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ARTICLE

Relationship between virus traffic and nitric oxide (NO) production in tobacco roots

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ABSTRACT Nitric oxide (NO) was proved to have several roles in plant-pathogen interactions from the contribution to the local and systemic induction of defence genes to the infection signalling. The aim of this paper was to prove the involvement of NO as signalling molecule during virus infection and to point out that determination of NO levels can be used as a new method of virus detection in plants. For detection of NO generation in tobacco plants infected by potato viruses X, Y and A, 4,5 diaminofluorescein-diacetate (DAF-2DA) was used. It was found that the infected tobacco roots showed two to three times higher NO accumulation, compared to control. Our results indicated that long distance virus movement through roots occured in the stele using phloem sieve elements, but most of the viruses did not reach meristem and root cap cells. It is suggested that NO is a proper signalling molecule during virus infection and fluorescent detection of NO makes possible to demonstrate the presence of viruses within the plant tissues.

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KEY WORDS

nitric oxide
potato virus X
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virus trafficking

Nitric oxide is a highly reactive molecule that rapidly diffuses and permeates cell membranes. This novel bioactive molecule is a free radical that can either gain or lose an electron to reach energetically more favourable structures, namely nitrosonium cation (NO^+) and nitroxyl anion (NO^-). Because of its unique chemistry, which permits both its stability and reactivity, NO and its exchangeable redox-activate forms are now recognized as intra- and intercellular signalling molecules (Wilson et al. 2008). During the last few years NO has been detected in several plant species and the increasing number of reports on its function in plants have implicated NO as a key molecular signal, that participates in the regulation of several stress responses (Erdei and Kolbert 2008 and references therein). NO has been also implicated in disease resistance to virulent and avirulent pathogen attack (Delledonne et al. 1998). In particular, NO has a significant role in plant resistance to pathogens by contributing to the local and systemic induction of defence genes (Romero-Puertas et al. 2004; Wendehenne et al. 2004; Hong et al. 2008). Most of the experimental data available on NO detection during plant-pathogen interactions come from studies on infections by biotrophic pathogens (Romero-Puertas et al. 2004; Foissner et al. 2000), but there are no reports available on NO production in roots as pathogen stress response.

There are two basic routes by which a virus can move through the plant to give a full systemic infection: cell-to-cell movement through plasmodesmata and long distance movement (Scholthof 2005). Cell-to-cell or short distance movement means, that the virus spreads from the initially infected cell, which is usually epidermal or mesophyll cell to the vascular bundle and during long distance movement the virus travels then through the vascular tissue, usually the phloem sieve-tubes. As known from literature, the virus needs three days to pass into the vascular system and the first part infected is the root followed by the youngest leaves of the plant (Hull 2001).

According to our best knowledge, there are no reports reflecting on development of virus infection-induced NO generation in roots. Since after infection in the leaf, viruses appear first in roots before returning to the shoot system, our aim was to follow the NO production in time and in location in virus- infected primary roots. Therefore we carried out *in vivo* and *in situ* determination of NO levels in tobacco roots infected by potato virus X, Y and A using fluorescence microscopic method.

Materials and methods

Plant material and growth conditions

Six-weeks-old *Nicotiana tabacum* L. SR1 plants (Medgyesy et al. 1980) were transferred from soil to modified Hoagland nutrient solution for another week and were grown under controlled conditions in greenhouse at photo flux density of 240 μmol m^{-2} s^{-1} (16/8 h day/night period) at relative humidity of 55-60%, and 25 ± 2°C temperature. After a week, plants were mechanically inoculated at the second leaf counted from the

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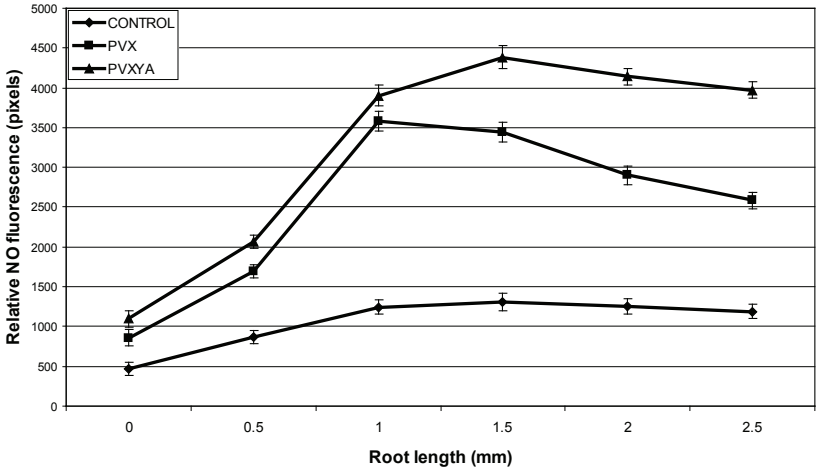

Figure 1. Localization of NO fluorescence along the length of primary roots of Nicotiana tabaccum L. SR1 plants three days after inoculation through the leaves with Potato virus X (PVX, ■), or a mixture of three viruses Potato virus X, Y and A (PVXYA, ▲). Untreated plants served as control (♦). NO levels were determined as described in Materials and Methods. Vertical bars are standard errors (n= 3-5).

basis with *Potato virus X* or with a mixture of three viruses: *Potato virus X, Potato virus Y* and *Potato virus A* and three days later NO fluorescence was detected in roots. Healthy, non-infected plants constituted the control.

