property. This hypothesis could be tested in further yeast two-hybrid experiments.

Heterologous interaction between begomovirus AL2 proteins provides additional evidence for a conserved sequence motif

In addition to self-interaction properties, the begomovirus AL2 proteins were also shown to display heterologous interaction with one another in yeast two-hybrid assays. This result supports the hypothesis that the AL2 proteins may contain a highly conserved structural feature. This conserved structural feature could result from a conserved sequence motif that causes oligomerization and consequently, interaction between all the begomovirus AL2 proteins. Significant sequence differences between the begomovirus AL2 proteins supports the idea that this structural feature may be under selective pressure to be conserved, perhaps because it is necessary for *in vivo* function of AL2.

Since plants are commonly found harboring multiple begomovirus infections, due to their sharing a single insect vector species, this would allow many opportunities for heterologous AL2 proteins to interact. However, interaction between heterologous AL2 proteins within a single plant may not be important with regards to the biological function of the protein. In order to determine this, one plant could be inoculated with two separate wild type begomoviruses, while another was inoculated with two begomoviruses containing mutant AL2 proteins lacking heterologous interaction activity while still maintaining their biological function. Then, the interaction between the heterologous wild type AL2 proteins would have to be verified *in planta*, and the effects, if any, of this interaction on the biological function of the protein would be determined by comparison to the plant harboring the mutated AL2 proteins. Lack of phenotypic differences between the virus infections in the two plants would support the idea that heterologous interaction between AL2 proteins is not important for the biological function of the protein.

Lack of interaction between LexA-TAL2ΔC and proteins encoded by candidate host cDNAs Nb#51 and Nb#62

Data from the yeast two-hybrid assays revealed that TAL2 fused to the N-terminus of LexA interacted with the Nb#51 and Nb#62 'prey' proteins but TAL2 fused to the C-terminus of LexA did not. There are several possibilities as to why this result may have occurred. One is that perhaps the interaction domain of TAL2 was blocked or not folded in its native configuration when the protein was fused to the C-terminus of LexA, which would prevent the interaction. Another possibility is that a post-translational modification that might be required for interaction, such as phosphorylation, was blocked when the AL2 protein was fused to the C-terminus of LexA. A study of TGMV AL2 revealed that the

protein is phosphorylated in insect cells and is likely to be phosphorylated in plant cells as well (Hartitz *et al*., 1999). Therefore, lack of post-translational phosphorylation might disturb the protein's interaction potential.

Because the C-terminal TAL2 'bait' did not interact with Nb#51 and Nb#62 'preys' using the yeast two-hybrid system, the C-terminal BAL2 and CAL2 'baits' could not be used in further two-hybrid assays. Therefore, N-terminal BAL2 and CAL2 'baits' were constructed. However, unlike the N-terminal TAL2 'bait' protein, these 'bait' proteins were not detected on a western blot of yeast whole cell extracts. One of the reasons that the N-terminal BAL2 and CAL2 'baits' may not have accumulated protein at levels high enough to be detected by western blot was that no mRNA may have been transcribed. This hypothesis could be tested by performing a northern blot of RNA extracted from cells transformed with the N-terminal 'bait' plasmids. A negative result for RNA accumulation could indicate one of two things. The first is that no mRNA is being transcribed and the second is that the level of RNA degradation may be high enough to make the steady-state level of RNA undetectable by the northern blot. However, the amount of mRNA may not be the most likely problem since the promoter sequences of all the N-terminal 'bait' plasmids were found to be cloned correctly.

Another reason that the N-terminal BAL2 and CAL2 'bait' proteins may not have been detected on a western blot is perhaps the proteins are degraded more rapidly than the TAL2 protein fused to the N-terminus of LexA. One explanation for a difference in degradation levels between two proteins is the N-end rule. The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (Varshavsky, 1992). In

eukaryotes, the N-end rule pathway is a part of the ubiquitin system; certain N-terminal amino acids promote targeting of the protein by ubiquitin for degradation by the proteasome (Varshavsky, 1992). In yeast, methionine, serine, threonine, alanine, valine, cysteine, glycine, or proline protect proteins from ubiquitin tagging, but all other N-terminal amino acids promote degradation (Alberts *et al*., 1994). However, the nucleotide sequences show that the N-terminal amino acid for TAL2 and BAL2 is arginine and the N-terminal amino acid of CAL2 is glutamine, indicating that all three N-terminal fusion proteins should have been equally targeted for degradation.

A third reason that the N-terminal BAL2 and CAL2 'baits' may not have accumulated a high enough level of protein to be detected by western blot is that translation of the proteins was not efficient. One possibility for why this may have occurred was a poor match with Kozak's consensus sequence around the start codons of the N-terminal 'baits'. A poor Kozak's sequence surrounding the start codon of the N-terminal 'baits' could be preventing the ribosome from recognizing the start codon efficiently so translation can initiate. Analysis of the sequences of the N-terminal TAL2 'bait' protein and the N-terminal BAL2 'bait' protein revealed that they share the same Kozak's sequence surrounding their start codons indicating that they should have been translated at the same level of efficiency. However, the Kozak's sequence of *CAL2* differs from that of *TAL2* and *BAL2*. Perhaps this difference in the Kozak's sequence surrounding the start codon of the N-terminal CAL2 'bait' is preventing its efficient translation. Another possibility could be that there may be additional, out of frame start codons upstream of the start codon where translation of the AL2 protein begins. If alternative start codons were present then translation may be starting there

which would result in the expression of a different protein. However, analysis of the sequences of the N-terminal BAL2 and CAL2 'bait' proteins did not reveal any additional start codons in the 5' leader region upstream of the start codon where translation of AL2 begins, indicating that this was not the problem leading to the lack of protein detection for these N-terminal 'baits'.

One method that could be used to map regions of BAL2 and CAL2 that prevent protein accumulation when fused to the N-terminus of LexA is to create hybrid proteins. For example, the N-terminal region of TAL2 could be fused to the C-terminal region of BAL2 or CAL2 and the resulting hybrid protein could be fused to the N-terminus of LexA. The hybrid proteins could then be tested for protein accumulation by western blot. If the BAL2 and CAL2 proteins are expressed when present in this hybrid form, then some portion of their N-terminal regions must be preventing their accumulation when fused to the N-terminus of LexA. Then, additional hybrid proteins could be made by fusing progressively smaller N-terminal portions of TAL2 to progressively larger C-terminal portions of BAL2 and CAL2 to more precisely map the areas that are preventing BAL2 and CAL2 accumulation when fused to the N-terminus of LexA.

II. Evaluation of a curtovirus C2 protein using the yeast two-hybrid system

The transcription activation potential of BCTV C2

Previous studies revealed that BCTV C2 protein cannot *trans*-activate the TGMV coat protein gene promoter so it was speculated to lack any transcriptional activation activity (Sunter *et al*., 1994). However, the same result can also be explained if C2 lacks the ability to recognize the coat protein gene promoter of TGMV, even if it does contain transcriptional

activation activity that might be directed to the promoter of another gene. Alignment of the BCTV Logan C2 and TGMV AL2 proteins shows that both contain a C-terminal region that is composed of a high number of acidic amino acids (figure 11). This region has been shown to contain an activation domain in the TAL2 protein (Hartitz *et al*., 1999). As yeast two-hybrid data presented in this thesis demonstrated, full-length C2 also autoactivated the *LEU2* reporter gene when fused to the LexA DNA-binding domain, but LexA-C2ΔC did not. Therefore, C2 not only might function as a transcriptional activator *in vivo*, but like the AL2 protein, it has a C-terminally located activation domain.

AL2 and C2 are believed to have diverged from a common ancestral protein. This idea explains how C2 could still retain some transcriptional activation activity if some acidic amino acids have not converted to less acidic ones during the divergence of the AL2 and C2 proteins. In addition, it is possible that C2 is unable to recognize the TGMV coat protein gene promoter, not because of a lack of activation potential, but instead because C2 does not possess the same interaction capabilities as the AL2 protein. It is known that transcriptional activator proteins of several mammalian DNA viruses do not bind directly to specific DNA sequences, but instead are targeted to them by specific interactions with host transcription factors that recognize sequence elements within certain promoters. Examples of viral proteins that exhibit this type of binding include adenovirus E1A, VP16 of herpes simplex virus, and EBNA2 of Epstein-Barr virus (Gerster and Roeder, 1988; Flint and Shenk, 1989; Hsieh and Hayward, 1995). It has been suggested that AL2 may be targeted to the coat protein gene promoter by similar host factor protein interaction (Sunter and Bisaro, 1997). If

so, then it is possible that C2 cannot recognize the host factor that targets AL2 and therefore cannot *trans*-activate the TGMV coat protein gene promoter.

In order to determine the importance of the transcription activation potential of C2, the C2 homologues from other curtoviruses could be tested to determine if they also autoactivate in the yeast two-hybrid system. If the homologous proteins do possess transcription activation activity, it would suggest that the C2 protein could be under selective pressure to retain this activity. Alternatively, it is possible that an N-terminal portion of the C3 protein could be under the selective pressure and not the C-terminal portion of C2, because DNA that codes for the C-terminus of C2 also codes for the N-terminus of C3 (figure 2). In this case, maintenance of transcription activation activity of C2 may be an evolutionary artifact, and unrelated to the biological functions of C2 during BCTV infection.

Lack of C2 self-interaction

Data from the yeast two-hybrid assays revealed that while TAL2, BAL2, and CAL2 all possess the property of self-interaction, C2 does not. One possibility for why C2 doesn't self-interact is perhaps changes have occurred in its sequence to disrupt the ability of the protein to oligomerize. If C2 lacks the ability to oligomerize, it would not be expected to self-interact in the yeast two-hybrid assay. Consequently, if C2 cannot self-interact, maybe it also cannot interact with host factors and thus cannot bind to the coat protein gene promoter in the manner that AL2 has been proposed to do (Sunter and Bisaro, 1997). Additionally, perhaps C2 has not been under selection pressure to retain the self-interaction property that AL2 has because self-interaction isn't necessary for its *in vivo* function in host defense suppression (Sunter *et al*., 2001).

Evaluation of the C2 N-terminal region

As shown in figure 11, a sequence alignment of BCTV Logan C2 and TAL2 reveals that the C2 protein contains a 33 N-terminal amino acid extension not seen in TAL2. Data from previous studies have shown that mutations in the sequence after the second methionine codon result in recovery from symptoms of plants infected with the mutant BCTV, while mutations within the N-terminal extension of C2 have no such effect (Hormuzdi and Bisaro, 1995). A possible reason for these results may be that translation of the C2 protein starts at the second in-frame methionine codon and the putative N-terminal extension is never expressed (Hormuzdi and Bisaro, 1995). However, yeast two-hybrid data show that C2ΔC 'bait' interacts with AL2 'prey' while C2ΔNΔC 'bait' does not, which could suggest that expression of the N-terminal extension may be important for the protein's function. However, this result could also be an artifact of the yeast two-hybrid system. In order to determine if the N-terminal extension of C2 is expressed, sequences of other curtoviruses could be examined to see if their C2 homologues also contain a similar N-terminal extension. However, to determine definitively if the N-terminal extension is expressed, the 5'-ends of transcripts from this region of the BCTV Logan genome should be mapped. If the 5'-end of the mRNA includes the N-terminal extension region, then it is likely expressed during virus infection and may be important for the function of C2. On the other hand, if the 5'-end of the mRNA lies within the coding region for the putative N-terminal extension, then only C2 protein corresponding to the C2ΔN form could be expressed during virus infection *in vivo*.

The yeast two-hybrid data are inconclusive with regard to possible interaction between C2 and AL2, since all but one pairwise combination of AL2 and C2 'bait' and

'prey' proteins do not interact. In fact, the only time C2 and AL2 could interact is if they were found in a plant simultaneously, and the only way this could occur is if the plant was infected with both a begomovirus and BCTV. However, the probability of a simultaneous infection is quite low due to the fact that their insect vectors inhabit different climates (leafhoppers thrive in temperate climates while whiteflies thrive in tropical climates). Therefore, the preponderance of evidence suggests that interaction between the C2ΔC 'bait' and the AL2 'preys' was most likely an artifact of the yeast two-hybrid assay.

III. Evaluation of the immunocapture assay

The purpose of the immunocapture assay was to biochemically test the ability of TAL2 to self-interact. Prior to conducting this assay, it can only be inferred that the TAL2 'bait' and 'prey' proteins were physically interacting with one another in yeast. However, it is possible that the TAL2 'bait' and 'prey' proteins were not actually physically interacting but binding through a yeast bridging protein instead. Purifying the TAL2 'bait' and 'prey' proteins to homogeneity prior to conducting the immunocapture assay would be a way to ensure that direct physical interaction occurs between the fusion proteins.

Extracting protein from yeast cells grown in minimal galactose medium

Extracting protein from yeast cells grown in galactose medium was one of the biggest obstacles in being able to perform the immunocapture assay. However, growth of the yeast cells in galactose medium was required for the expression of the B42AD fusion proteins. The yeast cell wall consists of a highly cross-linked system of sugar polymers and proteins, and the density of this cross-linking determines its strength (Walker, 1998). In addition, yeast preferentially metabolize glucose over galactose (Walker, 1998). Therefore, perhaps

when yeast cells are forced to metabolize galactose, the structure of the polysaccharide cross-linking of its cell wall is altered and it becomes more difficult to lyse. However, if this was the problem then removal of the cell wall using lyticase should have increased detection of soluble protein on a western blot, since approximately 50% of the cells were fully converted into spheroplasts prior to lysis, but this did not occur. Another possibility that could explain the difficulties in detecting protein in cleared lysates of yeast cells grown in medium containing galactose is that maybe there is less protein yield from cells grown in galactose than glucose. To avoid growing the yeast cells in galactose, expression vectors containing the B42 activation domain under the control of the *ADH1* promoter were constructed but protein detection in cleared lysates of yeast cells transformed with the vectors was never observed by western blot, so they could not be used (see Appendix A).

