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پژوهش‌های کاربردی در گیاه‌پزشکی ۱۳(۲): ۱۶۴-۱۵۹ (۱۴۰۳)- مقاله پژوهشی

Journal of Applied Research in Plant Protection 13(2): 159-164 (2024)-Research Article

DOI: https://dx.doi.org/10.22034/arpp.2024.17488

Peanut stunt virus symptom attenuation is associated with an unusual mutation in the virus genome

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Received: 28 October 2023 Revised: 9 December 2023 Accepted: 9 December 2023

Abstract

Plant viruses may behave differently in glasshouse than in nature, especially those that are naturally transmitted by vector. Biological assays, as the oldest and still used virus detection methods, involve inoculation of host plant and monitoring symptoms in glass- or green-house. While maintaining *Peanut stunt virus* (PSV) in glasshouse we came across with symptom attenuation of PSV strain B (PSV-B) in *Nicotiana benthamiana* after eight successive passages. To determine if any changes have occurred in the PSV-B genome, the virus particles were purified from the plant and subjected to RNA extraction with phenol/ chloroform to remove the virus coat protein (CP), and followed by precipitation with ethanol to purify the virus RNAs. Then, electrophoresis of the virus RNAs on agarose gel showed that the RNA3 component of the PSV-B isolate was longer than that in the original reference PSV-B, suggesting occurrence of insertion(s) in the RNA3. To confirm this observation, the full length cDNA corresponding to the RNA3 was synthesized by RT-PCR with the use of the 5' and 3' terminal primers which were designed according to sequences of the PSV RNA3 terminal ends (accession PSU31366). The RT-PCR product was subjected to dideoxy cycle sequencing and the sequence data was compared with the corresponding PSV RNA3 sequences. This revealed occurrence of a 183- nucleotide repeat at the 3' untranslated region of the RNA3 and suggested an association between this mutation (repeat) and the symptom attenuation. This emphasizes that in glasshouse studies, sequential passages of a virus can lead to emergence of mutants. This observation also has implication as to plant protection because the mutated variant may be used in cross protection.

Keywords: PSV, attenuation, duplication, mutation, *Cucumovirus*, Bromoviridae

تضعیف علایم ناشی از ویروس کوتولگی بادام زمینی مرتبط با جهش غیرمعمول در ژنوم ویروس

نعمت سخندان بشیر✉

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دریافت: ۱۴۰۲/۰۸/۰۶ بازنگری: ۱۴۰۲/۰۹/۱۸ پذیرش: ۱۴۰۲/۰۹/۱۸

چکیده

ویروس‌های گیاهی، بویژه آنهایی که در طبیعت با ناقل منتقل می شوند، در گلخانه ممکن است متفاوت از محیط بیرون عمل کنند. آزمون‌های زیستی به عنوان قدیمی‌ترین روش‌های ردیابی ویروس که هنوز هم مورد استفاده قرار می‌گیرند شامل مایه زنی گیاه میزبان و بررسی بروز علایم در گلخانه یا آزمایشگاه می‌باشد. به هنگام نگهداری ویروس کوتولگی بادام زمینی ((*Peanut stunt virus* (PSV) در گلخانه با علایم تضعیف شدهٔ نژاد ب پس از هشت بار پاساژ در گیاه تنباکو (*Nicotiana benthamiana*) مواجه شدیم. برای تعیین اینکه آیا تغییراتی در ژنوم جدایهٔ ب بوجود آمده است ذرات ویروس از گیاه آلوده تخلیص و آر ان ای از ذرات ویروس با استفاده از تیمار با فنول/ کلروفرم استخراج گردید تا پروتئین پوششی ویروس حذف شود و به‌دنبال آن ترسیب با اتانول انجام گرفت تا آر ان ای ویروس مورد تخلیص قرار گیرد. آنگاه الکتروفورز آر ان ای های ویروسی روی ژل آگاروز نشان داد که قطعهٔ آر ان ای ۳ (RNA3) نژاد PSV-B بزرگ‌تر از قطعهٔ مشابه در جدایهٔ مرجع این نژاد می‌باشد که حکایت از بوجود آمدن نوکلئوتیدهای مضاعف درج شده در قطعه مورد نظر را داشت. برای تأیید این مشاهدات، cDNA منطبق بر RNA3 از طریق RT-PCR با آغازگرهای انتهای '۵ و '۳ ساخته شد که بر اساس ترادف‌های انتهایی RNA3 (رس شمار PSU31366) طراحی شده بودند. قطعهٔ حاصل از RT-PCR مورد ترادف‌یابی با روش "ترادف یابی چرخهای دی داکسی" قرار دادهشد و داده‌های حاصله مورد مقایسه با ترادف‌های PSV RNA3 قرارگرفتند. این بررسی آشکار نمود که تکراری به اندازهٔ ۱۸۳ نوکلئوتید در انتهای ۳ پریم ترجمه نشوندهٔ RNA3 بوجود آمده‌است که حاکی از رابطهٔ بین این جهش (تکرار) و تضعیف علایم بود. نتایج این تحقیق تأکید بر این دارد که در مطالعات گلخانه‌ای، پاساژهای پی در پی ویروس منجر به بروز جهش یافته‌های ویروسی می‌شود. این مشاهدات همچنین در رابطه با حفاظت گیاه نیز دارای پیآمد می‌باشد برای اینکه واریانت-های جهش یافته و تضعیف شدهٔ ویروس را می‌توان به عنوان نژاد ملایم در حفاظت گیاه به کار برد.

