RESEARCH ARTICLE

A putative antiviral role of plant cytidine deaminases [version 1; peer review: 2 approved]

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Abstract

Background: A mechanism of innate antiviral immunity operating against viruses infecting mammalian cells has been described during the last decade. Host cytidine deaminases (e.g., APOBEC3 proteins) edit viral genomes, giving rise to hypermutated nonfunctional viruses; consequently, viral fitness is reduced through lethal mutagenesis. By contrast, sub-lethal hypermutagenesis may contribute to virus evolvability by increasing population diversity. To prevent genome editing, some viruses have evolved proteins that mediate APOBEC3 degradation. The model plant Arabidopsis thaliana genome encodes nine cytidine deaminases (AtCDAs), raising the question of whether deamination is an antiviral mechanism in plants as well.

Methods: Here we tested the effects of expression of AtCDAs on the pararetrovirus Cauliflower mosaic virus (CaMV). Two different experiments were carried out. First, we transiently overexpressed each one of the nine A. thaliana AtCDA genes in Nicotiana bigelovii plants infected with CaMV, and characterized the resulting mutational spectra, comparing them with those generated under normal conditions. Secondly, we created A. thaliana transgenic plants expressing an artificial microRNA designed to knock-out the expression of up to six AtCDA genes. This and control plants were then infected with CaMV. Virus accumulation and mutational spectra where characterized in both types of plants.

Results: We have shown that the A. thaliana AtCDA1 gene product exerts a mutagenic activity, significantly increasing the number of G to A mutations in vivo, with a concomitant reduction in the amount of CaMV genomes accumulated. Furthermore, the magnitude of this mutagenic effect on CaMV accumulation is positively correlated with the level of AtCDA1 mRNA expression in the plant.

Conclusions: Our results suggest that deamination of viral genomes may also work as an antiviral mechanism in plants.

Open Peer Review

Reviewer Status ✔ ✔

Invited Reviewers

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Any reports and responses or comments on the article can be found at the end of the article.
Keywords
antiviral innate immunity, Cauliflower mosaic virus, error catastrophe, hypermutagenesis, mutational spectrum, plant-virus interaction, pararetrovirus, virus evolution

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**Introduction**

The human APOBEC (apolipoprotein B mRNA editing catalytic polypeptide-like) family includes enzymes that catalyze the hydrolytic deamination of cytidine to uridine or deoxycytidine to deoxyuridine. This family is composed of eleven known members: APOBEC1, APOBEC2, APOBEC3 (further classified as A3A to A3H), APOBEC4, and AID (activation induced deaminase). APOBEC proteins are associated with several functions involving editing of DNA or RNA (reviewed by Smith et al.). APOBEC1 mediates deamination of cytidine at position 6666 of apolipoprotein B mRNA, resulting in the introduction of a premature stop codon and the production of the short form of the protein. APOBEC2 is essential for muscle tissue development. APOBEC4 has no ascribed function so far. AID deaminates genomic ssDNA of B cells, initiating immunoglobulin somatic hypermutation and class switch processes. Most notably, APOBEC3 enzymes participate in innate immunity against retroviruses and endogenous retroelements. Sheehy et al. demonstrated that A3G also plays a role in immunity against human immunodeficiency virus type 1 (HIV-1). For its antiviral role, A3G is packaged along with viral RNA. Upon infection of target cells and during the reverse transcription process, A3G deaminates the cytosine residues of the nascent first retroviral DNA strand into uracils. The resulting uracil residues serve as templates for the incorporation of adenine, which at the end result in strand-specific C/G to T/A transitions and loss of infectivity through lethal mutagenesis. On the other hand, sub-lethal mutagenic activity of APOBEC3 proteins may end up being an additional source for HIV-1 genetic diversity, hence bolstering its evolvability. APOBEC3 proteins have been shown to inhibit other retroviruses (simian immunodeficiency virus, equine infectious anemia virus, human T-cell leukemia virus, and murine leukemia virus), pararetroviruses (hepatitis B virus and DNA viruses), and herpesviruses (herpes simplex virus, Epstein-Barr virus, HSV-1 and EBV respectively, and human papillomavirus). In the cases of HSV-1 and EBV, the antiviral role of deaminases has not yet been demonstrated. Evidence also exists that A3G significantly interferes with negative-sense RNA viruses lacking a DNA replicative phase. For example, the transcription and protein accumulation of measles virus, mumps virus and respiratory syncytial virus (RSV) was reduced 50–70%, whereas the frequency of C/G to U/A mutations was ~4-fold increased. In contrast, A3G plays no antiviral activity against influenza A virus despite being highly induced in infected cells as part of a general IFN-β response to infection.