Lack of LexA-TAL2ΔC fusion protein accumulation when co-expressed with the B42AD-TAL2 fusion protein

Western blot data revealed that LexA-TAL2ΔC accumulation could not be detected when it was co-expressed in yeast with B42AD-TAL2. There are two possibilities as to why this could occur. The first is that there may be a problem with expression of the LexA-TAL2ΔC fusion protein, or that it is not synthesized as efficiently as the B42AD-TAL2 fusion protein, when the proteins are co-expressed in yeast grown in medium containing galactose as the carbon source. Perhaps interaction between LexA-TAL2ΔC and B42AD-TAL2 is affecting the expression level of LexA-TAL2ΔC leading to less synthesis of the 'bait' protein than the 'prey' protein. As a result, detection of B42AD-TAL2 would be greater than LexA-TAL2ΔC on a western blot.

The second possibility that would explain the lack of LexA-TAL2ΔC detection on a western blot compared to B42AD-TAL2 is that there could be a problem with degradation of LexA-TAL2ΔC. It is possible that interaction between LexA-TAL2ΔC and B42AD-TAL2 is affecting the degradation of the LexA-TAL2ΔC, resulting in its enhanced turnover.

Improving the immunocapture assay

One of the first things that needs to be determined in order to utilize the immunocapture assay is whether the anti-HA antibody was actually coupled to the column. Evidence that the antibody was coupled to the column includes protein concentration assay results that undetectable levels of protein were observed for the unbound antibody fraction and the uncoupled antibody flow-through fraction. In addition, no protein was detected by western blot for either fraction under conditions where as little as 1.4 μg of anti-HA antibody were detected. Evidence that the antibody was never coupled to the column includes the lack of antibody detected by western blot in the boiled column material, even though boiling the column matrix material in mecaptoethanol should have reversed the N-hydroxysuccinimide ester DSS cross-linker and freed the anti-HA antibody. One possibility that could account for this contrary result is that perhaps the mecaptoethanol was not effective in reversing the cross-linking, and the anti-HA antibody remained bound to the column matrix.

Another problem that may have lead to the unsuccessful utilization of the immunocapture assay is that the anti-HA antibody may not bind to the column matrix as well as claimed by the manufacturer of the assay. However, if this was the case, it would be expected that the anti-HA antibody would be detected in the flow-through fraction after incubation of the antibody with the column matrix, but it was not. Nevertheless, to

circumvent this potential problem, other mouse IgG_{2a} antibodies could be bound to the column matrix, or anti-LexA antibody could be used since, according to the manufacturer of the assay, the binding capability of mouse IgG_1 antibodies to immobilized protein G is moderate. Also, classic immunoprecipitation experiments could be conducted as an alternative to the column assay using anti-HA antibody, protein A sepharose, and soluble fractions of yeast cell extracts that show good accumulation of protein on a western blot.

An additional problem that may have prevented the utilization of the immunocapture assay involves the HA tag. It is conceivable that the HA tag of the B42AD-AL2 fusion proteins may be restricted from binding to the anti-HA antibody on the column due its central location in the protein's native folded configuration. This problem could be avoided if the AL2 proteins were cloned instead so that the HA tag was on either end of the fusion protein instead of between the B42 activation domain and the AL2 protein. This way, the HA tag may be in a better position to bind to the anti-HA antibody immobilized on the column matrix. Additionally, other tagging systems could be used besides HA. An alternative approach to the HA tag is a histidine tag (His-tag) where a series of four to six histidine residues are fused to the protein of interest (Müller *et al*., 1998). These histidine residues can bind metal ions, such as nickel, that are immobilized to the column matrix (Müller *et al*., 1998). Also, a glutathione *S*-transferase (GST) tag could be used in place of the HA tag, which would allow the fusion protein to bind to glutathione immobilized on a column (Ding *et al*., 2001). With the utilization of a His tag, anti-His tag and anti-LexA antibodies could be used to detect the fusion proteins in eluted fractions, while with a GST tagged system, anti-GST and anti-LexA antibodies could be used. The use of either of these tagging systems

would eliminate the need to bind an antibody to column and thus eliminate the need to confirm whether this step occurred correctly in the first place. In addition, the binding capacities of either of these types of columns would be much greater than the anti-HA antibody column because of the smaller size of glutathione and nickel ions compared to the anti-HA antibody. The use of both the GST-tag and His-tag together could also allow for the purification of both proteins prior to conducting the capture assay and could lead to an improvement in their binding during the assay. In addition, using both tags together would allow the expression of the proteins in a system other than yeast which would circumvent the problem of growing cells in medium containing galactose as the carbon source.

If the anti-HA antibody did bind to the column matrix and the anti-HA tag is accessible for binding the antibody, several approaches could be taken to improve the binding between the HA-tagged proteins in the soluble fractions passed over the column and the anti-HA antibody. One approach is to alter the binding conditions of the assay. It is possible that the pH or salt concentration of the buffer being used during binding is not the optimum condition for binding of the HA-tagged fusion proteins. Troubleshooting experiments with varying pH levels and salt concentrations of the binding buffer could be conducted to determine if changing one of these parameters would aid in the binding of the HA-tagged AL2 protein to the HA-antibody immobilized to the column.

In conclusion, the biochemical verification of TAL2 self-interaction was unsuccessful due to multiple factors. Confirmation of antibody binding to the column must occur if an immunocapture approach is used to test this interaction. Alternative assays using glutathione or nickel ion columns that bind GST or His-tagged proteins could be used instead of

antibody-bound columns that bind B42AD or LexA fusion proteins. In addition, classic immunoprecipitation assays using anti-HA or anti-LexA antibody against the B42AD or LexA fusion proteins provide an excellent alternative to the immunocapture assays that should be explored in future experiments.

Table 1 Functions of the New World bipartite begomovirus and curtovirus proteins.

New World bipartite begomovirus proteins	
AL1	DNA replication
AL2	transcription control, host defense suppression
AL3	replication booster
AL4	unknown
AR1	coat protein
BR1	movement
BL1	movement

curotovirus proteins	
C1	DNA replication
C2	host defense suppression
C3	replication booster
C4	symptom determinant
V1	coat protein, movement
V2	viral DNA synthesis
V3	movement

Table 2 Non-reciprocal interactions among the heterologous combinations of C2 and AL2 'baits' and 'preys'

'bait'	'prey' TAL2	BAL2ΔC	CAL2ΔC
C2ΔC	+ [(a)]	+	+
C2ΔNΔC	- [(a)]	-	-

'bait'	'prey' C2ΔC	C2ΔNΔC
TAL2ΔC	-	-
BAL2ΔC	-	-
CAL2ΔC	-	-

a + indicates interaction; – indicates no interaction

Table 3 Lysis methods performed on yeast cells grown in galactose minimal medium

A	4 times the volume of Y-PER™[1] and an equal volume of glass beads added to cell pellet; lysis by mixing hard at RT; soluble fraction obtained by centrifugation at 14,000 rpm for 5 minutes at RT
B	4 times the volume of IP buffer[2] and an equal volume of glass beads added to cell pellet; lysis by mixing hard at RT; soluble fraction obtained by centrifugation at 14,000 rpm for 5 minutes at RT
C	4 times the volume of Y-PER™, an equal volume of glass beads, and protease inhibitors[3] added to cell pellet; lysis by mixing hard at 4ºC for 20 minutes; soluble fraction obtained by centrifugation at 14,000 rpm for 20 minutes at 4ºC
D	An equal volume of 2x Laemmli buffer[4] added to each cell pellet; lysis by boiling for five minutes in a water bath; soluble fraction obtained by centrifugation at 14,000 rpm for five minutes at RT
E	Freeze-thaw cell pellet 3 times in liquid nitrogen and 37ºC waterbath[5]; 4 times the volume of Y-PER™ and an equal volume of glass beads added to cell pellet; lysis by mixing hard at 4ºC for 20 minutes; soluble fraction obtained by centrifugation at 14,000 rpm for 20 minutes at 4ºC
F	Freeze-thaw cell pellet 3 times in liquid nitrogen and 37ºC waterbath; 4 times the volume of IP buffer and an equal volume of glass beads added to cell pellet; lysis by mixing hard at 4ºC for 20 minutes; soluble fraction obtained by centrifugation at 14,000 rpm for 20 minutes at 4ºC
G	Convert cells to spheroplasts using lyticase[6]; lysing of spheroplasts by adding 4 times the volume of Y-PER™ and an equal volume of glass beads and mixing hard at 4ºC for 20 minutes; soluble fraction obtained by centrifugation at 14,000 rpm for 20 minutes at 4ºC

1 Yeast Protein Extraction Reagent (Pierce)

2. 50mM Tris.HCl, pH7.5, 1mM EDTA, 10% glycerol, 30mM NaCl, 0.05% IGEPAL CA-630, 1mM DTT, 1mM PMSF, 1µg/ml leupeptin, and 1µg/ml pepstatin A (Miyoshi *et al*. 2002)

3. 1mM PMSF, 1µg/ml leupeptin, and 1µg/ml pepstatin A.

4. 0.125M Tris.HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mecaptoethanol (Laemmli 1970).

5. Cell pellet frozen 1minute in liquid nitrogen followed by thawing in 37ºC waterbath for l minute (Langlands and Prochownik, 1997)

6. Cells resuspended in 4 times the volume of lyticase buffer (50mM Tris-HCl, pH8.0, 1M sorbitol, and 150mM NaCl). 2000 U/ml of lyticase, 1mM PMSF, 1µg/ml leupeptin, and 1µg/ml antipain are added and cells are incubated in a 30ºC waterbath for 80 minutes. Spheroplasts are recovered by centrifugation at 4ºC at 14,000 rpm for 20 minutes (Milani *et al*. 2001; Jewell *et al.,* 2002)

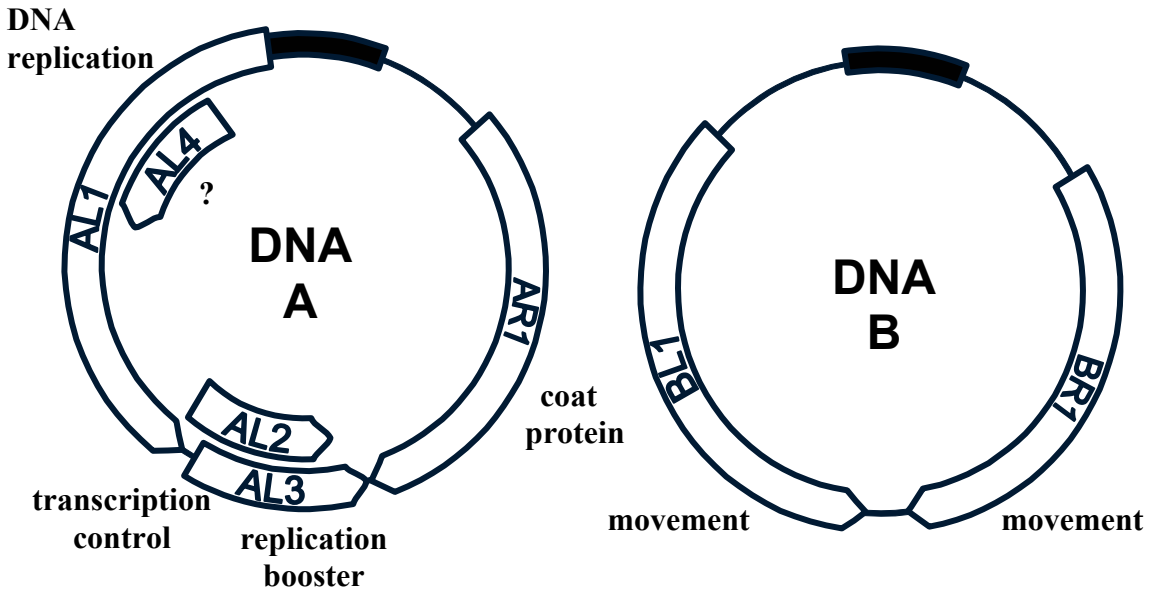


Fig. 1 Schematic of the bipartite geminivirus genome organization. The locations and functions of DNA coding sequences on the A and B genome are shown and their direction of transcription are represented by arrows.

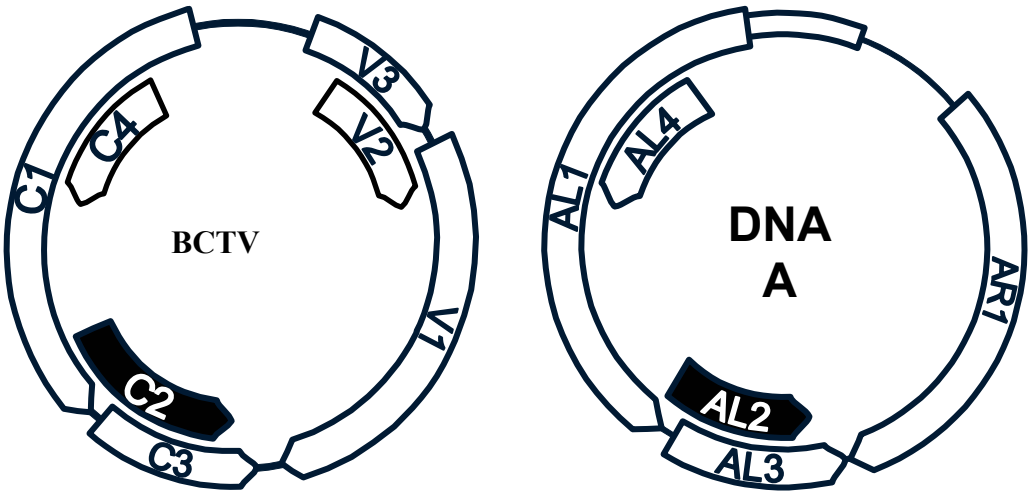


Fig. 2 Comparison of the curtovirus genome with the DNA A component of a New World begomovirus. The locations and functions of DNA coding sequences on the BCTV genome and the A component of the begomovirus are shown and their direction of transcription are represented by arrows. The *C2* and *AL2* ORFs are highlighted.

