DOI: 10.1007/s10535-014-0448-z BIOLOGIA PLANTARUM 58 (4): 783-787, 2014

BRIEF COMMUNICATION

Potato virus X induces DNA damage in leaf nuclei of the host plant *Nicotiana tabacum* L. var. *xanthi*

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Abstract

We employed the comet assay (single cell gel electrophoresis) to evaluate induced DNA damage in nuclei isolated from tobacco leaves (*Nicotiana tabacum* var. *xanthi*) inoculated with *Potato virus X* (PVX). The highest DNA damage, expressed by the tail moment value, was observed in the inoculated leaves and decreased in the 1st to 4th systemic leaves. DNA damage increased with the time after the inoculation (from day 3 to day 21) and was higher in nuclei isolated from a part of the leaf at the petiole compared to nuclei isolated from the leaf tip. A Pearson moment correlation (r = 0.94) between the induced DNA damage and the PVX titres expressed by ELISA absorbance values was observed. The PVX infection did not induce a significant increase in the rate of somatic mutations evaluated by appearance of dark green, yellow, and double green/yellow sectors on the heterozygous pale green leaves of *N. tabacum* var. *xanthi*.

Additional key words: Comet assay, ethyl methanesulphonate, single cell gel electrophoresis, somatic mutations.

Potato virus X (PVX) is a member of the genus *Potexvirus*, family *Flexiviridae*. PVX is frequently used as model to study the molecular mechanisms controlling virus replication and movement (Batten *et al.* 2003, Atabekov *et al.* 2007, Verchot-Lubicz *et al.* 2007). PVX forms filamentous particles of about 515 nm in length and 13 nm in diameter (Sonenberg *et al.* 1978). The particle consists of a 6.4 kb long positive sense single-stranded genomic RNA which is capped and polyadenylated. Approximately 1 300 copies of the PVX coat protein (XCP) encapsidate the viral RNA. XCP is also necessary for cell-to-cell and systemic movement in host plants (Verchot-Lubicz 2005). The viral genomic RNA contains a 84 nucleotide (nt) long 5´-untranslated region (UTR) followed by five open reading frames coding for 165 kDa replicase, triple gene block proteins TGBp1, TGBp2, and TGBp3 involved in virus movement, a 25 kDa coat protein (CP), and 72 nt long 3´-UTR (Huisman *et al.* 1988, Skryabin *et al.* 1988). A successful infection by PVX requires counteracting host defences as well as intimate interactions between the viral genomes/genome-encoded products and host cellular factors including host transcriptional, translational, and macromolecular trafficking machineries.

The objective of this study was to determine whether PVX induces *1*) DNA damage in nuclei, evaluated by the comet assay, and *2*) mutations on leaves, evaluated by the somatic mutation assay, in PVX inoculated tobacco leaves as well as in the 1st to 4th systemic leaves.

The alkaline version of the single cell electrophoresis

Submitted 6 March 2014, last revision 4 June 2014, accepted 11 June 2014.
Abbreviations: EMS - ethyl methanesulphonate; TM - tail moment; PT-ELISA - plate-trapped ELISA; PVX - *Potato virus X*; ROS - reactive oxygen species; TM - tail moment.
Acknowledgements: The authors wish to thank Mrs R. Hadamkova and D. Cibochova, the Institute of Experimental Botany, Prague, the Czech Republic, for their excellent technical assistance. This research was supported by grant No. P501/12/1761 of the Czech Science Foundation, OPPK CZ.2. 16/3.1.00/24014 of the City of Prague, and grant No. 631412 of the Charles University Grant Agency.
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This publication is dedicated to the memory of Mrs. Dagmar Cibochová.

assay (comet assay) can quantitatively measure DNA damage, including single strand breaks, double strand breaks, alkali labile sites (primarily apurinic and apyrimidinic sites), incomplete excision repair sites, and DNA crosslinks (Tice *et al.* 2000). Although this technique has been primarily applied to animal cells, the adaptation of the comet assay to plant tissues (Koppen and Verschaeve 1996, Gichner and Plewa 1998, Gichner *et al.* 2009, Procházková *et al.* 2013) significantly extends its utility in basic and applied studies in environmental mutagenesis.

The comet assay detects acute DNA damage in nuclei of leaf and root cells shortly after the treatment. By contrast, the leaf primordial cells within the apical meristem are the target cells of the somatic mutations (Dulieu and Dalebroux 1975).