Detection of NO

Visualization of NO was performed according to the highly sensitive *in situ* and *in vivo* method of Kojima et al. (1998) with some modifications. About 2.5 cm long root segments were cut and dyed with 10 μM 4,5-diaminofluorescein-diacetate (DAF-2DA) for 20 minutes at 25 ± 2°C in darkness. Cross- sections were cut with a sharp razor. After dyeing, the samples were washed 4 times within 20 minutes with 2-(N-morpholino) ethanesulfonic acid (MES) buffer (10^{-3}M, pH 6.15). To detect fluorescence intensity, Zeiss Axiowert 200M-type fluorescent microscope (Carl Zeiss, Germany) equipped with filter set 10 (exc.: 450-490 nm, em.: 515-565 nm) was used. To measure the fluorescence intensity, Axiovision Rel. 4.5 software was applied. The same camera settings were recorded for each digital image.

Detection of NO fluorescence was performed at days 3, 5, 7, 9, 11 and 13 after the infection of plants at their second leaf counted from the basis. In order to understand the virus traffic within the plant, measurements were done every 0.5 mm from the root tip to 2.5 mm distance on a 0.4 mm diameter circle. In each treatment, at least 3 samples were measured. When time-dependence of NO intensity was followed, measurements were done at the 2.5 mm of the root as from the tip.

Results and Discussion

The long-distance movement of viruses after mechanical infection of the leaves is immediately directed to the roots where it turns back towards the shoot apex and afterwards the infection spreads to the whole shoot system (Hull 2001). Therefore, in our experiments, first the expected changes in the supposedly concomitant NO accumulation were localized in space and time. In roots, intensity of NO levels varied basipetally, showing maxima from 1 to 2 mm distance from the tip, depending on treatments (Fig. 1). The lowest NO levels were seen within the first 0.5 mm of the root tip, which includes root cap and meristem and the maximal values, were detected between 1 and 2 mm from the tip. After 2 or even 2.5 mm from the tip NO fluorescence intensity slowly decreased or reached a constant value. Comparing the results of infected samples with control ones it was observed that infected roots showed at least two times higher level of NO than the control (Fig. 1). Thus, it was concluded that under our experimental conditions an increased NO production was involved in the response reaction to virus infection (Gould et al. 2003) or as a plant defence mechanism against the pathogens (Delledone et al. 1998). Other reports showed that root tips of plants infected with one of several viruses have been found to be free of detectable virus (Appiano and D'Agostino 1983). Smith and Schlegel (1964) studied the distribution of *Clover yellow mosaic virus* in root tip of *Vicia faba* and found that within the limit of the assay method, the first 0.4 mm of the root tip (root cap and meristem) was virus free. According to these results in our experiments, NO production in virus infected root tip regions was not higher than that in the root tips of the healthy control plants (Fig. 2 abc), leading to the conclusion that the majority of the viruses are not entering the meristematic cells (Hull, 2001). But, in the cases of mixed infections (PVX, PVY and PVA) some of the viruses were trafficking to the tip of the root infecting also the meristematic cells. This process was accompanied by NO accumulation