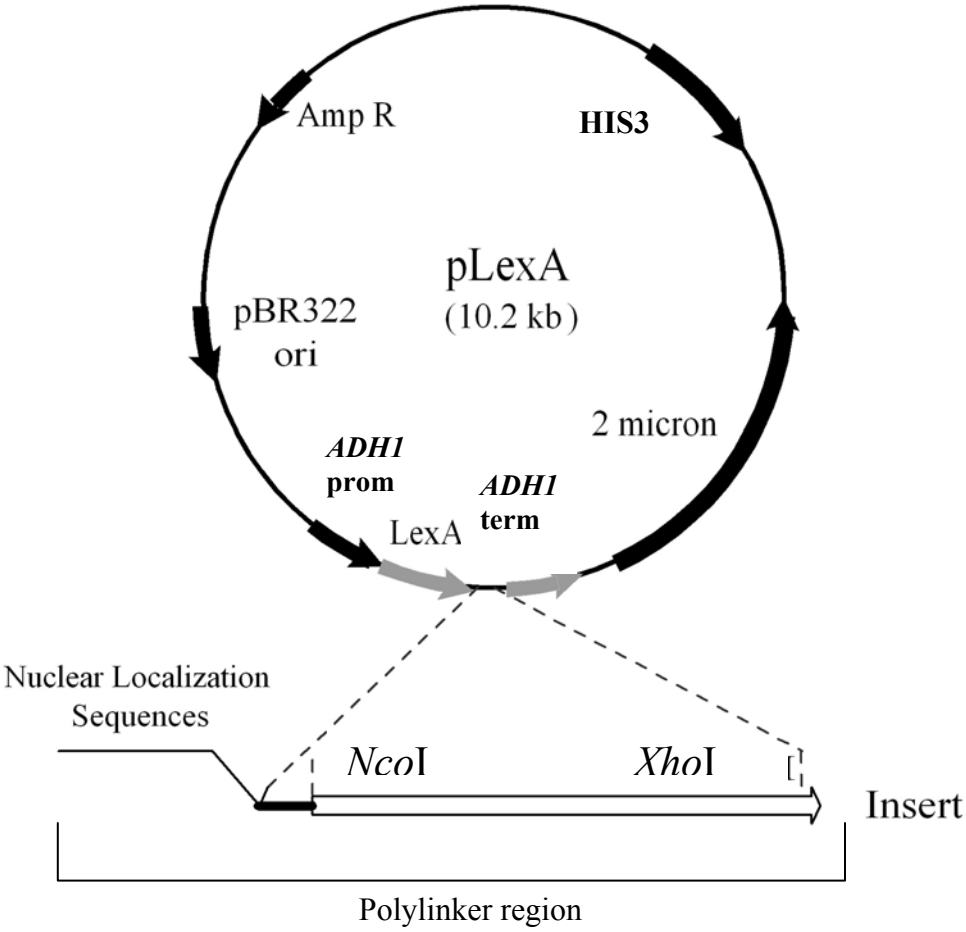


Fig. 3 Schematic illustration of the pLexA vector. This vector contains a yeast selectable marker *HIS3*, an ampicillin resistance marker for selection in *E. coli*, a pBR322 origin of replication for maintenance in *E. coli*, a 2 μm origin of replication for high copy number in yeast, an *ADH1* promoter, and an *ADH1* terminator. The vector's *ADH1* promoter allows for the constitutive expression of fusion proteins in yeast grown in medium containing glucose as the carbon source. DNA fragments encoding the proteins of interest are inserted into the polylinker region of the pLexA vector between *Nco*I and *Xho*I restriction sites and are fused to the LexA DNA binding domain.

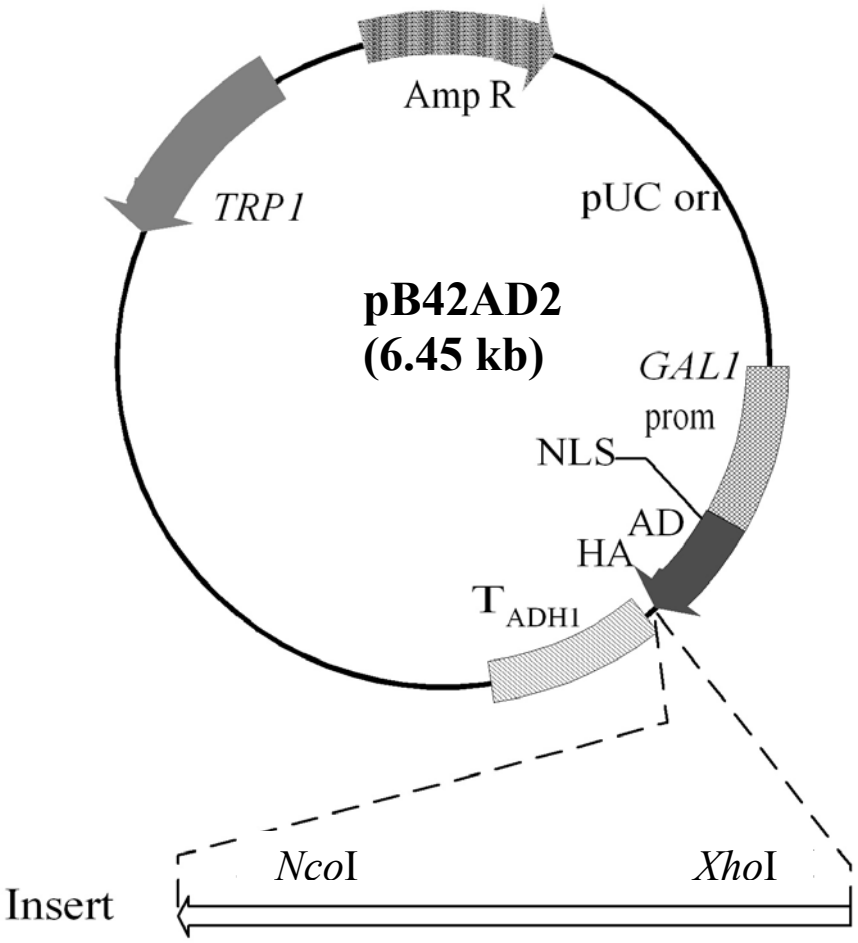


Fig. 4 Schematic illustration of the pB42AD2 vector. This vector contains the yeast selectable marker *TRP1*, an ampicillin resistance marker for selection in *E. coli*, a nuclear localization sequence (NLS) to target the fusion protein to the nucleus, a pUC origin of replication for maintenance in *E. coli*, a *GAL1* promoter, and an *ADH* terminator. The vector's *GAL1* promoter allows for the expression of its fusion proteins in yeast grown in medium containing galactose as the carbon source. This vector also contains the activation domain which embodies a hemagglutinin (HA) epitope tag. DNA fragments encoding the proteins of interest are cloned into the pB42AD2 vector between *Nco*I and *Xho*I restriction sites and fused to the activation domain containing the HA tag.

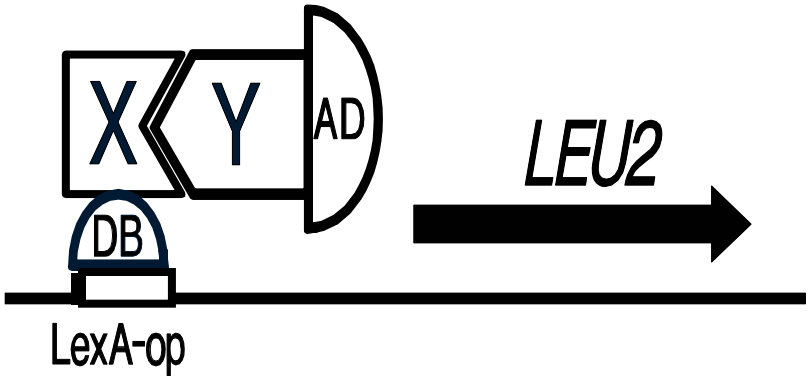
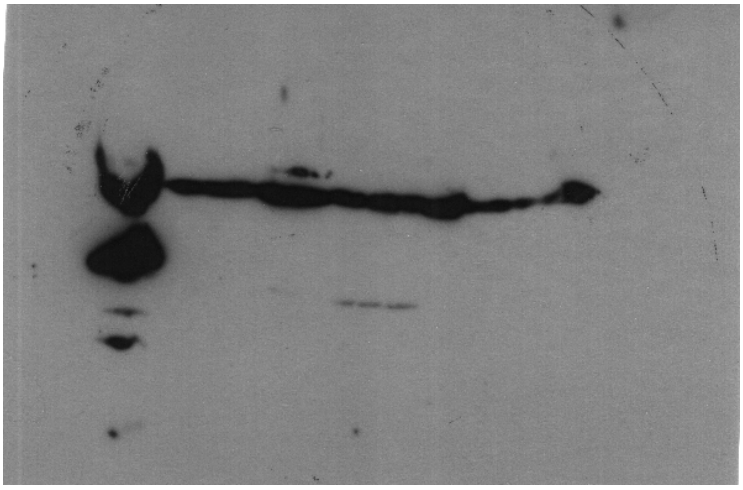


Fig. 5 Schematic of the yeast two-hybrid system. When 'bait' protein X fused to the DNA-binding domain (DB) interacts with 'prey' protein Y fused to the activation domain (AD), this activates the LEU2 reporter gene and enables yeast transformed with both 'bait' and 'prey' proteins to grow on medium lacking leucine.



1 2 3 4 5 6

Fig. 6 Western blot of whole yeast cell extracts demonstrating protein accumulation of LexA-BAL2ΔC and LexA-CAL2ΔC. Protein accumulation was detected using anti-LexA primary antibody. Soluble fractions of yeast cell lysates demonstrating LexA and LexA-TAL2ΔC accumulation were used as positive controls. All of the strains expressing the BAL2ΔC and CAL2ΔC 'bait' proteins exhibited fusion protein accumulation.

Lane 1 - LexA (positive control)
Lane 2 - LexA-TAL2ΔC (positive control)
Lane 3 - LexA-BAL2ΔC.1
Lane 4 - LexA-BAL2ΔC.2
Lane 5 - LexA-CAL2ΔC.1
Lane 6 - LexA-CAL2ΔC.2

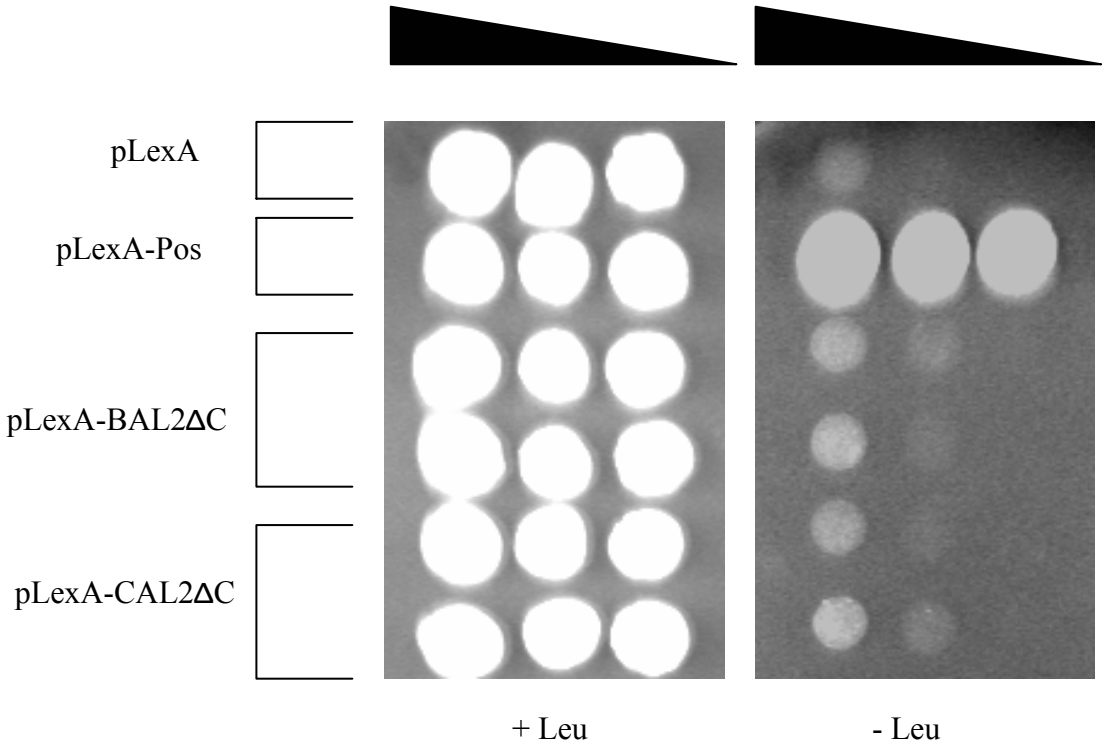


Fig. 7 Autoactivation tests of the BGMV and CabLCV AL2 'bait' proteins. Yeast strains containing the autoactivation positive control, pLexA-Pos, or the negative control, pLexA, were tested together with pLexA-BAL2ΔC and pLexA-CAL2ΔC. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each culture were spotted onto minimal medium containing leucine (+ Leu), or lacking leucine (- Leu), with glucose as the carbon source. Plates were incubated at 30ºC for three days. Only the autoactivation positive control, pLexA-Pos, allowed yeast growth on – Leu medium.