Human APOBEC belongs to a superfamily of polynucleotide cytidine and deoxycytidine deaminases distributed throughout the biological world. All members contain a zinc finger domain (CDD), identifiable by the signature (H/C)-x-E-x25-30P-C-x-x-C. Plants are not an exception and, for example, the Arabidopsis thaliana genome encodes nine putative cytidine deaminases (with genes named AtCDAl to AtCDAl9). Whilst the AtCDAl gene is located in chromosome II, the other eight genes are located in chromosome IV. In the case of rice and other monocots, only one CDA has been identified. Interestingly, this CDA expression was highly induced as part of the general stress response of rice against infection of the fungal pathogen Magnaporthe grisea, resulting in an excess of A to G and U to C mutations in defense-related genes. Edited dsRNAs might be retained in the nucleus and degraded, generating miRNAs and siRNAs. Given the relevance of deamination as an antiviral innate response in animals, we sought first to determine whether any of the AtCDAs proteins encoded by plants can participate in deaminating the genome of the pararetrovirus, cauliflower mosaic virus (CaMV; genus Caulimovirus, family Caulimoviridae) and, second, we sought to explore whether this deamination may negatively impact viral infection. We hypothesize that deamination may take place mainly at the reverse transcription step. The CaMV genome is constituted by a single molecule of circular double-stranded DNA of 8 kbp. The DNA of CaMV has three discontinuities, Δ1 in the negative-sense strand (or a strand), and Δ2 and Δ3 in the positive-sense strand (yielding the b and g strands). In short, the replication cycle of CaMV is as follows: in the nucleus of the infected cell, the a strand is transcribed into 35S RNA, with terminal repeats, that migrates to the cytoplasm. Priming of the 35S RNA occurs by the annealing of the 3' end of tRNA* to the primer-binding site (PBS) sequence, leading to the synthesis of the DNA a strand by the virus’ reverse transcriptase. Then, the RNA in the heteroduplex is degraded by the virus’ RNaseH activity, leaving purine-rich regions that act as primers for the synthesis of the positive-sense DNA b and g strands.

Our results show that AtCDAl significantly increases the number of G to A mutations in vivo, and that there is a negative correlation between the amount of AtCDAl mRNA present in the cell and the load reached by CaMV, suggesting that deamination of viral genomes may also constitute a significant antiviral mechanism in plants.

**Methods**

**Transient overexpression of AtCDAs in Nicotiana bigelovii plants infected with CaMV**

AtCDAs cDNAs were cloned under the 35S promoter in a pBIN61 vector. N. bigelovii plants were inoculated with CaMV viroids purified from Brassica rapa plants previously infected with the clone pCaMVW260. Symptomatic leaves were agroinfiltrated with one of the nine AtCDAs and with the empty vector pBIN61, each on one half of the leaf. Samples were collected three days post-agroinfiltration.

**Inducible co-suppression of multiple AtCDAs by RNAi**

The design and cloning of the artificial micro-RNA (amiR) able to simultaneously suppress the expression of AtCDAs 1, 2, 3, 4, 7, and 8 was performed as described in ref. The amiRNA was cloned under the control of Aspergillus nidulans ethanol regulon and used to transform A. thaliana by the floral dip method. By doing so, we obtained the transgenic line amiR1-6-3. One-month-old seedlings of transgenic and wild-type A. thaliana were treated with 2% ethanol (or water for the control groups) three times every four days. Three days after the third treatment, plants were inoculated with the infectious clone pCaMVW260 as described in ref. 41. Infections were established by applying 1.3×10 molecules of pCaMVW260 to each of three leaves per plant. Subsequently, plants were subjected to two additional treatments with 2% ethanol (or water) one and five days post-infection. Finally, samples were taken eight days after inoculation and handled as previously described. For each genotype (transgenic or wild-type) and treatment (ethanol or water) combination, 22 plants were analyzed.
Detection of A/T enriched genomes

CaMV genomic DNA was purified using DNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instructions. For detection of edited genomes 3D-PCR was performed using primers HCa8Fdeg and HCa8Rdeg. PCRs were performed in a Mastercycler® (Eppendorf) at denaturation temperatures 82.1 °C, 82.9 °C, 83.9 °C, and 85.0 °C. PCR products obtained with the lowest denaturation temperature were cloned in pUC19 vector (Fermentas), transformed in Escherichia coli DH5α and sent to GenoScreen (Lille, France) for sequencing.

