Research Note

Increased Cytokinin-9-Glucosylation in Roots of Susceptible *Solanum tuberosum* Cultivar Infected by Potato Virus Y^{NTN}

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Infection with potato virus Y^{NTN} (PVY^{NTN}) changed root cytokinin metabolism in infected potato plants of the very susceptible cultivar Igor. There was a trend for a shift in concentrations of biologically active cytokinin free bases and ribosides toward the inactive 9-glucosides after primary infection. This change was even more marked after secondary infection and not observed in PVY^{NTN}-resistant cultivar Sante.

Etiological studies have indicated that recently characterized potato tuber necrotic disease (PTRND) is always associated with the presence of potato virus YV^{NTN} (PVY^{NTN}), a new strain of PVY^N. In last few years this disease has been reported in several European countries (Le Romancer *et al.* 1994), including Slovenia (Buturović and Kus 1990), and has already reached an epidemic state in some of these countries (Kus 1992). The primary infection with PVY^{NTN} causes interveinal mosaics and crinkles on the potato leaves and ring necrotic areas on the tubers. Secondarily infected plants initially displayed a clear mosaic on the leaves, and a severe necrosis gradually developed on the stems and veins that eventually resulted in accelerated leaf senescence and leaf drop (Le Romancier *et al.* 1994; Kus 1992). There is evidence that a development of light-green and dark-green areas in the leaves systematically infected with tobacco mosaic virus correlates with changes in the host's cytokinin metabolism, expressed in increased concentration of cytokinin-*O*-glucosides (Whenham 1989). Regarding the role of cytokinins in leaf senescence (Van Staden *et al.* 1988) an association between PVY^{NTN} and cytokinins could be also expected. In this paper, we report the influence of PVY^{NTN} infection on distribution of nine endogenous cytokinins in healthy, primarily and secondarily infected potato plants of the very susceptible cultivar Igor and in the only known PVY^{NTN}-resistant potato cultivar Sante (Kus 1992).

Tubers of potato (*Solanum tuberosum* L.) cultivars, PVY^{NTN}-resistant cv. Sante and PVY^{NTN}-susceptible cv. Igor were analyzed by ELISA using antibodies against potato viruses PVY, PVA, PVM, PVS, PVX, and PLRV. Primarily PVY^{NTN} infected plants of cv. Igor originated from virus-free

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tubers. Secondarily infected Igor plants were derived from the PVY^{NTN} infected stock. The symptoms of PTRND were fully expressed on the secondarily infected potatoes. The plants were kept in a constant environment chamber at 25° C, 70% humidity, and a 16-hr photoperiod with light intensity of 10–12 W m^{-2}. Four-week-old healthy plants were inoculated with virus inoculum prepared from secondarily-PVY^{NTN}-infected leaves as described elsewhere (Whenham *et al.* 1986). On the 10th day after the inoculation the presence of PVY^{NTN} was unambiguously confirmed by ELISA in the leaves of all inoculated plants of cv. Igor. Plants were harvested 4 days after virus inoculation. The roots, stems, and leaves of healthy primarily and secondarily infected plants were excised, blotted, weighed, and immediately frozen in liquid nitrogen. The cytokinin metabolites were isolated by a modified immunoaffinity chromatographic system based on polyclonal antibodies against *trans*-zeatin riboside (ZR)- and *iso*-pentenyladenosine (iPA)-bovine serum albumine conjugates (Nicander *et al.* 1993) exactly as described (Dermastia *et al.* 1994, Kovač and Žel 1994). After the HPLC step (Dermastia *et al.* 1994), the recoveries of cytokinin standards were as follows: *trans*-zeatin (Z), 100%; ZR, 100%; *trans*-zeatin-9-glucoside (Z-9-G), 90%; dihydrozeatin (DHZ), 80%; dihydrozeatin riboside (DHZR), 70%; dihydrozeatin-9-glucoside (DHZ-9-G), 80%; *iso*-pentenyl adenine (iP), 100%; iPA, 100%; and *iso*-pentenyl adenine-9-glucoside (iP-9-G), 90%. The identification and quantification of cytokinin samples were based on comparison with authentic standard compounds in different systems, i.e., HPLC, scanning of the interval among 220 and 300 nm, with a Hewlett Packard 8452A diode array spectrophotometer (Nicander *et al.* 1993) and modified *Amaranthus* bioassay (Biddington and Thomas 1973).