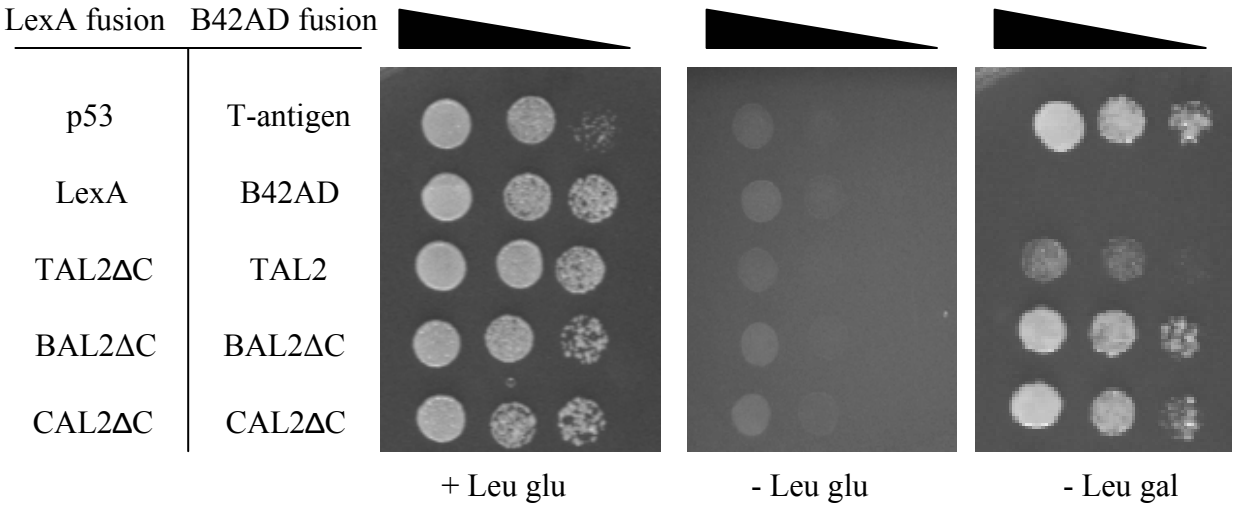


Fig. 8 Self-interaction tests of begomovirus AL2 proteins. The positive control strain contains pLexA-p53 plus pB42AD-T and the negative control strain contains pLexA vector plus pB42AD vector. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each yeast strain were spotted onto minimal medium lacking histidine and tryptophan, and either containing (+Leu) or lacking (-Leu) leucine. Medium contained either glucose (glu) or galactose plus raffinose (gal) as the carbon source. Plates were incubated at 30ºC for three days. Galactose-dependent growth on –Leu medium was observed for the interaction positive control (p53 plus T-antigen), as well as for TAL2, BAL2, and CAL2.

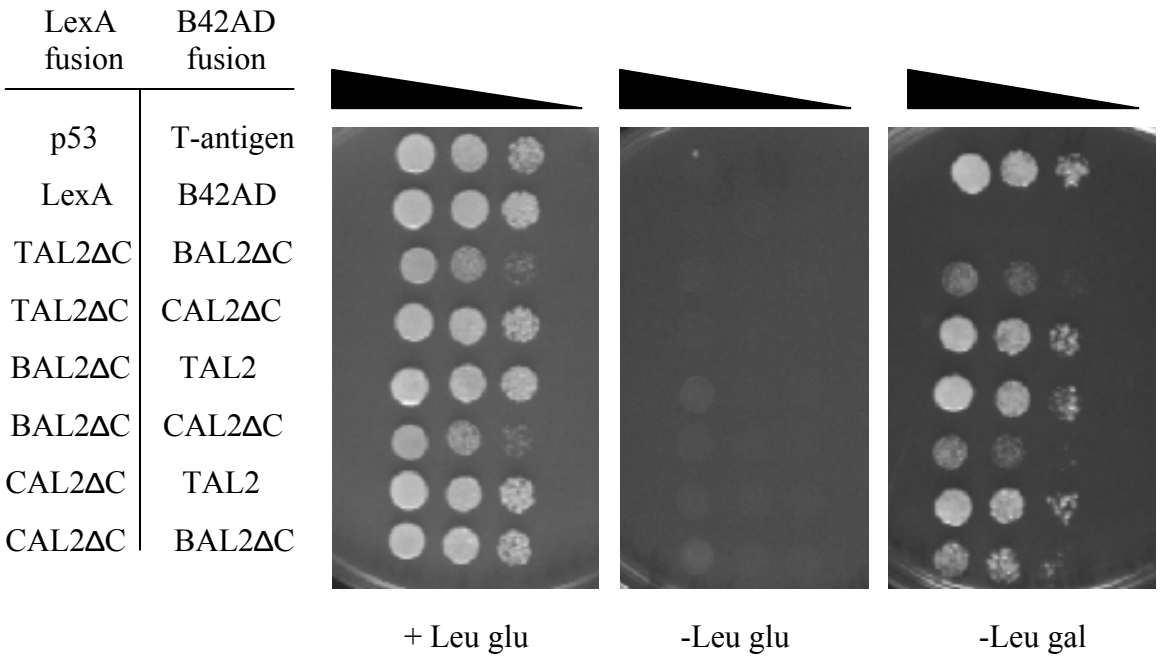


Fig. 9 Interaction tests between heterologous begomovirus AL2 proteins. The positive control stain contains pLexA-p53 plus pB42AD-T and the negative control strain contains pLexA vector plus pB42AD vector. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each yeast strain were spotted onto minimal medium lacking histidine and tryptophan, and either containing (+Leu) or lacking (-Leu) leucine. Medium contained either glucose (glu) or galactose plus raffinose (gal) as the carbon source. Plates were incubated at 30ºC for three days. Galactose-dependent growth on –Leu medium was observed for the interaction positive control (p53 plus T-antigen), as well as for all heterologous combinations of AL2 'baits' and AL2 'preys'.

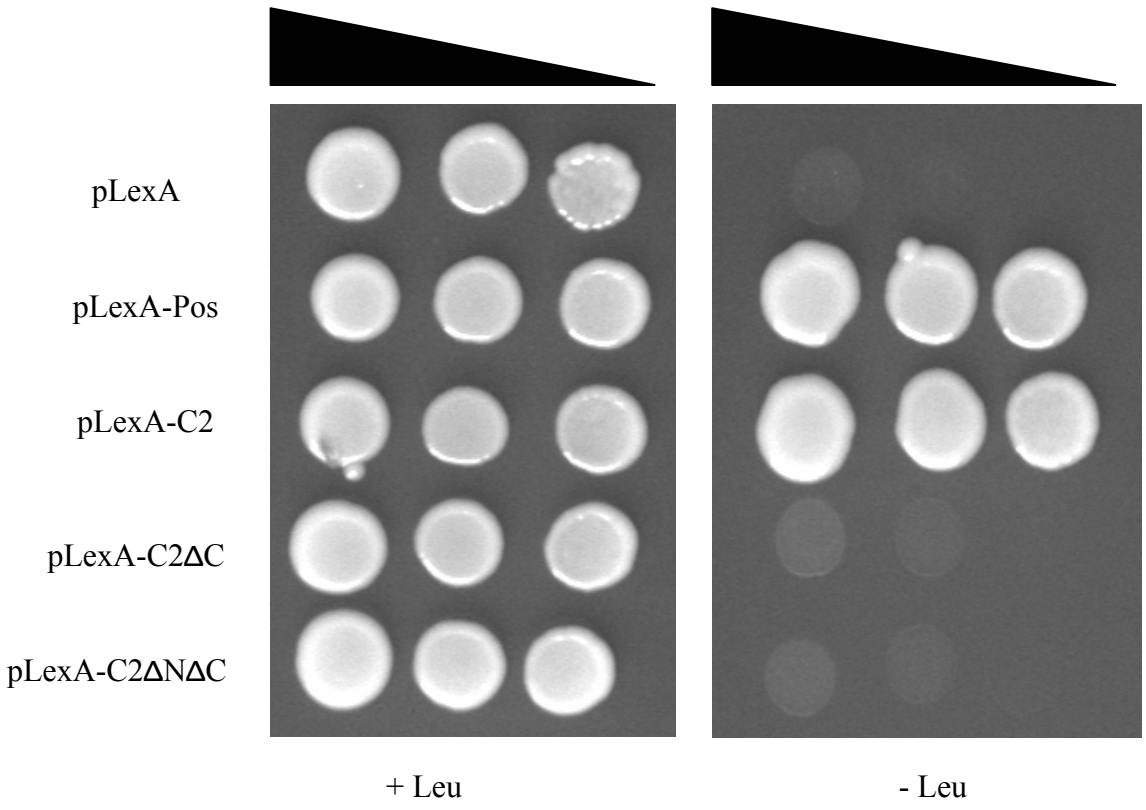


Fig. 10 Autoactivation tests of the C2 'baits'. Yeast strains containing the autoactivation positive control, pLexA-Pos, or the negative control, pLexA, were tested together with pLexA-C2, pLexA-C2ΔC, and pLexA-C2ΔNΔC. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each culture were spotted onto minimal medium containing leucine (+ Leu), or lacking leucine (- Leu), with glucose as the carbon source. Plates were incubated at 30ºC for three days. Only the autoactivation positive control, pLexA-Pos, and the full length C2 'bait' allowed yeast growth on – Leu medium.

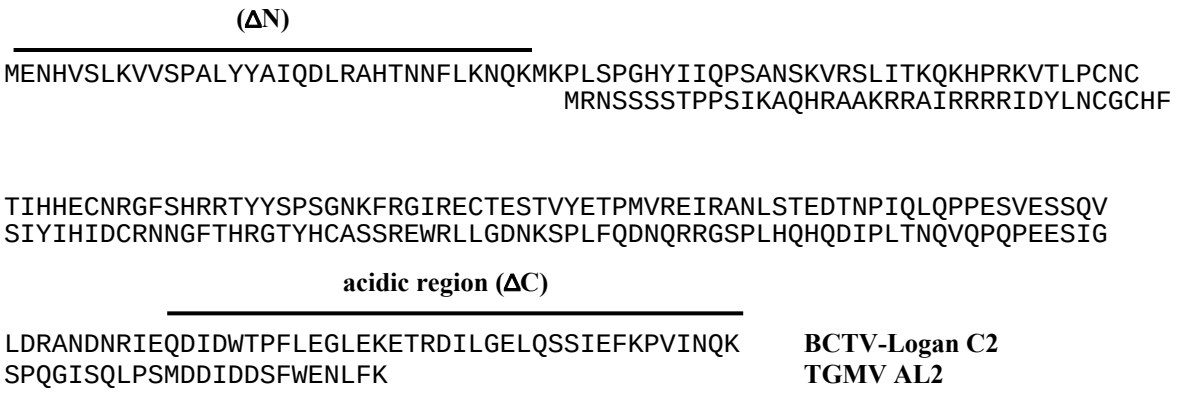
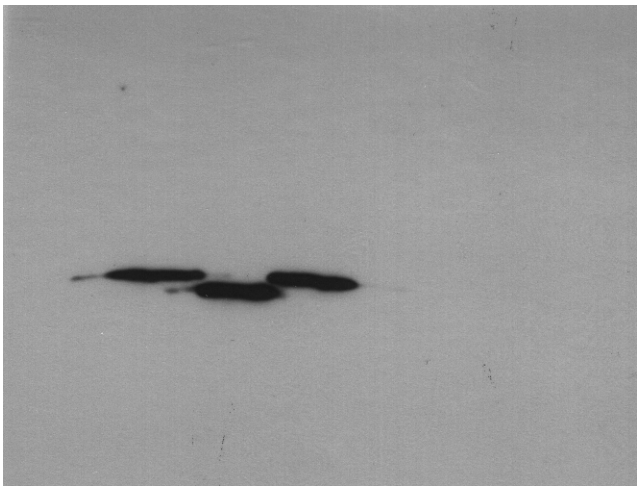


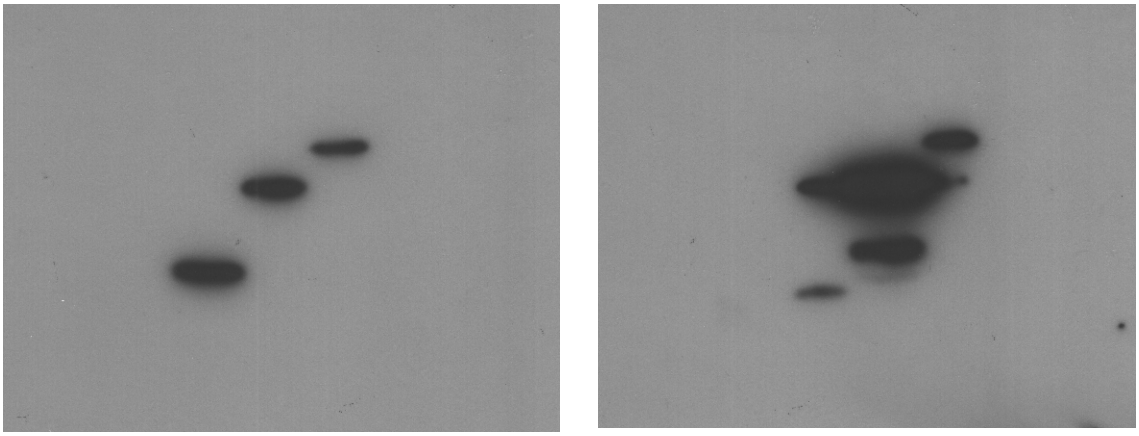
Fig. 11 Alignment of the TGMV AL2 and BCTV-Logan C2 proteins. The amino acid sequences of TAL2 and C2 are shown. The C2 protein contains a 33 amino acid N-terminal extension and a 22 amino acid C-terminal extension not seen in TAL2.