کلمات کلیدی: ویروس کوتولگی بادام زمینی، تضعیف، دوبله شدن، جهش، کوکوموویروس، بروموویریده

How to cite:
Sokhandan Bashir N, 2024. *Peanut stunt virus* symptom attenuation is associated with an unusual mutation in the virus genome. *Journal of Applied Research in Plant Protection* 13 (2): 159–164.

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Introduction

Plant viruses may behave differently in glasshouse than in nature. A biological assay is based on inoculation of host plant with viral inoculum and monitoring symptoms in glass- or green-house. *Peanut stunt virus* (PSV) is a member of the genus *Cucumovirus* in the family *Bromoviridae*. This genus also encompasses *Cucumber mosaic virus* (CMV) (e.g., Rakhshandehroo *et al*. 2021) with the broadest host range amongst plant viruses. It is important on plants especially on legumes as it causes economical crop losses. PSV is transmitted by aphids, seed and in glasshouse by mechanical inoculation of infected sap.

PSV is a single-stranded positive-sense RNA virus. A full description of *Bromoviridae* has been published by Bujarski (2021). Accordingly, PSV possesses three genomic fragments (tripartite) and exists as three particles (triparticulate). RNA1 and RNA2 code for 1a and 2a proteins, respectively, both being components of RNA-dependent RNA polymerase (RdRp). The earlier contains the so-called signatures methyl transferase and helicase domains whereas the latter carries the GDD domain. There is also a small overlapping open reading frame (ORF) on RNA2 coding for 2b protein that acts as a viral suppressor of RNA silencing (VSR) and it is also required for long-distance movement of the virus through phloem. The virus RNA3 is discistronic, i.e. containing two ORFs coding for the virus movement protein (MP) and coat protein (CP), from 5' to 3' direction. There is an intergenic region between these two ORFs which contains a promoter for the synthesis of the CP subgenomic RNA known as sgRNA4. Not to mention that the 2b protein is also expressed via a subgenomic RNA namely sgRNA4A. However, the other proteins including 1a, 2a and MP are translated directly from the virus genomic RNAs. In addition, all the genomic RNAs of PSV possess end-groups, cap and tRNA-like structures at the 5' and 3' ends, respectively, which are believed to contribute to stability of the RNA. The tRNA-like structure (TLS) is believed to act as a primer for synthesis of complementary RNA strand during replication of the virus.

