Plant Signaling & Behavior 5:6, 705-708; June 2010; © 2010 Landes Bioscience

Symptom induction and RNA silencing suppression by the cucumber mosaic virus 2b protein

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Key words: salicylic acid, RNA silencing suppressor, plant virus, disease resistance, counter-defense, argonaute

Abbreviations: AGO, argonaute; CMV, cucumber mosaic virus; NLS, nuclear localization sequence; miRNA, microRNA; sRNA, short RNA; siRNA, short interfering RNA

Submitted: 02/21/10

Accepted: 02/21/10

Previously published online: www.landesbioscience.com/journals/psb/article/11643

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Addendum to: Lewsey M, Surette M, Robertson FC, Ziebell H, Choi SH, Ryu KH, et al. The role of the *Cucumber mosaic virus* 2b protein in viral movement and symptom induction. Mol Plant-Microbe Interact 2009; 22:642–54; PMID: 19445589; DOI: 10.1094/MPMI-22-6-0642.

The 2b protein encoded by *Cucumber mosaic virus* (CMV) and other cucumoviruses is multifunctional, having roles in local and systemic virus movement, symptom determination, evasion of defense mediated by salicylic acid, and in suppression of antiviral RNA silencing. It also perturbs silencing-mediated regulation of host transcripts, suggesting that another function of 2b protein is to manipulate host gene expression and physiology in a way that may aid the virus. The 2b proteins encoded by the various cucumoviruses (CMV strains, as well as *Tomato aspermy virus* and *Peanut stunt virus*) share conserved amino acid sequence domains, suggesting that these might determine specific functions of the protein. We analyzed the effect of mutations in these domains on functions of the 2b protein during viral infection. This revealed that binding of short RNAs, the key determinants of RNA silencing specificity, correlates with RNA silencing suppression activity. Two putative phosphorylation sites were found to be required for virus symptom induction, despite having no influence on RNA silencing suppression. This indicates that the ability to suppress silencing is not the only factor affecting symptom induction by the 2b protein. In accordance with this, our studies also revealed that the 2b protein acts synergistically with some other CMV product(s) to induce symptoms, and that the role of the 2b protein in symptom determination is host species specific.

The 2b Counter-Defense Protein of Cucumber Mosaic Virus

Cucumber mosaic virus (CMV) can infect more than 1,200 plant species, which is the widest host range of any known plant virus.[1] CMV is the type-species of the Cucumovirus genus, which also includes *Tomato aspermy virus* and *Peanut stunt virus*.[1] The hosts of CMV include agronomically important crops, such as tobacco, tomato, banana and melon, and outbreaks of the disease can cause substantial economic losses.[1] Each cucumovirus encodes five proteins on various components of a tripartite positive-sense RNA genome (**Fig. 1A**): the 1a and 2a replication proteins, a coat protein, the movement protein, and the multifunctional 2b protein.[1] The 2b protein is a determinant of virus symptoms and movement, and consequently plays an important part in the virulence of CMV.[2,3] For example, by transferring the *2b* gene of a severe cucumovirus into a mild one, the symptoms induced by the mild strain were significantly enhanced.[3] The 2b protein is also a suppressor of two key plant antiviral defense systems: salicylic acid-mediated resistance,[4] and RNA silencing.[5]

Antiviral RNA silencing involves the degradation of invading viral RNA, or inhibition of its translation into protein, in a sequence-specific manner.[6] Sequence-specificity is conferred by virus-derived short (s)RNAs that direct the endonucleolytic activity of ARGONAUTE (AGO) proteins against complementary