1 2 3

Fig. 12 Western blot of whole yeast cell extracts demonstrating accumulation of the truncated C2 'bait' proteins. Protein accumulation was detected using anti-LexA primary antibody. A yeast cell lysate containing LexA-BAL2ΔC was a positive control. Both truncated C2 'bait' proteins were expressed.

Lane 1 - LexA-BAL2ΔC (positive control)
Lane 2 - LexA-C2ΔNΔC
Lane 3 - LexA-C2ΔC



1 2 3 4 5 6

Fig. 13 Western blot of whole yeast cell extracts demonstrating accumulation of the C2 'prey' proteins. Protein accumulation was detected using anti-HA primary antibody. Soluble fractions of yeast cell lysates containing B42AD or B42AD-BAL2ΔC proteins were used as positive controls. Accumulation was observed for all the C2 'prey' proteins.

Lane 1 - B42AD (positive control)
Lane 2 - B42AD-C2ΔNΔC
Lane 3 - B42AD-C2ΔC
Lane 4 - B42AD (positive control)
Lane 5 - B42AD-BAL2 (positive control)
Lane 6 - B42AD-C2

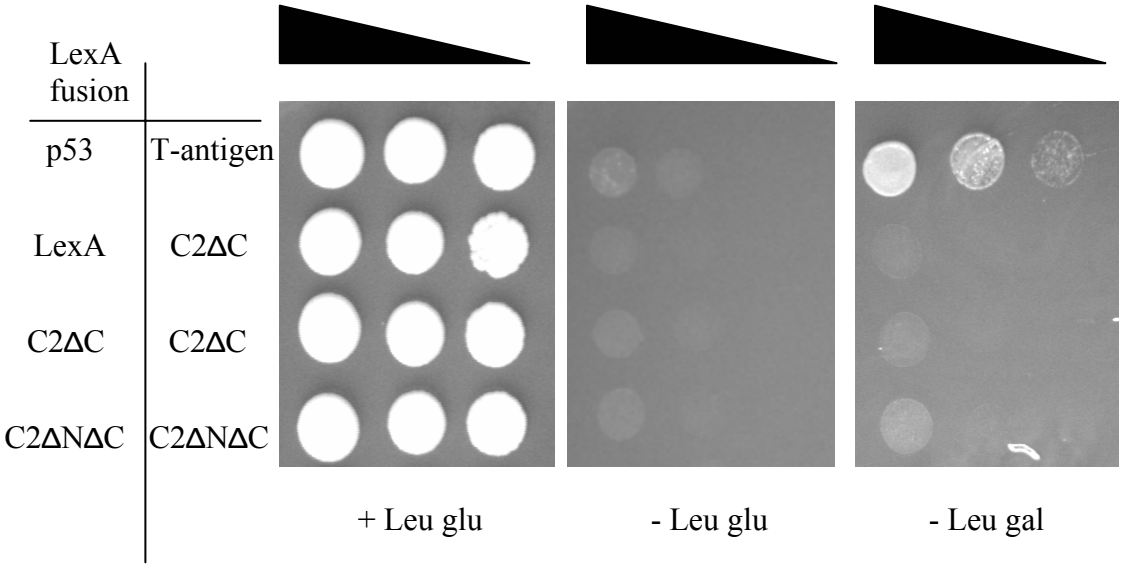


Fig. 14 Self-interaction test of the C2 protein. The positive control strain contains pLexA-p53 plus pB42AD-T and the negative control strain contains pLexA vector plus pB42AD-C2ΔC. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each yeast strain were spotted onto minimal medium lacking histidine and tryptophan, and either containing (+Leu) or lacking (-Leu) leucine. Medium contained either glucose (glu) or galactose plus raffinose (gal) as the carbon source. Plates were incubated at 30ºC for three days. Galactose-dependent growth on –Leu medium was observed only for the interaction positive control (p53 plus T-antigen).

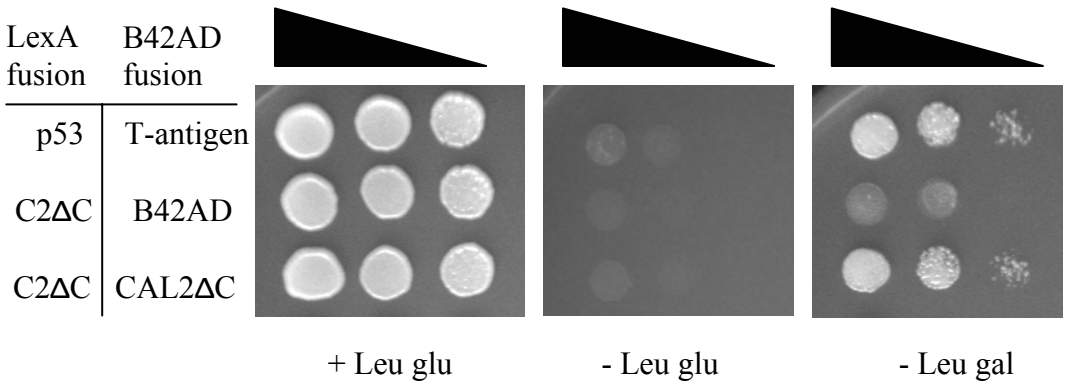


Fig. 15 Interaction tests with C2 'bait' proteins and AL2 'prey' proteins. The positive control strain contains pLexA-p53 plus pB42AD- T while the negative control strain contains pLexA-C2ΔC plus the empty pB42AD vector. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each yeast strain were spotted onto minimal medium lacking histidine and tryptophan, and either containing (+Leu) or lacking (-Leu) leucine. Medium contained either glucose (glu) or galactose plus raffinose (gal) as the carbon source. Plates were incubated at 30ºC for three days. Galactose-dependent growth on –Leu medium was observed for the interaction positive control (p53 plus T-antigen), as well as for LexA-C2ΔC plus B42AD-CAL2ΔC.

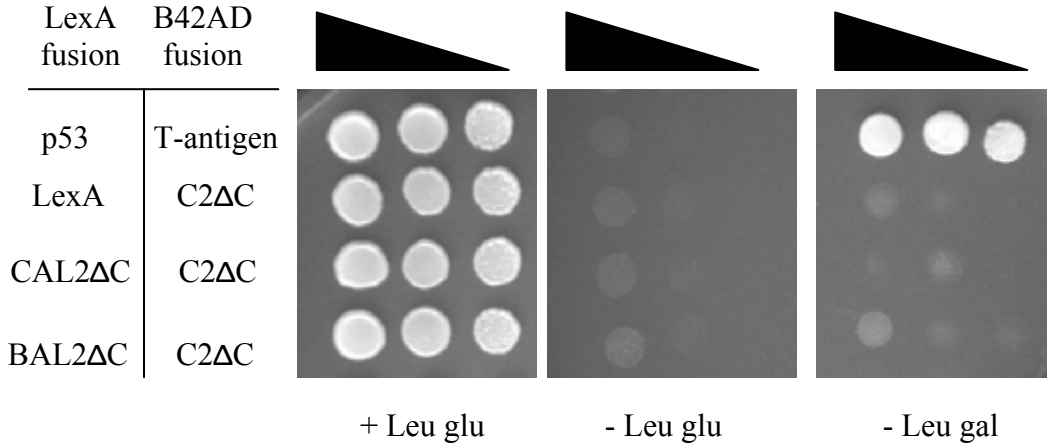


Fig. 16 Interaction tests with AL2 'bait' proteins and C2 'prey' proteins. The positive control strain contains pLexA-p53 plus pB42AD-T while the negative control strain contains pLexA vector plus pB42AD-C2ΔC. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each yeast strain were spotted onto minimal medium lacking histidine and tryptophan, and either containing (+Leu) or lacking (-Leu) leucine. Medium contained either glucose (glu) or galactose plus raffinose (gal) as the carbon source. Plates were incubated at 30ºC for three days. Galactose-dependent growth on –Leu medium was observed only for the interaction positive control (p53 plus T-antigen).

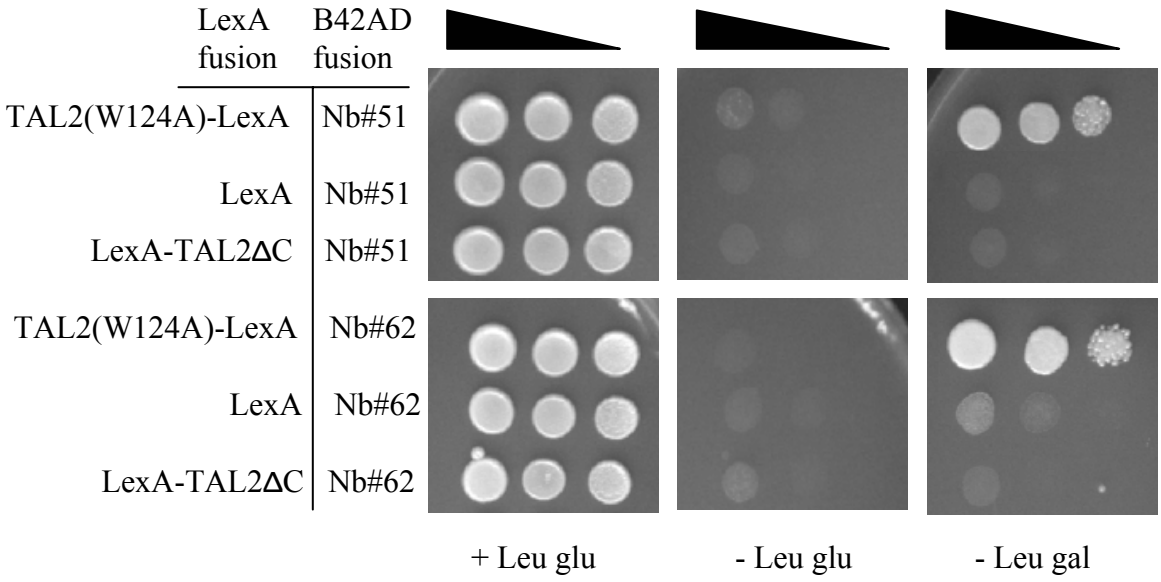


Fig. 17 Interaction tests with TAL2(W124A)-LexA and LexA-TAL2ΔC 'bait' proteins and Nb#51 and Nb#62 'prey' proteins. Strains expressing LexA plus Nb#51 or Nb#62 are used as the negative controls. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each yeast strain were spotted onto minimal medium lacking histidine and tryptophan, and either containing (+Leu) or lacking (-Leu) leucine. Medium contained either glucose (glu) or galactose plus raffinose (gal) as the carbon source. Plates were incubated at 30ºC for three days. Galactose-dependent growth on –Leu medium was observed only for TAL2(W124A)-LexA with either Nb#51 or Nb#62.

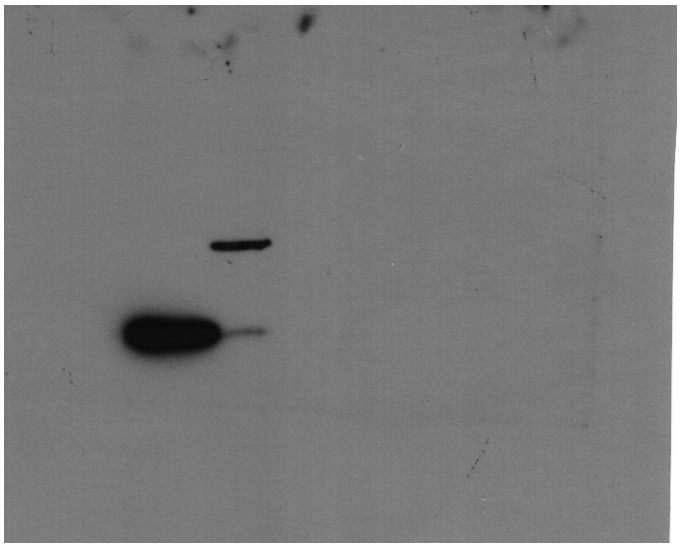


Fig. 18 Western blot of yeast whole cell extracts of strains containing plasmids encoding N-terminal AL2 and C2 LexA fusion proteins. Protein accumulation was detected using anti-LexA primary antibody. Yeast cell lysates containing LexA-BAL2ΔC and TAL2(W124A)-LexA proteins were used as positive controls. No protein accumulation was observed for BAL2(W124A)-LexA, CAL2(W124A)-LexA, C2ΔC-LexA, or C2ΔNΔC-LexA.

Lane 1 - LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 2 - TAL2(W124A)-LexA (N-terminal LexA fusion protein positive control)
Lane 3 - BAL2(W124A)-LexA
Lane 4 - CAL2(W124A)-LexA
Lane 5 - C2ΔC-LexA
Lane 6 - C2ΔNΔC-LexA

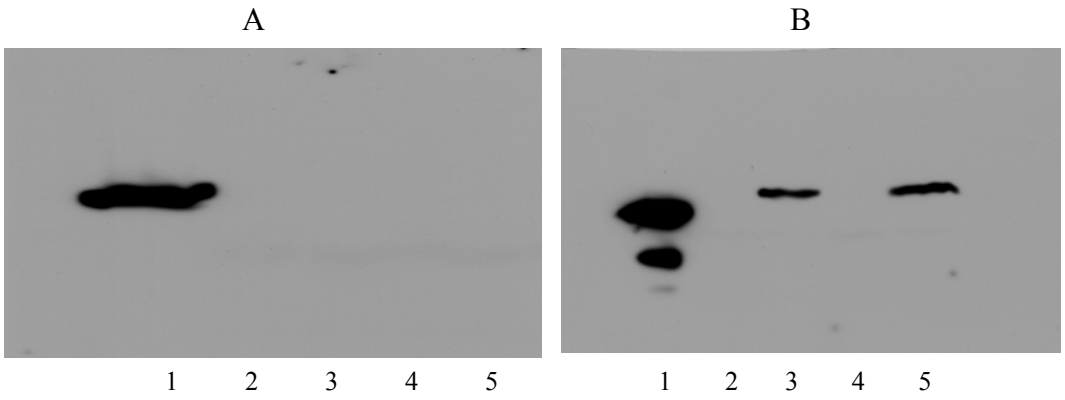


Fig. 19 Western blot of soluble fractions of yeast cell lysates generated by method A for cells grown in galactose minimal medium. Protein accumulation was detected on blot A using anti-LexA primary antibody and detected on blot B using anti-HA primary antibody. Soluble fractions of yeast cell lysates containing LexA-BAL2ΔC or B42AD-BAL2ΔC proteins were used as the positive controls. The results were positive for B42AD fusion protein accumulation for strains expressing LexA plus B42AD-TAL2 and LexA-TAL2ΔC plus B42AD-TAL2. None of the soluble fractions demonstrated LexA fusion protein accumulation.