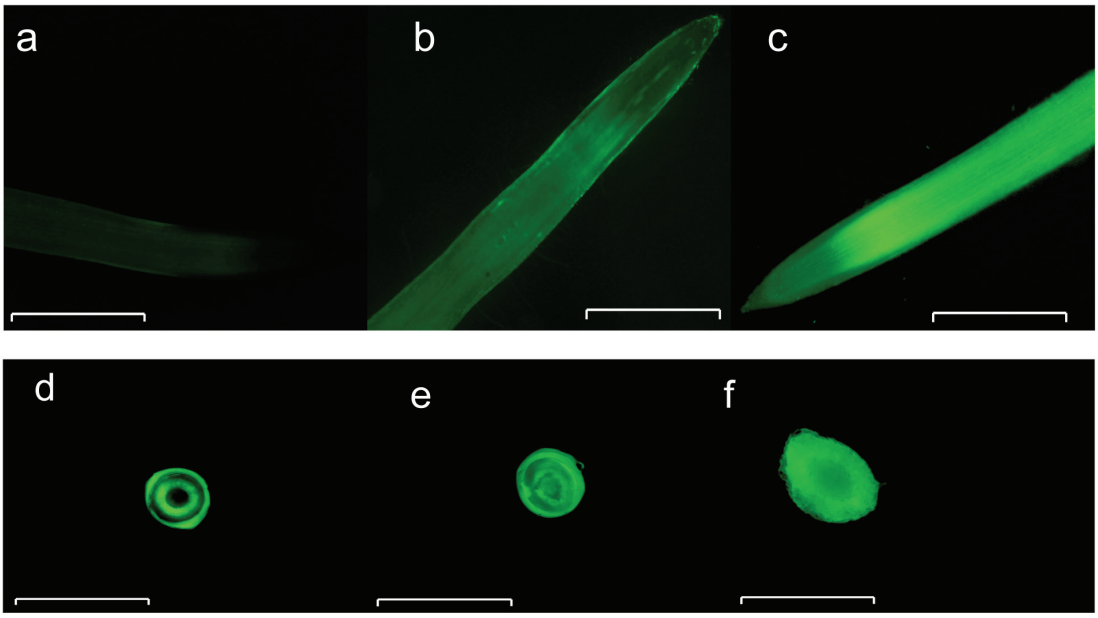

Figure 2. Fluorescence microscopic visualization of nitric oxide in the roots: a) control roots of healthy tobacco; b) PVX- infected root; c) PVX, PVY and PVA- infected root. Representative photographs of 831 pictures. Visualization of NO by fluorescence microscopy in root cross- sections: d) control roots of healthy tobacco; e) PVX- infected root; f) PVX, PVY and PVA- infected root. Representative photographs of 78 pictures. Samples were prepared as described in Materials and Methods. Bars= 1 mm.

(Fig. 1), since NO-related fluorescence intensity was higher in PVXYA- infected root tips than in those of control roots. This provides evidence that some viruses and also in mixed infections may invade the primary meristematic tissues. In order to localize the tissue used for virus transport, fine cross sections were cut from the root using a sharp razor. It was found that in control samples NO fluorescence was restricted only to the cortex cells, but in infected roots stele also showed intensive NO fluorescence (Fig. 2 def). These results suggest that the virus transport occured through the vascular bundle, which is in agreement with other literature data (Hull 2001).

NO fluorescence was determined in the 3rd, 5th, 7th, 9th, 11th and 13th day after the mechanical virus inoculation and it was found that with a single exception (7th day), the NO formation

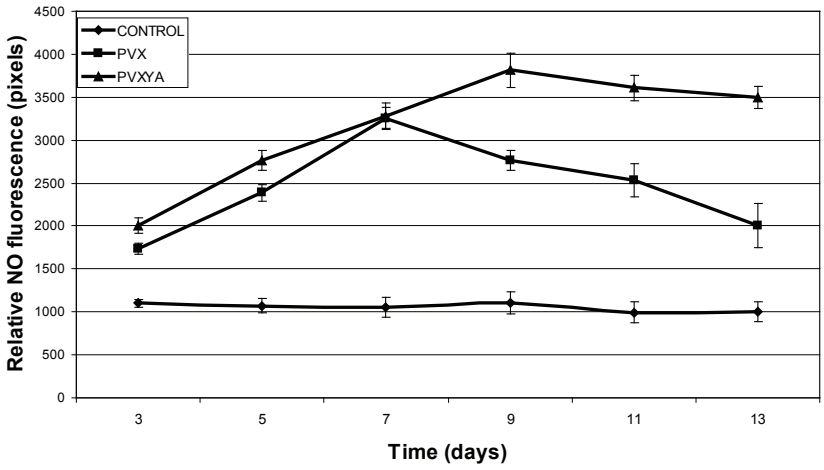

Figure 3. Time dependence of viral infection evolution in primary roots of Nicotiana tabacum L. SR1 plants after three days of inoculation their leaves with Potato virus X (PVX, ■), or a mixture of three viruses Potato virus X, Y and A (PVXYA, ▲). Untreated plants served as control (♦). NO levels were determined as described in Materials and Methods. Vertical bars are standard errors (n= 3-5).

was the highest in roots infected by the virus mixture (PVX, PVY and PVA), followed by those infected with PVX alone, but all showed higher levels of NO than the control. These are in accordance with other results showing that a plant sensibility increases with multiple infections (Faccioli and Zoffoli 1998). In the cases of PVX- infected roots NO fluorescence reached its maximum value on the 7th day, while in samples infected by virus mixture the highest NO level was detected later, on the 9th day after infection. This phenomenon can be due to the difference between their transport rates within the plant. We suppose that synergic interactions between viruses might result in lower speed of the viral complex in the mixed infection. The decrease of NO fluorescence after the seventh or the ninth day, respectively, may be due either to the development of the plant defence mechanisms against virus infection (Hull 2001) or to the feedback of high concentration of NO, which might be toxic for the pathogen (Mur et al. 2003). Curves of NO fluorescent values showed a minimum and a maximum, depending on the virus, while NO levels in control roots prooved to be constant during the whole experimental period (Fig. 3).

According to these results we conclude that NO is a proper signalling molecule during virus infections and investigation of NO-coupled fluorescence can be used as a method of virus detection in plants.

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