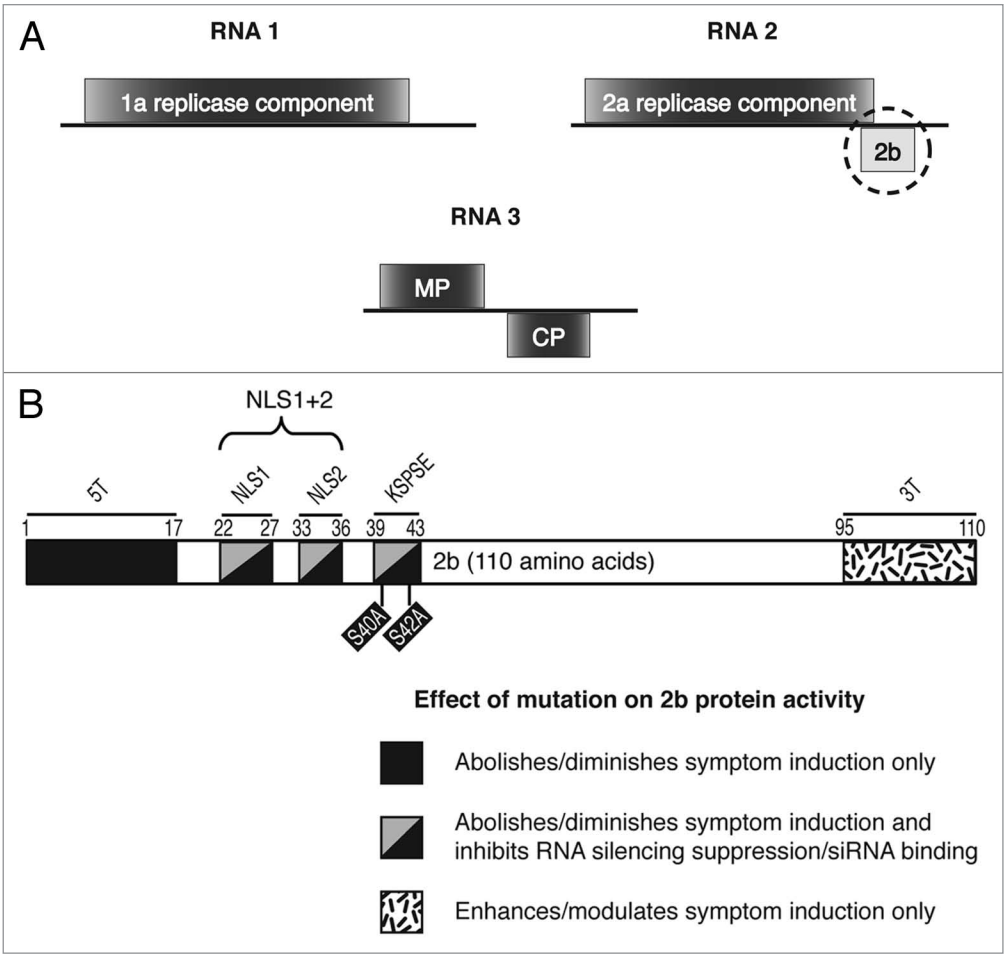

Figure 1. Targeted mutations in the CMV genome investigated in these studies. (A) A diagram illustrating the tripartite CMV genome. RNA 1 encodes the 1a component of the viral replicase. RNA 3 encodes the viral movement protein (MP) and the coat protein (CP). RNA 2 encodes the 2a component of the viral replicase and the 2b protein (ringed). The MP and 2b proteins are translated from sub-genomic mRNAs derived from RNAs 3 and 2, respectively, but which are not shown in this diagram. (B) The conserved amino acid sequences from the 2b protein targeted for mutation. The effects of these mutations on 2b protein activity are indicated by their shading patterns (see key). The N-terminal region of the protein (5T), nuclear localization signals 1 and/or 2 (NLS1, 2, 1 + 2), a putative phosphorylation motif (KSPSE), and the C-terminal region of the protein, were mutated by deletion. The putatively phosphorylated serine residues 40 and 42 were mutated by substitution with alanine (S40A and S42A). Note that mutations S40A and S42A (black boxes, white writing) diminished symptom induction but did not affect siRNA binding/silencing suppression. Note also that the siRNA binding properties of the 5T and 3T mutants were not examined in the studies described here, and their roles in siRNA binding remain to be investigated.

sequences within the viral RNA.[7,8] RNA silencing also plays an important role in regulating accumulation of host mRNAs, including several that encode factors controlling plant growth and development.[9,10] The RNA silencing pathways directed against viral RNAs and host transcripts have many shared or closely related components.[6] Consequently, the symptom inducing properties of the CMV 2b protein may be attributable to its ability to interfere with the activity of components shared between the silencing pathways involved in virus resistance and the regulation of gene expression.[11] However, whereas the ability of 2b proteins to disrupt the regulation of host mRNA accumulation by a class of sRNAs known as microRNAs varies between CMV strains and correlates with symptom induction, the degree to which 2b proteins inhibit silencing directed by the class of sRNAs known as short-interfering (si) RNAs (which includes antiviral silencing) does not.[11,12] This suggests that symptoms may not after all be an incidental effect of suppressing antiviral silencing and conceivably interference with microRNA-mediated silencing may provide some unknown fitness advantage to the virus. The 2b protein can bind double-stranded sRNAs and interact directly with the AGO 1 and 4 proteins.[7,13-15] The 2b protein's ability to suppress silencing has been attributed to both of these biochemical properties, but only recently was their relative importance in silencing suppression activity analyzed.[15]