RT-qPCR analysis of AtCDA1 mRNA and qPCR analysis of CaMV load in transgenic plants

Total RNA was extracted from A. thaliana plants using the RNeasy® Plant Mini Kit (Qiagen), according to manufacturer’s instructions. AtCDA1 specific primers qCDA1-F and qCDA1-R were designed using Primer Express software (Applied Biosystems). RT-qPCR reactions were performed using the One Step SYBR PrimeScript RT-PCR Kit II (Takara). Amplification, data acquisition and analysis were carried out using an Applied Biosystems Prism 7500 sequence detection system. All quantifications were performed using the standard curve method. To quantify AtCDA1 mRNA, a full-ORF runoff transcript was synthetized with T7 RNA polymerase (Roche) using as template a PCR product obtained from cloned AtCDA1 and primers T7-CDA1F and qCDA1-R. CaMV qPCR quantitation was performed as described in ref. 46.

Primers

All primers used are listed in Supplementary Table S3.

Results

Effect of AtCDAs overexpression on CaMV mutational spectrum

To test the mutagenic activity of A. thaliana CDAs, nine N. bigelovii plants were inoculated with CaMV. After systemic infection was established, we performed transient AtCDA overexpression experiments. To do so, the same leaf was agroinfiltrated twice; one half of the leaf was infiltrated with one of the nine AtCDA genes and the other half of the leaf was infiltrated with the empty vector. This test was done for all nine AtCDA genes in different plants. The presence of AtCDA mRNAs was verified by RT-PCR from DNase-treated RNA extracts. DNA was extracted from agroinfiltrated areas for 3D-PCR amplification of a 229 bp fragment in the ORF VII of CaMV. 3D-PCR uses a gradient of low denaturation temperatures during PCR to identify the lowest one, which potentially allows differential amplification of A/T rich hypermutated genomes47. There were no differences in the lowest denaturation temperature that could result in differential amplification of controls and the AtCDA-agroinfiltrated samples, suggesting that hypermutated genomes should be at low frequency, if present at all.

PCR products obtained at the lowest denaturation temperature were cloned and sequenced. In a preliminary experiment, we sequenced 25 clones from each AtCDA/negative control pair (Supplementary Table S1). At least one G to A transition was detected in clones from areas infiltrated with AtCDA1, AtCDA2 and AtCDA9 genes. For these three genes, we further increased the number of sequenced clones up to 106. The CaMV mutant spectra was significantly different between plants overexpressing AtCDA1 and their respective negative controls (Figure 1a: \( \chi^2 = 25.760, 7 \text{ d.f.}, P = 0.001 \)). This difference was entirely driven by the 471.43% increase in G to A transitions observed in the plants overexpressing AtCDA1. A thorough inspection of alignments showed that most of the G to A mutations (65.6%) detected in the different samples were located at the nucleotide position 181 (Supplementary Table S1). By contrast, no overall difference existed between the mutant spectra of CaMV populations replicating in plants overexpressing AtCDA2 (Figure 1b: \( \chi^2 = 8.944, 6 \text{ d.f.}, P = 0.177 \)) or AtCDA9 (Figure 1c: \( \chi^2 = 6.539, 8 \text{ d.f.}, P = 0.587 \)) and their respective controls. Consistently, the mutant spectra from the three AtCDA-overexpressed

![Figure 1](image_url)

**Figure 1.** Number of mutations in CaMV genomes isolated from plant tissues agroinfiltrated with different AtCDAs. (a) AtCDA1, (b) AtCDA2 and (c) AtCDA9. The pBIN61 empty vector was agroinfiltrated in the same leaves than their corresponding AtCDAs (mock). For each sample 20,034 nucleotides were sequenced.
samples were significantly heterogeneous ($\chi^2 = 41.063$, 16 d.f., $P = 0.001$), again due to the enrichment in G to A transitions observed in the case of AtCDA1. By contrast, the three independent control inoculation experiments showed homogeneous mutant spectra for CaMV ($\chi^2 = 14.605$, 18 d.f., $P = 0.689$), undistinguishable from the mutant spectra previously reported for natural isolates of this virus. The consistency of the mutant spectra observed for the three control experiments and for a natural isolate of the virus suggests that in absence of a perturbation such as the overexpression of AtCDA1, the CaMV mutant spectrum is rather stable.