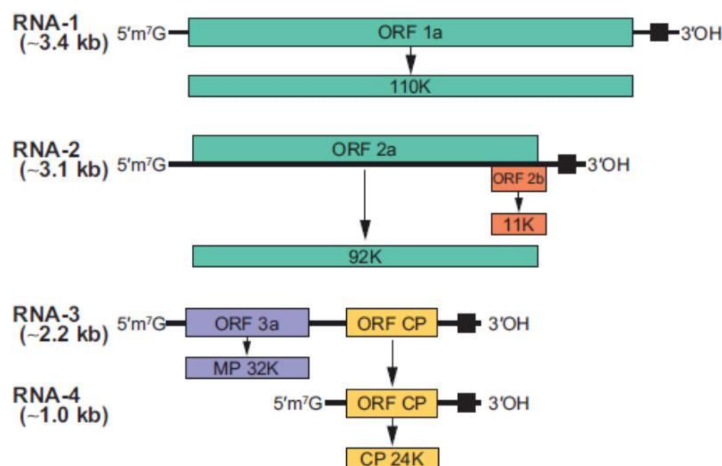

Figure 1. Genome organization of PSV, Courtesy of King *et al*. (2011).

There is evidence showing that under glasshouse conditions viruses can go under greater mutations than the normal mutation rate of virus that occur in nature (e.g., Rodríguez-Cerezo *et al*. 1991). This is especially true when plant viruses that are transmitted by vectors in nature are propagated by infected sap and preserved actively in glasshouse. Also, occurrence of a repeat in the virus genome and attenuation of virus has been reported for *Japanese encephalitis virus* (JEV).

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The aim of this study was to look at RNA3 sequence of an attenuated variant of PSV-B to explore any changes may have occurred in it.

Materials and methods

Plant inoculations and virus purification

PSV-B was maintained in *Nicotiana benthamiana* successively, i.e., when the inoculated plant became older, leaf material from such a plant was subjected to sap extraction and inoculation on a 6-leaf-stage *N. benthamiana* plant. The extraction was prepared in 0.1 M potassium phosphate buffer pH 7.4 and rubbed with a gloved hand on carborundum-dusted leaves. The inoculated leaves were washed with spray water 10 min after inoculation and the plants were kept in glasshouse and the development of symptoms was monitored on an every-other-day basis up until 2 weeks from inoculation.

The attenuated variant was purified according to Ghabrial *et al*. (1977) and the purified preparation was subjected to phenol-chloroform extraction to release viral RNA, and followed by precipitation by ethanol. The released RNAs were run on a low-melting agarose gel and the RNA3 was purified from gel.

cDNA synthesis, cloning and sequence analysis

The cDNA library representing RNA3 from attenuated PSV-B was synthesized using components of the Amersham cDNA synthesis kit. Poly(A) tail was added enzymically to the RNA3 by treatment with poly(A) polymerase (Sippel 1973) and synthesis of first strand cDNA was primed with oligo$(dT)_{18}$. The second strand was synthesized according to Gubler and Hoffman (1983). Double-stranded (ds) cDNA was blunt-ended with T4 DNA polymerase and ligated into *Sma*I-linearized pUC119. Selection of ampicillin-resistant cDNA clones containing inserts was made by blue/ white colony screening on X-Gal/IPTG medium (Green & Sambrook, 2012).

Multiple independent cDNA clones of appropriate size were used for sequencing by dideoxy cycle sequencing method (Sanger 1977). At least two independent clones were sequenced from both orientations for each region of the cDNAs. BioEdit software (Hall 1999) was used to align and compare nucleotide (nt) and deduced amino acid sequence identities (and amino acid sequence similarities) between RNA3s of the attenuated variant of PSV-B and previously reported PSV-B sequences.

RNA secondary structure analysis

The sequences for 3'UTR right after the CP region respective to nucleotides 1888-2161 (accession PSU31366) containing the 183-nucleotide repeat (1888-2069) or lacking the repeat (ordinary 3'UTR sequence) were separately subjected to RNAfold Web Server (Lorenz *et al*. 2011) with the default settings.

Results

The symptoms with so-called wildtype PSV-B included severe mosaic, curling of leaf edges, and stunting whereas the attenuated PSV-B exhibited only mild mosaic.