Lane 1 - Blot A- LexA-BAL2ΔC (LexA fusion protein positive control)
Blot B- B42AD-BAL2ΔC (B42AD fusion protein positive control)
Lane 2 - LexA-p53 + B42AD-T
Lane 3 - LexA + B42AD-TAL2
Lane 4 - LexA-TAL2ΔC + B42AD
Lane 5 - LexA-TAL2ΔC + B42AD-TAL2

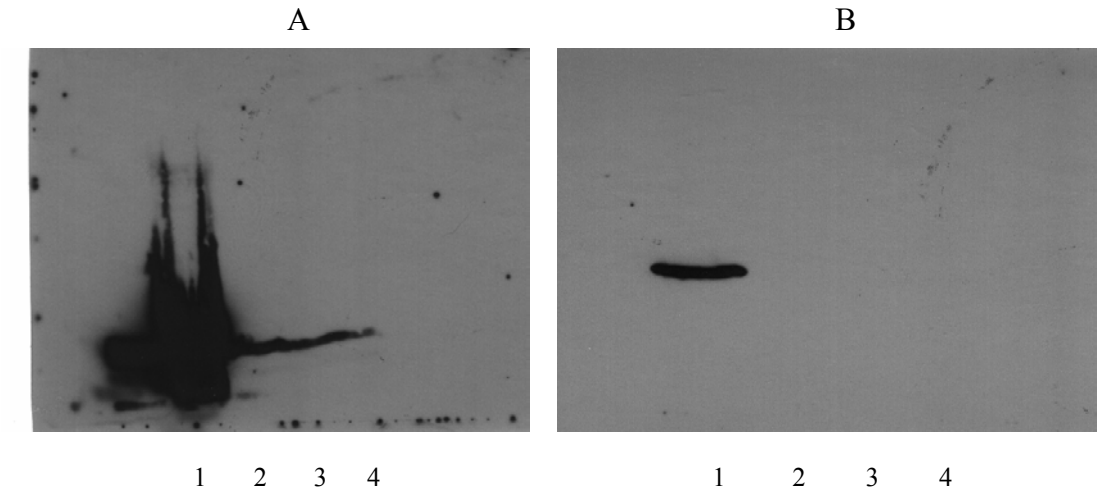


Fig. 20 Western blot of soluble fractions of yeast cell lysates of the strain containing pLexA-TAL2ΔC plus pB42AD-TAL2 generated by method A by mixing for 20, 40, or 60 minutes. Protein accumulation was detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC or LexA-BAL2ΔC proteins were used as positive controls. B42AD-TAL2 protein accumulation was seen in all the soluble fractions, while LexA-TAL2ΔC accumulation was not observed in any of the soluble fractions.

Lane 1 - Blot A- B42AD-BAL2ΔC (B42AD fusion protein positive control)
Blot B- LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 2 - Soluble fraction of the strain containing pLexA-TAL2ΔC plus pB42AD-TAL2 generated by method A for 20 minutes
Lane 3 - Soluble fraction of the strain containing pLexA-TAL2ΔC plus pB42AD-TAL2 generated by method A for 40 minutes
Lane 4 - Soluble fraction of the strain containing pLexA-TAL2ΔC plus pB42AD-TAL2 generated by method A for 60 minutes

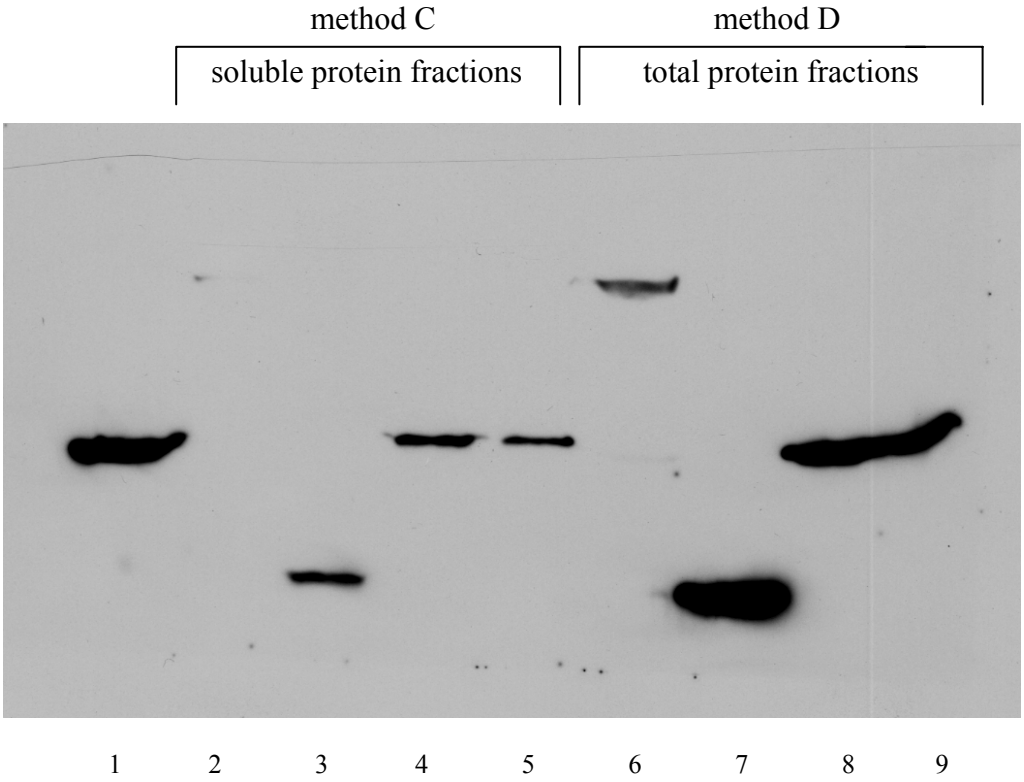


Fig. 21 Western blot of soluble and total protein fractions of yeast cell lystates generated by methods C and D from cells grown in glucose rich medium. Protein accumulation was detected using anti-LexA primary antibody. A soluble fraction of a yeast cell lysate containing LexA-BAL2ΔC protein was used as a positive control. The proteins expressed in each soluble and total protein fraction and the lysis method used to generated them were as follows:

Lane 1 - LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 2 - LexA-p53 + B42AD-T (method C)
Lane 3 - LexA + B42AD-TAL2 (method C)
Lane 4 - LexA-TAL2ΔC + B42AD (method C)
Lane 5 - LexA-TAL2ΔC + B42AD-TAL2 (method C)
Lane 6 - LexA-p53 + B42AD-T (method D)
Lane 7 - LexA + B42AD-TAL2 (method D)
Lane 8 - LexA-TAL2ΔC + B42AD (method D)
Lane 9 - LexA-TAL2ΔC + B42AD-TAL2 (method D)

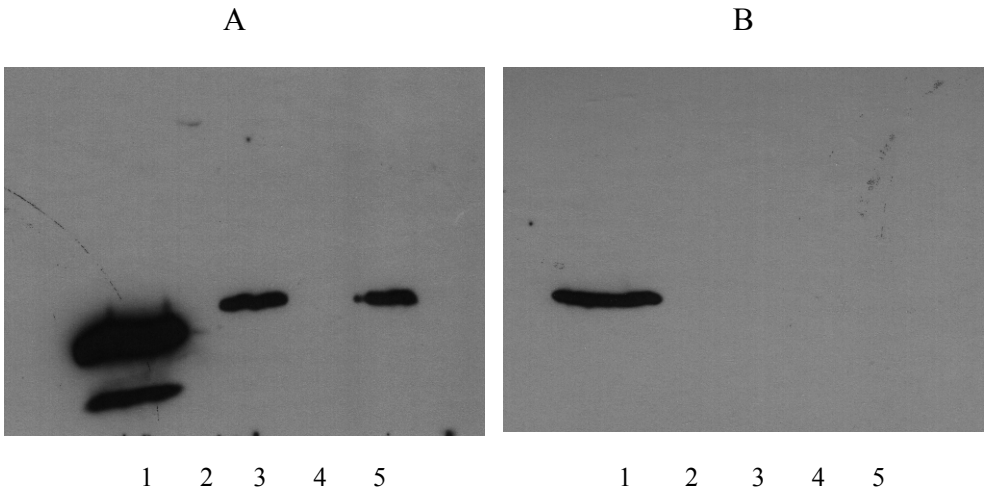


Fig. 22 Western blot of soluble fractions of lysates generated by method D from yeast cells grown in galactose rich medium. Protein accumulation was detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC or LexA-BAL2ΔC proteins were used as positive controls. B42AD fusion protein accumulation was seen only for the strains containing pLexA plus pB42AD-TAL2 and pLexA-TAL2ΔC plus pB42AD-TAL2. None of the soluble fractions showed LexA fusion protein accumulation.

Lane 1 - Blot A - B42AD-BAL2ΔC (B42AD fusion protein positive control)
Blot B - LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 2 - soluble fraction of the strain containing pLexA-p53 plus pB42AD-T
Lane 3 - soluble fraction of the strain containing pLexA plus pB42AD-TAL2
Lane 4 - soluble fraction of the strain containing pLexA-TAL2ΔC plus pB42AD
Lane 5 - soluble fraction of the strain containing pLexA-TAL2ΔC plus pB42AD-TAL2

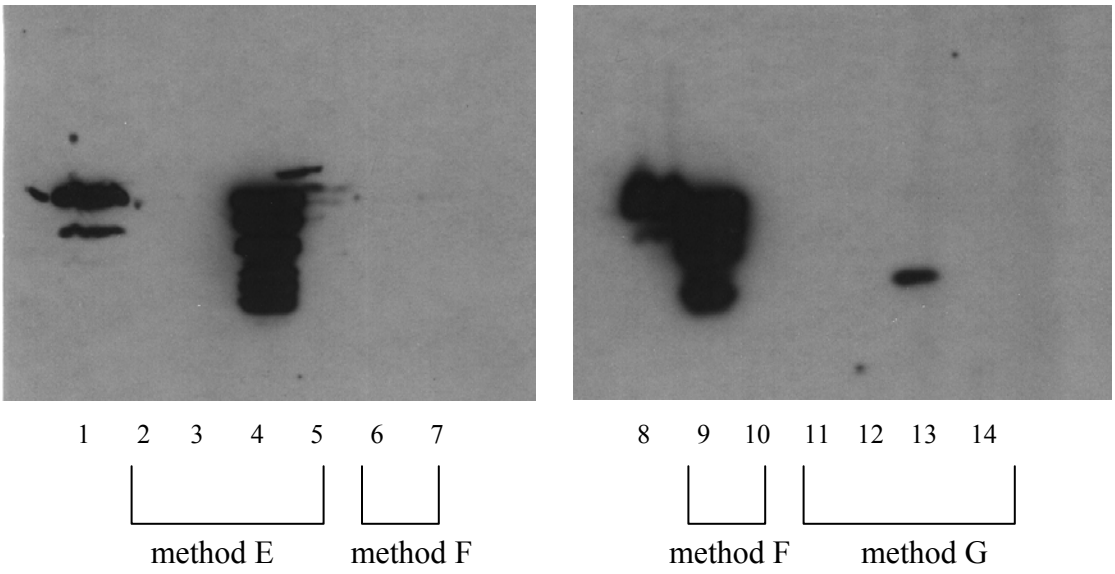


Fig. 23 Western blot of soluble fractions of yeast cell lysates generated by lysis methods E, F, and G of cells grown in galactose minimal medium. Protein accumulation was detected using anti-HA primary antibody. A soluble fraction of a yeast cell lysate containing B42AD-BAL2ΔC protein was used as a positive control. The Target protein accumulated more than the other B42AD fusion proteins when made by all three methods, but showed substantially higher protein accumulation by methods E and F than method G. The soluble fraction from cells expressing B42AD-TAL2 made by method E also contained detectable fusion protein. The proteins expressed and the methods used to generate the soluble fractions were as follows:

Lane 1 - B42AD-BAL2ΔC (positive control)
Lane 2 - B42AD (method E)
Lane 3 - B42AD-T (method E)
Lane 4 - Target (method E)
Lane 5 - B42AD-TAL2 (method E)
Lane 6 - B42AD (method F)
Lane 7 - B42AD-T (method F)
Lane 8 - B42AD-BAL2ΔC (positive control)
Lane 9 - Target (method F)
Lane 10 - B42AD-TAL2 (method F)
Lane 11 - B42AD (method G)
Lane 12 - B42AD-T (method G)
Lane 13 - Target (method G)
Lane 14 - B42AD-TAL2 (method G)