We conclude that overexpressing the AtCDA1 gene results in a significant shift in CaMV genome composition towards G to A mutations, as expected from cytidine deaminase hypermutagenic activity.

Effect of suppressing AtCDA expression on the viral load and mutational spectrum of CaMV

To test the effects of suppressing the expression of AtCDA on viral accumulation we produced a transgenic line of A. thaliana Col-0, named amiR1-6-3. This line was stably transformed with an amiR, controlled by the A. nidulans ethanol regulon to achieve ethanol-triggered RNAi-mediated simultaneous suppression of AtCDAs 1, 2, 3, 4, 7, and 8 expression. Transgenic and wild-type plants were subjected to periodical treatment with 2% ethanol (or water for the control groups). Subsequently, plants were inoculated with the infectious clone pCaMVW260 that expresses the genome of CaMV. Samples were taken eight days after inoculation and AtCDA1 mRNA and CaMV viral load were quantified by real time RT-qPCR and qPCR, respectively, in the same samples. For each genotype and/or treatment, 22 plants were analyzed.

The expression of AtCDA1 mRNA depended on the plant genotype (Figure 2a; GLM: $\chi^2 = 28.085$, 1 d.f., $P < 0.001$) as well as on the interaction of plant genotype and treatment ($\chi^2 = 26.037$, 1 d.f., $P < 0.001$), suggesting a differential accumulation of AtCDA1 mRNA on each plant genotype depending on the amiR1-6-3 induction state. Ethanol treatment reduced the amount of AtCDA1 mRNA by 24.01% in transgenic plants, proving that triggering the expression of the amiR1-6-3 significantly and efficiently silences the expression of AtCDA1. Unexpectedly, the effect was the opposite in wild-type plants, for which we observed 23.76% increase in AtCDA1 mRNA accumulation (Figure 2a) upon treatment with ethanol. This increase in expression of AtCDA1 in wild-type plants after ethanol treatment and the underlying mechanisms certainly deserve to be investigated further. However, for the purpose of this study, its relevance is that it may increase the number of G to A mutations in the CaMV genome, thus making the antiviral effect stronger to some extent.

More interestingly, the relative accumulation of CaMV in ethanol-treated plants was significantly different, depending on the plant genotype being infected (Figure 2b; Mann-Whitney U test, $P = 0.002$): silencing the AtCDA1 gene bolstered CaMV accumulation to 103.10% compared to the accumulation observed in wild-type plants. Furthermore, there was a significant negative correlation between the number of molecules of AtCDA1 mRNA and viral load (partial correlation coefficient controlling for treatment: $r = −0.299$, 86 d.f., $P = 0.005$).

Given the significant increase of viral load in plants with lower levels of AtCDA1 mRNA, we sought the molecular signature of deamination in transgenic plants. For this, we selected three biological replicates from each treatment group (ethanol or control) and sequenced between 39–45 clones of the CaMV fragment from each replicate. As shown in Figure 3, silencing of the AtCDA1 gene affects the composition of CaMV mutant spectrum by reducing...
the number of G to A transitions by 69.23%. Nevertheless, overall, both mutational spectra were not significantly different (Figure 3: \( \chi^2 = 9.108, 6 \) d.f., \( P = 0.168 \)), prompting caution against making a definite conclusion on the role of deamination in the observed increase in CaMV accumulation.

We conclude that suppressing the expression of the AtCDAs 1, 2, 3, 4, 7, and 8 significantly reduces the accumulation of CaMV. However, the characterization of the mutant spectrum of the same CaMV populations provides no strong enough support to the cytidine deamination hypothesis.

**Discussion**

Lethal mutagenesis through deamination of RNA/DNA by cytidine deaminases has been proven to work as an antiviral mechanism against retroviruses\(^{16-19,23-27}\), and some DNA\(^{28-31}\) and RNA\(^{32}\) viruses infecting mammals. Our results show that the *A. thaliana* CDA1 gene has some degree of mutagenic activity on the pararetrovirus CaMV genome. Moreover, simultaneously suppressing the expression of a subset of AtCDAs, including AtCDA1, increased CaMV load, strongly suggesting an antiviral role for AtCDAs.