Electrophoresis of extracted genomic RNAs from particles of attenuated PSV-B revealed that its RNA3 was larger than that of wildtype (non mutated) PSV-B. By cloning and subjecting the RNA3 cDNA to sequencing and sequence analysis it was shown that there was a 183-nt repeat at the 3'UTR (Figure 2). When the 3' UTR region sequence from the attenuated and normal PSV-B were subjected to secondary structure analysis at RNAfold web server it came out that these two variants of PSV-B had quite different structures so that the repeated 3'UTR from the attenuated variant was much more structured compared to the normal strain (Figure 3).

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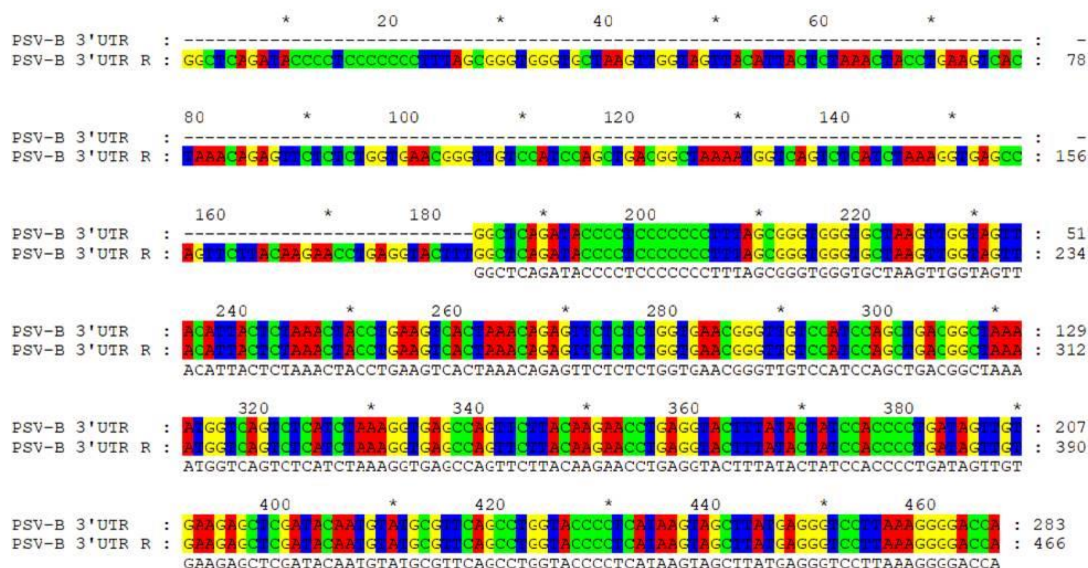

Figure 2. An alignment generated by GeneDoc (Nicholas & Nicholas 1997) representing 3'UTR of PSV-B RNA3 from normal strain (PSV-B 3'UTR) and attenuated variant (PSV-B 3'UTR R). The repeated 183 nt stretch does not exist in the normal strain.