Mutations are induced in these primordial cells and as the leaf grows, division of the mutant cells leads to development of individual clones of cells that appear as dark green, yellow, and green/yellow sectors on yellow-green leaves of the heterozygous *Nicotiana tabacum* var. *xanthi*. Thus, the earliest time when somatic mutation frequency can be evaluated on newly formed leaves is about one to two weeks after treatment. The tested line of *N. tabacum* var. *xanthi* has been demonstrated to be very sensitive to ionizing radiation (Ptacek *et al.* 2001), and various kinds of chemical mutagens (Dulieu and Dalebroux 1975, Gichner *et al.* 1999).

Seeds (a_1^+/a_1; a_2^+/a_2) of the double heteozygous tobacco (*Nicotiana tabacco* L. var. *xanthi*) used in the somatic mutation assay were generated by controlled genetic crossings according to Dulieu and Dalebroux (1975). Tobacco plants were cultivated in plastic pots (a diameter of 7 cm and a depth of 9 cm) filled with 150 cm^3 of soil (Chernozem, pH 7.2) at day/night temperatures of 28/22 °C, a relative humidity of 60 %, a 16-h photoperiod, and an irradiance of 150 μmol m^{-2} s^{-1}.

The 4th or 5th leaf, counted from cotyledons, of 5- to 6-week-old tobacco seedlings was inoculated with PVX (strain UK3) obtained from frozen infected leaves. The leaves dusted with carborundum powder were rubbed with a suspension of virus infected tissue in 0.057 M K_2HPO_4, pH 8.0. As negative control, healthy leaves were treated only with 0.057 M K_2HPO_4, pH 8.0. The plants were further cultivated as described above.

The comet assay and the image analysis using a fluorescence microscope and a computerized image-analysis system were performed according to Procházková *et al.* (2013). The frequency of dark green, yellow, and green/yellow twin sectors (Dulieu and Dalebroux 1975) were scored using a stereomicroscope on inoculated and 1st to 4th systemic leaves 3 weeks after the PVX inoculation. The mutation frequency was expressed as the mean number of sectors (dark green, yellow and green/yellow) *per* leaf. For each experiment, eight seedlings were inoculated and each treatment was repeated twice. Plate-trapped (PT) ELISA was done as described previously (Cerovska *et al.* 2008).

Data were analyzed using the statistical and graphical functions of *SigmaPlot 8.0* and *SigmaStat 3.0* (*SPSS Inc*., Chicago, IL, USA). In case a significant ($P < 0.05$) *F*-value was obtained in one-way *ANOVA*, the Dunnett's multiple comparison test between the treated and control groups was conducted. Differences between two groups were statistically evaluated by the paired *t*-test.

In 3 or 4 d intervals, a small piece of leaf tissue (about 1.5 cm^2) was taken near the petiole or tip of the inoculated leaf and of the 1st to 4th systemic leaves. In the control plants, DNA damage, expressed by tail moment (TM) values, varied from 2 to 4 μm. The inoculation of the tobacco leaves with PVX led to serious damage as expressed by crinkled leaves that finally led to leaf lethality. DNA damage detected in the nuclei of the inoculated leaves increased with the time after inoculation. At day 3, a TM value was 6 ± 0.8 μm compared to 75 ± 1.6 μm at day 14. Due to severe leaf damage, leaf nuclei could not be isolated at day 17. In the 1st systemic leaf, there was a similar increase in a value of TM depending on the time after inoculation. This increase was higher when the leaf nuclei were isolated

Table 1. DNA damage expressed by average median tail moment values [μm] in leaf nuclei of *Nicotiana tabacum* var. *xanthi* following inoculation with PVX. Nuclei were isolated from a small part of leaf (about 1.5 cm^2) at the petiole (A) or at the leaf tip (B) at different time periods after inoculation. $^+$ - leaf too small, the whole leaf used for the isolation of nuclei, $^{++}$ - nuclei could not be isolated due to severe leaf damage. Means ± SE, n = 150 - 200, * - values significantly different ($P \leq 0.05$) from the control.