Our data show that AtCDAs probably restrict CaMV replication through a process similar to the restriction of HIV-1 by APOBEC3. CaMV replicates in the cytoplasm by reverse transcription using the positive-sense 35S RNA as template. As for HIV-1, the first strand negative-sense cDNA could be deaminated during reverse transcription, transforming deoxycytidine into deoxyuridine. Then, when the positive-sense strand is produced, an A is incorporated instead of a G, increasing the proportion of G to A mutations. In the case of HIV-1, this G to A mutational bias is explained by A3G and A3H specificity for single negative stranded DNA: during HIV-1 replication, C to G transitions are rare and restricted to the PBS site and U3 regions in the 5’ long terminal repeat, where positive-stranded DNA is predicted to become transiently single stranded\(^{49}\). Similarly, during CaMV replication the negative strand remains single stranded, while the positive is copied from it and remains double stranded\(^{50}\). Surprisingly, for AtCDA1, C to T mutations were also increased; the region studied here is close to the 5’ end of CaMV, which contains the PBS for negative-strand synthesis and the ssDNA discontinuity Δ1. The observed C to T transitions could reflect transient positive-stranded ssDNA in the 5’ terminal region during reverse transcription, nevertheless a different substrate specificity of *A. thaliana* CDAs cannot be ruled out.

Most of the G to A transitions detected in agroinfiltration experiments were located in the G at position 181. HIV-1 hypermutated genomes show mutational hot spots as well, which are due to preference of A3G and A3F for deamination of the third C in 5’-CCC (negative-strand) and 5’-GGC, respectively\(^{51,52}\). The context of the C complementary to G181 (5’-G) differs from what has been described for APOBEC3, suggesting that if AtCDAs had context preference, it would be different from the one described for A3G. However, given the low number of mutations found, we should be cautious when concluding whether AtCDAs have a possible sequence-context preference. Since our experiments were

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Number of mutations found pooling the CaMV sequences from ethanol-treated and control amiR1-6-3 plants (3 replicates). The number of nucleotides sequenced was 23,436 for control and 24,003 for ethanol-treated plants. Ethanol-treated plants turn on the expression of amiR1-6-3 that was designed to silence the expression of the AtCDA1 gene.
performed in vivo, negative selection is expected to purge genomes carrying deleterious mutations. This limitation could account for our failure to detect largely hypermutated genomes, and demonstrates the need for developing new selection-free assays to further characterize AtCDA-induced mutagenesis.

Although there is not a demonstrated correlation between the expression of APOBEC3 and mutational bias of viruses infecting mammals, caulimoviruses have an excess of G to A transitions in synonymous positions. In *A. thaliana* plants, we found that silencing of *AtCDA1* reduced the frequency of G to A transitions in the CaMV genome, suggesting a contribution of AtCDAs to the nucleotide bias found in caulimoviruses. The increased viral load in CDA-silenced *A. thaliana* plants strongly suggests that deamination of viral genomes may work as an antiviral mechanism in plants, leading to questions about how general this mechanism might be, and how it may contribute to viral evolution. Describing a new natural antiviral mechanism in plants opens new research avenues for the development of new durable control strategies.

**Data availability**

All datasets that support the findings in this study are available at LabArchives with DOI: 10.6070/H4TD9VD5.

‘File Sequence_data_for_Figure_1.zip’ contains the FASTA files with the sequence data used to generate the mutational spectra shown in Figure 1.

‘Data_for_Figure_2a.xlsx’ contains the *AtCDA1* expression data used to generate Figure 2a.

‘Data_for_Figure_2b.xlsx’ contains the CaMV accumulation data used to generate Figure 2b.

‘Sequence_data_for_Figure_3.zip’ contains the FASTA files with sequence data used to generate the mutational spectra shown in Figure 3.

**Author contributions**

SFE conceived the study, designed the experiments and analyzed the data. SM, JMC and AG-P performed the experiments and contributed to experimental design. SM, JMC and SFE wrote the paper. All authors revised and approved the manuscript.

**Competing interests**

No competing interests were disclosed.

**Grant information**

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**Supplementary material**

**Supplementary Table S1.** Nucleotide substitutions detected in the overexpression experiments. For each of the nine infiltrated plants, the substitutions observed in the clonal sequences analyzed at the overexpressed (*AtCDA1* to *AtCDA9*) and control (pBIN61-infiltrated) regions are shown. In some cases, a given substitution is present in several clonal sequences from the same sample and the number of times it appears is indicated between parentheses. Nucleotide positions are given according to CaMV isolate W260, GenBank accession JF809616.1.