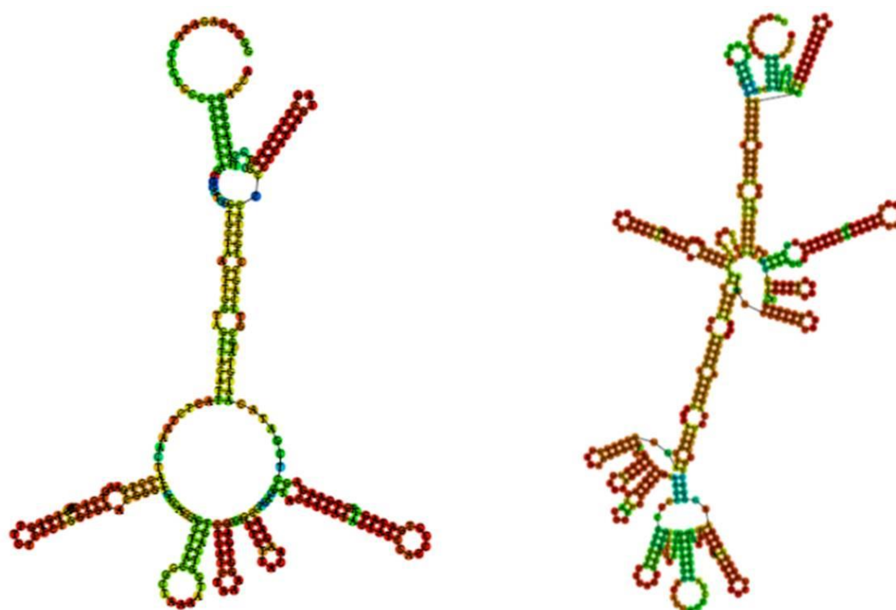

Figure 3. Secondary structures at the 3'UTR of PSV-B RNA, left: normal strain, right: attenuated variant with a 183 nt repeat.

Discussion

In this study a variant of PSV-B with attenuated symptoms on *N. benthamiana* was subjected to sequencing and RNA secondary structure analyses. The attenuated variant exhibited a 3'UTR region crowded with several additional stem loops (Figure 3) compared to that of the ordinary strain of PSV-B. This cucumovirus possesses TLS at the 3'UTR. The significance of TLS for virus genome translation and replication have been reported for *Brome mosaic virus* (BMV), as another member of *Bromoviridae*, and also for *Turnip yellow mosaic virus* (TYMV) of *Tymoviridae* (Bastin & Hall 1976; Dreher 1999

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cited by Rao & Kao 2015). It has been demonstrated that the regulation of BMV RNAs translation by the TLS is possibly done by helping to recruit ribosomes to provide efficient translation and to regulate the timing of translation and replication (Barends *et al*. 2004). Moreover, it has been reported that mutation at the 3' UTR of the potyvirus *Tobacco vein mottle virus* (TVMV) is associated with attenuation of the virus symptom. Putting together all these findings, it can be suggested that occurrence of the 183-nt repeat in PSV-B 3'UTR might interfere with the replication and/ or translation of PSV. Accordingly, the TLS at 3'UTR involves with the virus RNA polymerase to synthesize the opposite RNA strand. But, by the occurrence of further structures at the PSV-B 3'UTR as a result of the repeat, the association of TLS with the enzyme may somehow be compromised so that less virus protein products are produced. In studies with JEV (Zhang *et al*. 2022) and *Tobacco vein mottling virus* (TVMV) (Rodríguez-Cerezo *et al*. 1991) it has come out that virus quantity in the plant infected with the attenuated variant is not significantly reduced in comparison with non-attenuated variants. Whether or not this is the case for the PSV-B remains to be answered by further investigation. However, occurrence of the further structures at the 3' UTR and the symptom attenuation seems to be associated. This study signifies a consequence of repeated passages of plant virus in glasshouse which is manifested as attenuation of symptoms and occurrence of mutation in the virus genome. Such structural changes occurring in the non-translated region further emphasize the importance of the UTRs in virus activity. They do not code for proteins but directly affect protein synthesis of virus through cooperation with virus replicase or even possibly host replication enzyme(s). Therefore, in order to maintain virus in a stable state in terms of its genome integrity, especially viruses that are transmitted by vectors (i.e., aphids) in nature, they should not be subjected to serial mechanical passages in glasshouse.

It is also noteworthy that successive passages have been used to obtain mild strains for cross protection which could be regarded as a beneficial aspect of long term maintenance of virus in host plant. For instance, a mild strain of *Zucchini yellow mosaic virus* (ZYMV) known as strain wk has been obtained for cross protection (Lecoq & Raccah 2001).

Abbreviations

PSV = Peanut stunt virus; RdRp = RNA-dependent RNA polymerase; TLS = t-RNA like structure; JEV = Japanese encephalitis virus; UTR=untranslated region; nt = nucleotide; BMV = *Brome mosaic virus; TYMV = Turnip yellow mosaic virus; TVMV* = Tobacco vein mottle virus; ZYMV = Zucchini yellow mosaic virus

Acknowledgement- Part of this work was performed in late Professor S. A. Ghabrial’s lab at University of Kentucky.

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