Time after inoculation [d]	Control	Inoculated leaf	1st systemic leaf		2nd systemic leaf		3rd systemic leaf		4th systemic leaf	
		A	A	B	A	B	A	B	A	B
3	2 ± 0.5	6 ± 0.8*	6 ± 0.7*	3 ± 0.4	3 ± 0.5	4 ± 0.3	4 ± 0.5$^+$	---	---	---
7	4 ± 0.4	19 ± 1.7*	10 ± 1.1*	5 ± 0.5	4 ± 0.4	4 ± 0.5	4 ± 0.7	4 ± 0.6	---	---
10	4 ± 0.4	38 ± 3.4*	25 ± 0.9*	11 ± 1.4*	12 ± 1.2*	9 ± 1.1*	8 ± 0.8*	4 ± 0.5	4 ± 0.5	4 ± 0.4
14	4 ± 0.2	75 ± 1.6*	35 ± 3.8*	23 ± 1.4*	22 ± 1.4*	15 ± 1.4*	12 ± 1.0*	8 ± 0.7*	7 ± 1.1	4 ± 0.6
17	3 ± 0.4	$^{++}$	43 ± 1.8*	35 ± 1.8*	34 ± 2.1*	19 ± 1.4*	15 ± 1.8*	9 ± 1.0*	7 ± 1.2	6 ± 1.0
21	3 ± 0.4	$^{++}$	41 ± 1.5*	29 ± 1.9*	19 ± 1.0*	15 ± 0.9*	16 ± 1.3*	12 ± 1.2*	10 ± 1.2*	5 ± 0.4

Table 2. DNA damage expressed by average median tail moment (TM) values [μm] in leaf nuclei, and absorbance values evaluated by the ELISA method in leaves of *Nicotiana tabacum* var. *xanthi* three weeks after inoculation with PVX. For the comet assay and ELISA analysis, a small part of leaf (about 1.5 cm^2) at the petiole was used. Means ± SE, for TM values n = 150 - 200, for ELISA absorbance values n = 2.

Leaves	TM		ELISA absorbance values	
	PVX	control leaves	PVX	control leaves
1st systemic leaf	46.1 ± 4.2	4.8 ± 0.8	1.32 ± 0.07	0
2nd systemic leaf	32.8 ± 2.1	4.9 ± 0.2	1.02 ± 0.06	0
3rd systemic leaf	17.5 ± 1.3	4.1 ± 0.5	0.66 ± 0.08	0
4th systemic leaf	15.0 ± 2.3	4.0 ± 0.4	0.24 ± 0.01	0

from the leaf petiole (denoted as A in Table 1) compared to that at the leaf tip (denoted as B in Table 1). DNA damage in the 2nd, 3rd, and 4th systemic leaves was markedly lower when compared to the inoculated and the 1st systemic leaf.

Further, the tobacco leaves were inoculated with PVX and after cultivation of the treated seedlings for 3 weeks, the comet and PT-ELISA assays were performed from a piece of leaf at the petiole. DNA damage correlated with the quantity of PVX measured by PT-ELISA. DNA damage in the inoculated leaf could not be evaluated three weeks after the PVX inoculation due to severe damage to the nuclei. DNA damage in the 1st systemic leaf expressed by a TM value was 46.1 μm and an ELISA absorbance value was 1.32 (Table 2). In the 4th systemic leaf, a TM value decreased significantly to 15.0 μm and an ELISA absorbance to 0.24.

Table 3. Frequency of somatic mutations (a number of mutant sectors *per* leaf) in leaves of *Nicotiana tabacum* var. *xanthi* after inoculation of seedlings with PVX and three-week cultivation in soil. Means ± SE, n = 16, * - values significantly different ($P < 0.05$) from the control. For comparison, the effect of EMS is shown.

Evaluated leaves	Mutant sectors *per* leaf
Control leaves	1.6 ± 0.3
1st systemic leaf	1.5 ± 0.3
2nd systemic leaf	1.5 ± 0.2
3rd systemic leaf	1.9 ± 0.4
4th systemic leaf	1.4 ± 0.4
3 mM EMS for 24 h	16.0 ± 0.6*

Frequency of somatic mutant sectors *per* leaf scored on the 1st to 4th systemic leaves 3 weeks after inoculation with PVX ranged from 1.4 ± 0.4 to 1.9 ± 0.4 *per* leaf. This was not significantly different from the control value (Table 3). Mutation frequency on the inoculated leaf could not be evaluated due to the severe damage of the leaf. After the application of 3 mM EMS for 24 h on roots of tobacco seedlings followed by 14 d cultivation of the seedlings in a Hoagland solution, frequency of somatic mutations *per* leaf reached a value of 16.0 ± 0.6 (Fig. 1 Suppl.) that was significantly different from the control value (1.6 ± 0.3).