Click here to access the data.

**Supplementary Table S2.** Nucleotide substitutions found in *A. thaliana* transgenic plants with or without inducing the expression of amiR3-1-9 that silences the expression of several AtCDAs. In some cases, a given substitution is present in several clonal sequences from the same sample and the number of times it appears is indicated between brackets. Nucleotide positions are given according to CaMV isolate W260, GenBank accession JF809616.1.

Click here to access the data.

**Supplementary Table S3.** Primers used in this study.

Click here to access the data.
References


Israel Pagán
Center for Biotechnology and Plant Genomics (CBGP); E.T.S.I. Agrónomos (Higher Technical School of
Agricultural Engineering), Technical University of Madrid, Madrid, Spain

Martin et al. present an interesting work on the role of plant cytidine deaminases (CDA) as a defense
mechanism against virus infection through the increase of mutational load in the viral genome during
replication. CDAs are known to increase the frequency of G to A transitions. Although such mutational
load has been shown to be a effective defense mechanism against some animal viruses (mainly
retroviruses), this paper shows for the first time evidence that support a similar role in plants against a
plant pararetrovirus. As such, I consider the paper scientifically sound.

I find the paper well written and easy to read, and I would like to acknowledge the effort made by the
authors on this aspect. The methodology is well described and all the information necessary to
understand the experiments is provided. On this sense, I would just suggest adding complementary
information on the number of leaves from the N. bigelovii
At
to understand the degree of biological variation considered in the study.

The main conclusion of the manuscript is that overexpression of AtCDA leads to a decrease of viral load. I
think that this conclusion is robustly supported by the data presented in the result section, and statistics
are flawlessly performed and described as is the rule in the work from Prof. Elena’s group. A second main
conclusion of this work is that higher viral load may be associated with the trend towards reduced
frequency of G to A transitions in plants with silenced AtCDA. The authors are careful on drawing
conclusions from this observation, given that the observed trend is not statistically significant. I was
wondering whether the effect of the bias in G to A transitions might not be quantitative but rather
qualitative. In other words, it might be interesting some discussion about the existence of a threshold in
the frequency of G to A transitions bias that may lead to the reduction in viral load.

My last suggestion relates to the observation that mutations at position 181 accounts for most of the G to
A transitions. This makes me wonder about the spatial distribution of mutations (especially G to A
transitions) across the viral genome. I think that including some information on whether mutations are
mainly localized in coding or non-coding regions, and on whether mutations located in coding regions
results mainly in synonymous and non-synonymous changes may be a nice addition. Perhaps this information may help to understand the effects of G to A transitions in the genome “functionality”.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  
Yes

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Yes

Are all the source data underlying the results available to ensure full reproducibility?  
Yes

Are the conclusions drawn adequately supported by the results?  
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Plant virus evolution

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Author Response (Member of the F1000 Faculty) 09 Jun 2017**

**Santiago F Elena,** Evolutionary Systems Virology Group, Instituto de Biología Integrativa de Sistemas (CSIC-UV), Spain

Dear Dr. Pagán,

Thank you very much for your time in reviewing the manuscript and also for your very constructive comments. Below we provide detailed responses to each one of them.

1. We now mention the number of half-leaves per plant (3) that were agroinfiltrated with each one of the nine *AtCDAs.*

2. We have added a brief text to the Discussion on the possibility of whether low a threshold number of G to A transitions needs to be reached in order to have a significant effect on CaMV accumulation.

3. Please, recall that we have sequenced only a region within ORF VII, thus all mutations observed are in a coding sequence. Nonetheless, we have added extra text to the Discussion commenting on the synonymous/nonsynonymous nature of all the observed mutations, in particular for the G to A transitions most relevant for our study. Furthermore, Supplementary Table S1 now indicates the nonsynonymous substitutions for the case of *At CDA1* in the agroinfiltration experiments.
Pedro Gomez

Department of Stress Biology and Plant Pathology, CEBAS - CSIC (Centre for Applied Soil Science and Biology of the Segura) - (Spanish National Research Council), Murcia, Spain

Comments of the manuscript entitled "A putative antiviral role of plant cytidine deaminases".