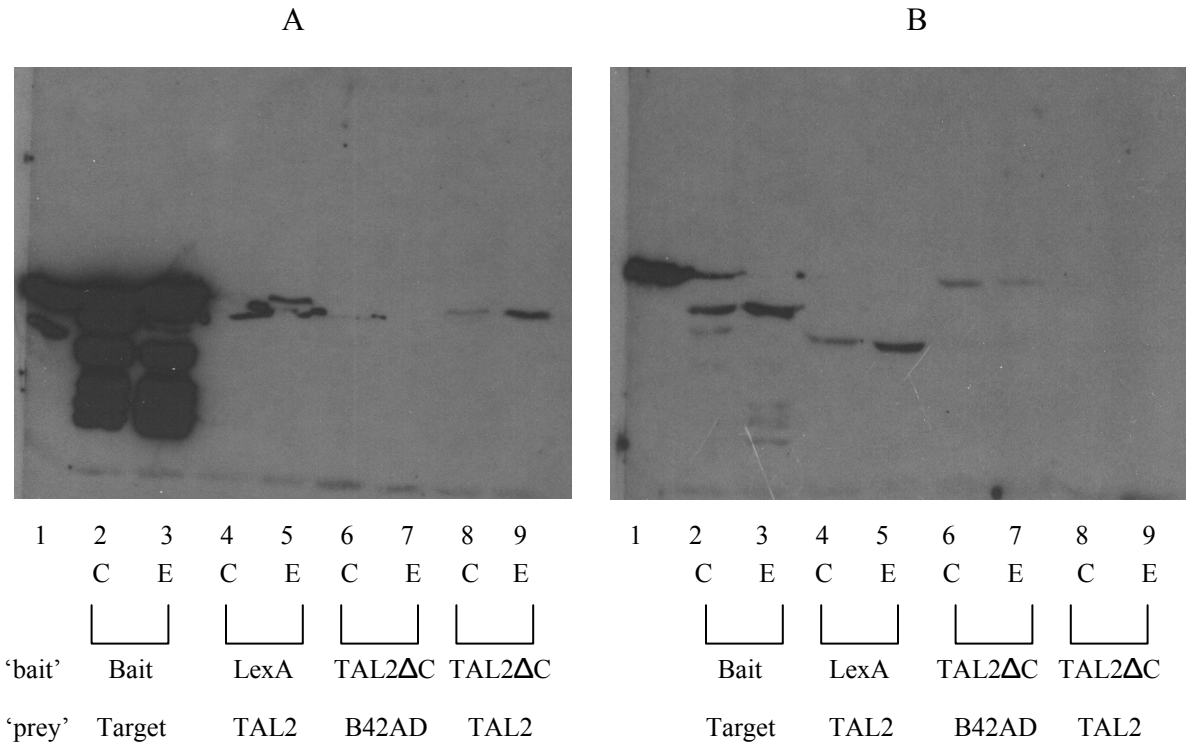


Fig. 24 Western blot of soluble fractions of yeast cell lysates generated by methods C and E of cells grown in galactose minimal medium. Protein accumulation was detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC or LexA-BAL2ΔC proteins were used as the positive controls. All of the soluble fractions exhibited B42AD fusion protein accumulation and all of the soluble fractions except for the one from cells expressing LexA-TAL2ΔC plus B42AD-TAL2 showed LexA fusion protein accumulation. The proteins expressed and the methods used to generate each soluble fraction were as follows:

Lane 1 - Blot A- B42AD-BAL2ΔC (B42AD fusion protein positive control)
Blot B- LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 2 - Bait + Target (method C)
Lane 3 - Bait + Target (method E)
Lane 4 - LexA + B42AD-TAL2 (method C)
Lane 5 - LexA + B42AD-TAL2 (method E)
Lane 6 - LexA-TAL2ΔC + B42AD (method C)
Lane 7 - LexA-TAL2ΔC + B42AD (method E)
Lane 8 - LexA-TAL2ΔC + B42AD-TAL2 (method C)
Lane 9 - LexA-TAL2ΔC + B42AD-TAL2 (method E)

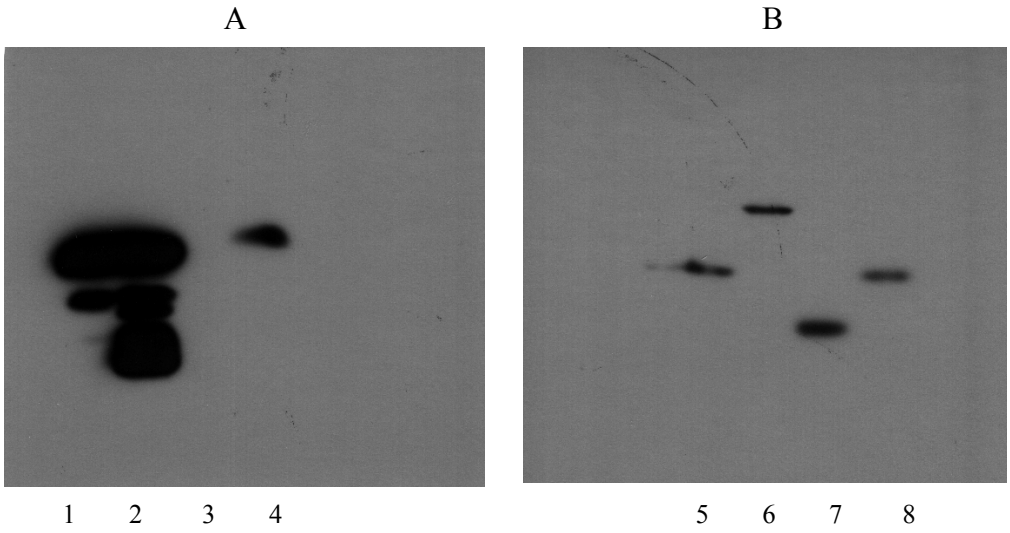


Fig. 25 Western blot of yeast cell cleared lysates from strains expressing LexA or B42AD fusion proteins separately. Protein accumulation is detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC or LexA-BAL2ΔC proteins were used as positive controls. All of the cleared lysate fractions demonstrated soluble protein accumulation except for that from the strain expressing B42AD.

Lane 1 - B42AD-BAL2ΔC (B42AD fusion protein positive control)
Lane 2 - Target
Lane 3 - B42AD
Lane 4 - B42AD-TAL2
Lane 5 - LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 6 - Bait
Lane 7 - LexA
Lane 8 - LexA-TAL2ΔC

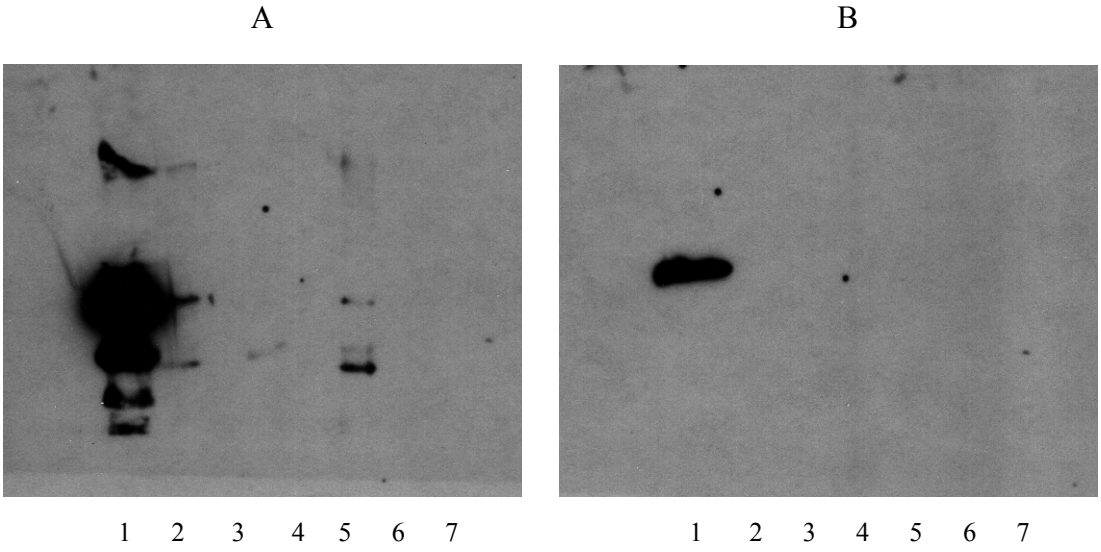


Fig. 26 Western blot of eluted fractions after passing positive and negative controls for interaction over the anti-HA antibody column. Protein accumulation was detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC or LexA-BAL2ΔC proteins were used as positive controls. The positive control for interaction is combined soluble fractions expressing Bait and Target and the negative control is combined soluble fractions expressing LexA and Target. A very small amount of B42AD fusion protein was seen in the first eluted fractions after binding of the combined soluble fractions expressing Bait and Target and after binding of the combined soluble fractions expressing LexA and Target. However, LexA fusion protein was not seen in any of the eluted fractions.

Lane 1 - Blot A – B42AD-BAL2ΔC (B42AD fusion protein positive control)
Blot B – LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 2 - eluted fraction 1 after incubation of soluble fractions expressing Bait and Target
Lane 3 - eluted fraction 2 after incubation of soluble fractions expressing Bait and Target
Lane 4 - eluted fraction 3 after incubation of soluble fractions expressing Bait and Target
Lane 5 - eluted fraction 1 after incubation of soluble fractions expressing LexA and Target
Lane 6 - eluted fraction 2 after incubation of soluble fractions expressing LexA and Target
Lane 7 - eluted fraction 3 after incubation of soluble fractions expressing LexA and Target

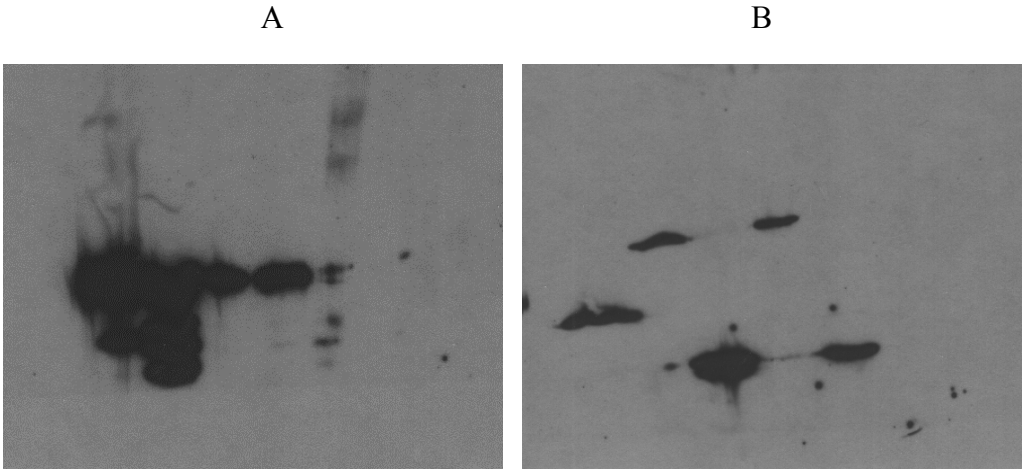


Fig. 27 Western blot of yeast cell cleared lysates incubated with the anti-HA antibody column matrix, flow-through fractions after incubation, and the column matrix itself. Protein accumulation was detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC or LexA-BAL2ΔC proteins were used as positive controls. B42AD and LexA fusion proteins were detected in the soluble fractions passed over the column as well as the flow-through fractions after incubating the soluble fractions with the column matrix. There is only a very small amount of protein trapped in the column matrix after incubation of the soluble fractions expressing Bait and Target.

Lane 1 - B42AD-BAL2ΔC (B42AD fusion protein positive control)
Lane 2 - soluble fraction expressing Target
Lane 3 - flow-through fraction after incubation of the soluble fractions expressing Bait and Target
Lane 4 - flow-through fraction after incubation of the soluble fractions expressing LexA and Target
Lane 5 - column material after incubation of the soluble fractions expressing Bait and Target
Lane 6 - column material after incubation of the soluble fractions expressing LexA and Target
Lane 7 - LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 8 - soluble fraction expressing Bait
Lane 9 - soluble fraction expressing LexA
Lane 10 - flow-through fraction after incubation of the soluble fractions expressing Bait and Target
Lane 11 - flow-through fraction after incubation of the soluble fractions expressing LexA and Target
Lane 12 - column material after incubation of the soluble fractions expressing Bait and Target
Lane 13 - column material after incubation of the soluble fractions expressing LexA and Target

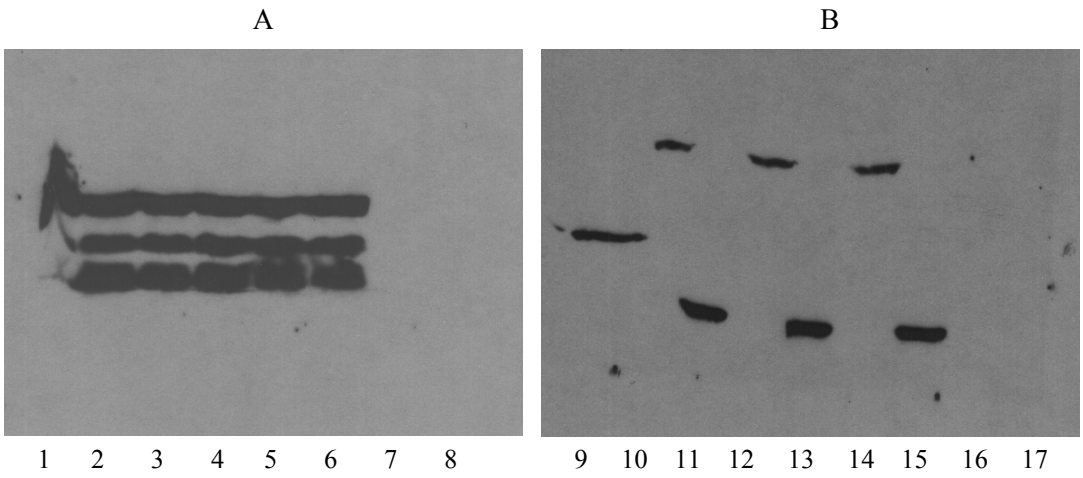


Fig. 28 Western blot of soluble fractions incubated with the anti-HA antibody column at 4ºC or room temperature, flow-through fractions after incubation, and the column matrix. Protein accumulation was detected on blot A using anti-HA primary antibody and on blot B using anti-LexA primary antibody. Soluble fractions of yeast cell lysates containing B42AD-BAL2ΔC and LexA-BAL2ΔC proteins were used as positive controls. Roughly equal amounts of B42AD and LexA fusion protein accumulation were seen in the soluble fractions incubated with the column matrix and in the flow-through fractions after incubation. There was no detectable B42AD or LexA fusion protein trapped in the column matrix.