The 2b proteins of different CMV strains and other cucumoviruses share a number of conserved amino acid sequence domains, suggesting they are required for important protein functions. Known or suspected functional domains include nuclear localization sequences (NLS),[16,17] an RNA binding domain (overlapping the NLSs),[13,14] putative phosphorylation sites,[17] as well as the N and C termini (involved in DNA binding and,

potentially, transcriptional activation[18,19]). These domains are depicted in the map of the 2b protein shown in **Figure 1B**. There is also a conserved domain required to inhibit transcription of the *Nicotiana benthamiana AGO4* mRNA, a component of the epigenetic RNA silencing machinery, in subgroup 1 (A and B) but not subgroup 2 CMV strains.[20] Here, we draw together the results of two recent studies in which we examined the effects of mutations in several of these conserved domains to investigate their functions in CMV biology.[15,21]

Functional Domains of the 2b Protein

Mutant versions of the 2b protein of the CMV strain Fny in which conserved amino acid domains were deleted or modified by replacement of amino acids were made by in vitro mutagenesis. Variants of these were expressed in a variety of contexts depending upon the application required: from a modified CMV RNA2 during virus infection of plants, in *Escherichia coli* as hexahistidine-tagged protein for in vitro protein-RNA interaction assays and, from a T-DNA for transient *Agrobacterium tumefaciens*-mediated expression (agroinfiltration) of wild-type and mutant 2b proteins in leaf tissue of *Nicotiana benthamiana*. Agroinfiltration was used for the in vivo expression of 2b variants to assess their silencing suppressor activity when co-infiltrated with a construct expressing a green fluorescent protein gene. It was also used to express fusion proteins of 2b with fluorescent protein sequences in vivo, permitting microscopic examination of the tagged proteins' subcellular localization, as well as bimolecular fluorescence complementation analyses of 2b protein self-interaction and interactions with correspondingly tagged variants of AGO1 and AGO4.[15,21] Results from these studies are amalgamated in **Figure 1B**.

We found that the N-terminal domain of the 2b protein and two NLSs were required for symptom induction, whilst serine residues 40 and 42 modulated symptom severity.[21] The C-terminal domain was revealed as a possible negative regulator of symptom severity.[21] We also found that 2b protein accumulation does not determine symptom severity, since mutation of NLS1 created a mild symptom inducing virus with a decreased titer but which expressed levels of the mutant 2b protein comparable to those seen in a wild-type CMV infection. The subsequent study[15] showed that the 2b protein was present in the nucleolus. Additionally, it demonstrated that all mutants tested, except those with serine to alanine substitutions at residues 40 and 42, had reduced siRNA binding activity, altered subcellular localization, and had lost the ability to suppress local RNA silencing.[15] In contrast, all these mutant 2b proteins retained their ability to bind to plant-encoded AGO proteins. Importantly, the proteins mutated at serine residue 40 or 42 retained their ability to suppress local RNA silencing, as well as their ability to bind siRNAs. Thus, the ability of the 2b protein to bind siRNAs is essential for its RNA silencing suppressor activity, whereas AGO binding alone is not sufficient to suppress RNA silencing.[15] Furthermore, replacement of serine residues 40 or 42 yielded mutant proteins with unaltered AGO and siRNA binding capacity but that elicited somewhat attenuated symptoms when expressed during viral infection (**Fig. 1**).[15,21] Therefore, in line with earlier suggestions,[11] RNA silencing suppression is not the only function of the 2b protein that determines virus symptom induction.

Another conclusion we may draw is that the formation of homodimers by 2b protein does not determine virus symptom induction or accumulation, since this ability remained unchanged in all 2b mutants.[15] It should be noted that this does not mean homodimerization is dispensable for RNA silencing suppression; it has been demonstrated that 2b protein homodimers are the functional unit that binds siRNAs.[14] This indicates that homodimerization is necessary, but not sufficient, for siRNA binding.

2b Protein is Not the Only Symptom Determinant Encoded by CMV

We have also obtained evidence that the 2b protein is a host-specific symptom inducer and is likely not the only symptom determinant encoded by CMV.[21] We found that a mutant of CMV (strain Fny) that did not express the 2b protein (CMVΔ2b) did not induce symptoms during infection of *Arabidopsis thaliana* ecotype Col-0, but did induce severe symptoms in ecotype C24.[21] These data indicate that the 2b protein has a species-specific role in symptom induction. Additionally, we found that in transgenic *A. thaliana* (ecotype Col-0) expressing constitutively the 2b protein from a mild strain of CMV (LS), infection with CMVΔ2b (derived from the severe strain Fny) resulted in clear viral symptoms. This demonstrates that the 2b protein acts synergistically with some other CMV component in order to promote viral symptom induction, which is in accordance with previous reports implicating all five CMV genes in symptom determination.[1] We conclude that symptom induction by CMV is a complex, multi-layered process, in which the 2b protein may act directly to induce symptoms and may also act synergistically with some other CMV product(s).

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