This manuscript reports how plant cytidine deaminases, particularly AtCDA1, might contribute to the deamination of Cauliflower mosaic virus (CaMV) genome, and hence, affect its viral accumulation in plants. The work has merit and seems to be a good contribution. Whereas this potential antiviral response has been assessed in human and animals, virtually nothing is known about this mutagenic activity in plants.

The experimental methods are overall solid, and the manuscript is very well-written, clear and easy to follow.

The authors first examined which AtCDA proteins encoded by Arabidopsis thaliana have an effect on the CaMV mutational spectrum by performing an AtCDA overexpression in Nicotiana bigelovii. While results are consistent with the expected AtCDA mutagenic activity, I would suggest to them to describe the reasoning behind performing it in N. bigelovii plants in order to clarify whether there would be any potential host AtCDAs background-noise effect that could affect or not the transient genes activity results. It may not matter, but I have not found the answers to this question within the text. If that is so, I am guessing that results from the mutant analysis spectra could even improve by providing strong results from the AtCDA1 analysis or even some differences to the other AtCDA genes could be found, as consequence of buffering those effects from negative control samples.

Secondly, they sought to evaluate the effect of suppressing AtCDAs in transgenic A. thaliana plants on the accumulation and mutant spectrum of CaMV. Here, I am a bit concerned whether the general claim that the authors are making with this study is properly warranted. Considering that all results of this section are only based on the AtCDA1, this seems to overstate the final conclusion and perhaps this can be slightly tempered. I would recommend either to moderate this conclusion (and title) to only the atCDA1 results or to show evidence of the CaMV load reduction when suppressing the expression of the other AtCDAs. This should then be accompanied by the full description of primers and expression patterns of AtCDA2-8 mRNA analysis in the methods section, in addition to inclusion of statistics data of the mutant spectrum in the results section. This could also increase the appeal of the manuscript.

In this sense, thinking about the general-nature of the findings, it would be very interesting and nice to read any thoughts/perspectives (in the discussion section) about this cytidine deaminase mutagenic
activity in some other plant viruses (i.e. RNA virus), which could be infecting through different replicating strategies.

Specific minor comments:
- Please, double check this % … 471.43% increase in G to A transitions
- Colouring treatments of the Fig 3 is a bit confusing. Please keep that as previous figures.
- Table S1: Please, describe that G to A substitutions detected here are shaded in the table.

Is the work clearly and accurately presented and does it cite the current literature? 
Yes

Is the study design appropriate and is the work technically sound? 
Yes

Are sufficient details of methods and analysis provided to allow replication by others? 
Yes

If applicable, is the statistical analysis and its interpretation appropriate? 
Yes

Are all the source data underlying the results available to ensure full reproducibility? 
Yes

Are the conclusions drawn adequately supported by the results? 
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Viral Evolutionary Ecology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Author Response (Member of the F1000 Faculty) 09 Jun 2017**

**Santiago F Elena,** Evolutionary Systems Virology Group, Instituto de Biología Integrativa de Sistemas (CSIC-UV), Spain

Dear Dr. Gómez,

Thank you very much for your time in reviewing the manuscript and also for your very constructive comments. Below we provide detailed responses to each one of them.

1. We justify the choice of *N. bigelovii* for our agroinfiltration experiments. Basically, it was a practical choice: the clone of CaMV used in this study does not infect *N. tabacum* nor *N. benthamiana* efficiently and we needed a plant with large enough leaves to be agroinfiltrated.

2. It is true that our results only provide suggestion that AtCDA1 may be involved in C deamination of CaMV genome. We have edited the text to avoid making any unsubstantiated claim. We have also added a paragraph in the Discussion putting our results in the context of recent findings that suggest that only AtCDA1 may be relevant for
the homeostasis of pyrimidines while the other eight members of the gene family may be pseudogenes.

3. We did not quantified the expression levels of AtCDAs 2 – 9, since we decided to focus our attention in AtCDA1 after observing that the expected bias in mutation spectrum was only found in this case.

4. We have added a new paragraph to the Discussion on the potential antiviral role of plant CDAs for other viruses. Unfortunately, possible evidences are only limited to one Potyvirus.

5. The three specific minor comments have been considered: the percentage was correct, coloring in Fig. 3 is right and the legend of Supplementary Tables S1 and S2 have been modified to indicate that G to A transitions are shaded in grey.

**Competing Interests:** No competing interests were disclosed.