Generally, two explanations for the DNA damaging effect of PVX on nuclei of the host tobacco plants can be suggested: *1*) PVX directly reacted with DNA of host nuclei, and *2*) PVX directly or indirectly produced metabolites that caused DNA damage in nuclei of the host plant.

Viruses or their genomic nucleic acids are capable to enter the animal or human cell membrane and to react directly with DNA or with DNA repair machineries of the host cell (Weitzman *et al.* 2010, Turnell and Grand 2012). At present, two main strategies for plant virus cell-to-cell movement are known: *1*) the viral genome moves in the form of intact virions, and *2*) the viral nucleic acids are trafficked through plasmodesmata in the form of a nucleoprotein complex (NPC; Ritzenthaler 2011).

Nuclear import and export are crucial processes for any eukaryotic cell, as they govern substrate exchange between the nucleus and the cytoplasm. Proteins involved in the nuclear transport network are generally conserved among eukaryotes, from yeast and fungi to animals and plants. Various pathogens, including some plant viruses, need to enter the host nucleus to gain access to its replication machinery or to integrate their DNA into the host genome. The newly replicated viral genomes then need to exit the nucleus to spread among host cells. To gain the ability to enter and exit the nucleus, these pathogens encode proteins that recognize cellular nuclear transport receptors and utilize host nuclear import and export pathways (Krichevsky *et al*. 2006). Other proteins of cytoplasmically replicating viruses were also found in the cell nucleus (Carrington *et al.* 1991, Li and Carrington 1993, Anindya and Savithri 2004, Ryabov *et al.* 2004, Vetter *et al*. 2004, Gao *et al.* 2012). Viral proteins that are imported into the host cell nucleus participate in the transport of viral genomes. Nuclear import of viral proteins is also involved in other stages of viral infection cycle, such as suppression of a RNA silencing defense response of the host plant (Krichevsky *et al.* 2006).

The mutational analysis of PVX TGBp3 by Ju *et al*.

(2008) demonstrated that wild-type and mutant TGBp3:GFP fusions are degraded by the 26S proteasome. In PVX infected cells, the TGBp3:GFP fusion is also seen in the nucleoplasm indicating that events during PVX infection trigger entry into the nucleus. The mutational analysis failed to identify a nuclear targeting domain. Mutations inhibiting a TGBp3 association with endoplasmatic reticulum (ER) and inhibiting virus movement do not block the TGBp3:GFP presence in the nucleoplasm. A mutation disrupting the N-terminal transmembrane domain of TGBp3 causes the fusion accumulates in the nucleus indicating that nuclear import is regulated by ER interactions. Thus, the direct reaction of PVX or its genomic particles with nuclear DNA of the tobacco host cannot be excluded.

The predominant reactive oxygen species (ROS) detected in plant-pathogen interactions are the superoxide anion, hydrogen peroxide, and hydroxyl radical (Mehdy 1994). Among these ROS, H_2O_2 was demonstrated to induce DNA damage in tobacco leaf nuclei and in tobacco cell cultures as evaluated by the comet assay (Stavreva and Gichner 2002, Gichner 2003, Cvjetko *et al*. 2014). Although direct effects of H_2O_2 on nuclei were shown, it is possible that H_2O_2 also triggers a signal-transduction cascade contributing to nuclear damage (Termini 2000).

Plants exhibit a range of responses to virus infections. Infected with a compatible pathogen, plants generate a systemic recombination signal that precedes the spread of pathogens and results in changes in somatic and meiotic recombination frequencies, DNA methylation patterns, and similar DNA changes (Boyko and Kovalchuk 2011, Yao *et al*. 2011). Infections of tobacco with *Tobacco mosaic virus* (Kovalchuk *et al*. 2003), and *Arabidopsis* with an oomycete pathogen *Peronospora parasitica* (Lucht *et al.* 2002) result in a significant increase in homologous recombination frequency. In contrast, we found no increase in the overall somatic mutation frequency in tobacco following the PVX infection, although a part of the somatic mutations may be attributed to a somatic recombination mechanism (Dulieu 1975). Indeed, our results imply that somatic recombination events occurring during pathogenic infections comprise only a very small portion of the total number of somatic mutations in a host plant.

As no increase in the frequency of somatic mutations (which represent genetic alterations), was observed after the PVX infection in tobacco var. *xanthi*, the increase of induced DNA damage in the leaf nuclei might be associated with a necrotic or apoptotic DNA fragmentation (Gichner *et al*. 2009).

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