Lane 1 - B42AD-BAL2ΔC (B42AD fusion protein positive control)
Lane 2 - soluble fraction expressing Target
Lane 3 - flow-through fraction after incubation at 4ºC of the soluble fractions expressing Bait and Target
Lane 4 - flow-through fraction after incubation at 4ºC of the soluble fractions expressing LexA and Target
Lane 5 - flow-through fraction after incubation at room temperature of the soluble fractions expressing Bait and Target
Lane 6 - flow-through fraction after incubation at room temperature of the soluble fractions expressing LexA and Target
Lane 7 - column matrix after incubation of the soluble fractions expressing Bait and Target
Lane 8 - column matrix after incubation of the soluble fractions expressing LexA and Target
Lane 9 - LexA-BAL2ΔC (LexA fusion protein positive control)
Lane 10 - soluble fraction expressing Bait
Lane 11 - soluble fraction expressing LexA
Lane 12 - flow-through fraction after incubation at 4ºC of soluble fractions expressing Bait and Target
Lane 13 - flow-through fraction after incubation at 4ºC of the soluble fractions expressing LexA and Target
Lane 14 - flow-through fraction after incubation at room temperature of the soluble fractions expressing Bait and Target
Lane 15 - flow-through fraction after incubation at room temperature of the soluble fractions expressing LexA and Target
Lane 16 - column matrix after incubation of the soluble fractions expressing Bait and Target
Lane 17 - column matrix after incbuation of the soluble fractions expressing LexA and Target

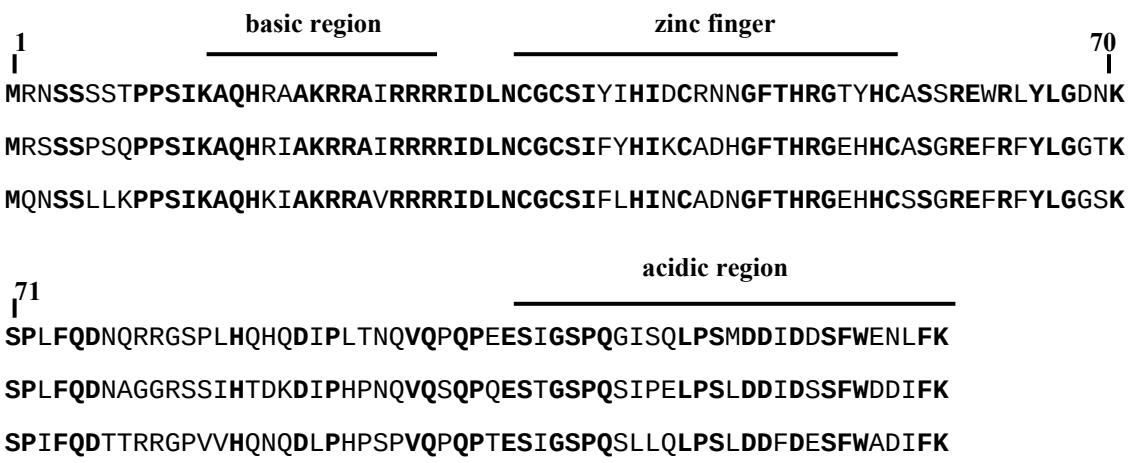


Fig. 29 Alignment of the TGMV, BGMV, and CabLCV AL2 proteins. The amino acid sequences of TAL2, BAL2 and CAL2 are shown. The proteins contain several regions often found in transcription factors including an N-terminal basic region, a zinc finger region, and a C-terminal acidic region. The conserved amino acids are bolded.

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Appendix

Appendix A - Generation of plasmids containing the B42AD coding region under the control of the constitutive ADH promoter

In order to avoid having to grow the yeast cells in galactose minimal medium to express the B42AD fusion protein, a new plasmid was made in which the B42AD coding region was placed under the control of the constitutive *ADH1* promoter. To do this, pB42AD2 was digested with *Aat*II to completion and then digested partially with *Hin*dIII to generate a 3860 bp fragment containing the B42AD coding region and the *ADH* terminator (figure 1A). This fragment was then ligated to the 3681 bp fragment of pLexA, containing the *ADH1* promoter, resulting from complete digestion with *Aat*II and *Hin*dIII. The resulting vector was designated pB42AD3. A western blot of whole cell extracts of three different clones was performed to test for detection of the B42AD protein in yeast strain EGY48 harboring pB42AD3. The results of the western blot were negative for protein detection when analyzed using anti-HA primary antibody. The western blot was repeated with a new anti-HA antibody dilution but the results remained negative for protein detection. The three pB42AD3 clones were checked by sequencing across their *ADH1* promoters and were found to be cloned correctly.

Since the whole cell extracts of yeast containing pB42AD3 did not show protein detection on a western blot, a second plasmid was made that was similar to the pB42AD3 plasmid previously made, but containing 18 additional nucleotides before the B42AD ORF (figure 2A). These 18 extra nucleotides were added in order to match the 5' leader of pLexA exactly since LexA fusion proteins have been shown be expressed at levels high enough to be detected by western blot. To construct this plasmid, a PCR product encoding B42AD with the 18 additional nucleotides was generated, cloned and sequenced. The clones with the

correct sequences were named pCR-AD2-18.1 and pCR-AD2-18.2. These plasmids was digested with *Hin*dIII and *Aat*II to generate the 383 bp DNA fragment that contained the B42AD ORF with the 18 additional nucleotides, the polylinker region, and the *ADH* terminator. Then, pLexA was digested with *Hin*dIII and *Aat*II to get a 3681 bp DNA fragment that contained the *ADH1* promoter in pBR322. Both the 383 bp fragment and the 3681 bp fragment were gel purified and ligated together to get pBR322-B42. The pBR322-B42 plasmid was digested with *Nco*I which cut the plasmid in the polylinker region and *Aat*II, and was ligated to a 3520 bp fragment of pB42AD2 that resulted from digestion with the same two enzymes. This plasmid was named pB42AD4. A western blot of whole cell extracts was performed to test for detection of the B42AD protein in yeast strain EGY48 harboring pB42AD4. The result of this western blot was also negative for protein detection.

One of the reasons that the pB42AD3 and pB42AD4 clones may not have expressed protein at levels high enough to be detected by western blot was that no mRNA may have been transcribed. This hypothesis could be tested by performing a northern blot of RNA extracted from cells transformed with these plasmids. A negative result for RNA expression could have indicated one of two things. The first is that no mRNA was being transcribed and the second is that the level of RNA degradation may have been high enough to make the steady-state level of RNA undetectable by the northern blot. However, the amount of mRNA may not be the most likely problem since the promoter sequences of both plasmids were found to be cloned correctly.

A second reason that the clones of the new yeast expression vectors may not have expressed a high enough level of protein to be detected by western blot is that the ribosomes

may not have been translating the mRNA efficiently. One possibility for why this may have occurred was that there was a problem with the capping of the mRNA. If the mRNA was not being capped properly then the ribosome would not bind it and begin scanning for the start codon to start translation. Another possibility is that translation may not be starting at the correct start codon. If there was a start codon upstream of the B42AD domain, then translation may be starting there which would result in the expression of a different protein. However, analysis of the sequences of the pB42AD3 and pB42AD4 plasmids did not reveal any additional start codons upstream of the B42AD domain indicating that this was not the problem leading to the lack of protein detection.

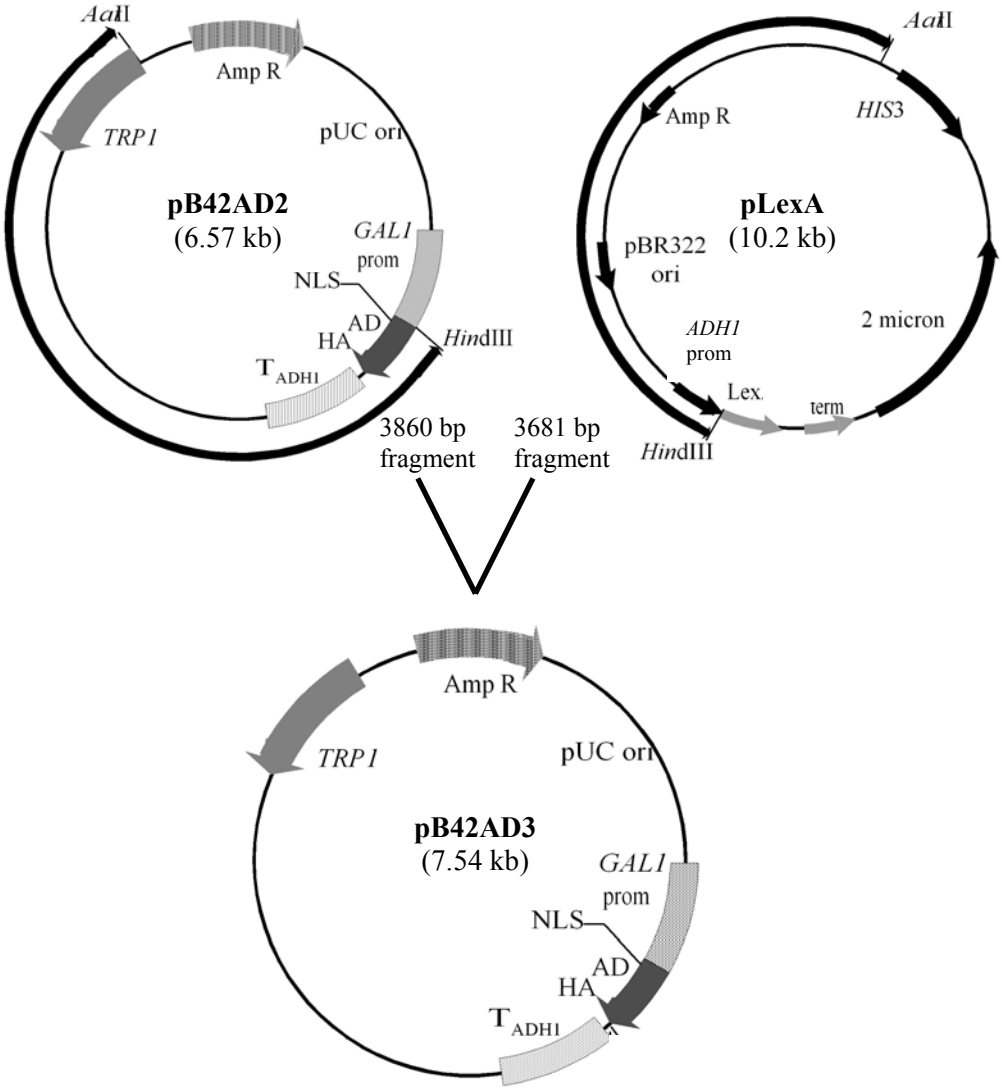
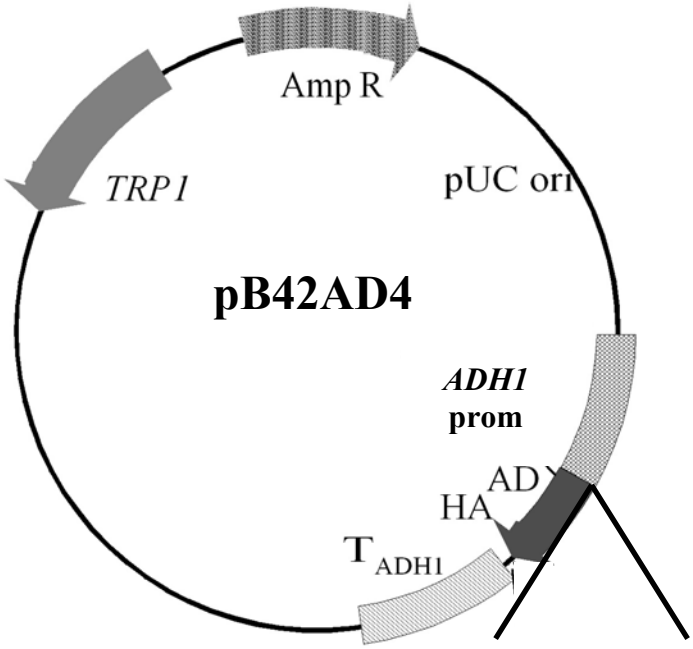


Fig. 1A Digestion of pB42AD2 and pLexA to get pB42AD3. pB42AD2 was digested with *Hin*dIII and *Aat*II to generate a 3860 bp fragment containing the B42 activation domain, the *ADH1* terminator, and *TRP1* marker. pLexA was digested with the same restriction enzymes to generate a 3681 bp fragment containing the *ADH1* promoter, pBR322 origin of replication, and ampicillin resistance marker. The resulting fragments were ligated together to generate pB42AD3.



GAA TTA ATT CCG *GGC* GGA

Fig. 2A Schematic of pB42AD4. The 18 extra nucleotides added before the B42 activation domain in order to match the 5' leader of pLexA exactly are shown.