The absolute level of measured cytokinins was relatively constant irrespective of the presence of the PVY^{NTN} in the plant (Tables 1 and 2). Qualitative determinations showed that three cytokinin bases Z, DHZ, iP, and their ribosides were generally present in the leaf and stem tissues, but their conjugates, 9-glucosides, were detectable only in roots (Tables 1 and 2). The resistant cultivar, Sante, was inoculated with PVY^{NTN}. As expected, the plants did not become infected and the changes in root cytokinins were not significant (Table 2, R; Fig. 1B). There were some small, but consistent alterations in the levels of the cytokinin free bases Z and DHZ toward

Table 1. Distribution of cytokinins in root (R), leaves and stems (LS) of virus-free, primarily and secondarily potato virus Y^{NTN} (PVY^{NTN})-infected potato cultivar Igor[a]

	Distribution of cytokinins (%)					
	Virus-free (4)		Primary infection (4)		Secondary infection (3)	
	R	LS	R	LS	R	LS
Z	3.7 ± 0.8	3.4 ± 2.1	2.3 ± 0.2*	1.2 ± 0.6	0.1 ± 0.03***	2.6 ± 0.9
ZR	20.0 ± 3.6	42.0 ± 5.8	18.6 ± 4.1	29.4 ± 6.9	9.1 ± 0.4**	44.5 ± 0.8
Z-9-G	6.6 ± 0.6	ND[b]	7.9 ± 1.2	ND	9.8 ± 0.5**	ND
DHZ	0.1 ± 0.1	17.1 ± 8.1	2.4 ± 0.7**	39.2 ± 18.9	9.1 ± 0.5***	23.2 ± 1.2
DHZR	6.8 ± 0.7	21.5 ± 9.1	3.6 ± 1.5*	23.7 ± 16.2	0.3 ± 0.1***	10.1 ± 0.4
DHZ-9-G	8.1 ± 0.3	ND	12.4 ± 0.6***	ND	27.0 ± 1.3***	ND
iP	2.4 ± 0.6	1.6 ± 0.6	0.7 ± 0.1**	1.1 ± 0.2	0.8 ± 0.2**	4.1 ± 1.0**
iPA	15.4 ± 1.9	14.5 ± 5.3	9.0 ± 0.3**	5.3 ± 1.2*	4.8 ± 0.2***	20.0 ± 1.0
iP-9-G	33.2 ± 1.4	ND	37.5 ± 2.8	ND	34.7 ± 1.7	ND
Absolute concn. (ng g DW^{-1})[c]	72.2 ± 16.5	32.0 ± 13.0	65.6 ± 17.9	28.4 ± 3.4	64.8 ± 2.5	29.4 ± 0.7

[a] Measurements represent the mean ± SE (n in parentheses) *: Statistical significance is based on the Student's t-test. Significantly different from healthy control at (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.
[b] ND, not detectable.
[c] The quantitation is based on the integration of HPLC peaks areas at 265 nm (Dermastia et al. 1994).

Table 2. Distribution of cytokinins in root (R), leaves and stems (LS) of virus untreated and virus treated plants of potato virus Y^{NTN} (PVY^{NTN})-resistant potato cultivar Sante[a]

	Distribution of cytokinins (%)			
	Untreated plants (3)		Virus treated plants (3)	
	R	LS	R	LS
Z	1.6 ± 0.7	4.0 ± 0.2	1.9 ± 0.7	2.3 ± 0.1***
ZR	16.2 ± 1.0	38.0 ± 0.4	18.0 ± 0.7	42.2 ± 0.4***
Z-9-G	5.4 ± 2.6	ND[b]	4.0 ± 1.0	ND
DHZ	1.2 ± 0.4	37.7 ± 0.3	1.2 ± 0.4	31.9 ± 0.4***
DHZR	9.2 ± 2.3	3.0 ± 0.1	9.9 ± 0.9	15.3 ± 0.3***
DHZ-9-G	20.4 ± 5.5	ND	20.4 ± 0.7	ND
iP	1.3 ± 0.1	ND	1.5 ± 0.2	ND
iPA	9.2 ± 5.0	13.2 ± 4.1	9.0 ± 0.1	8.3 ± 0.1
iP-9-G	35.7 ± 6.9	ND	34.1 ± 0.8	ND
Absolute concn. (ng g DW^{-1})[c]	31.4 ± 9.9	30.0 ± 6.4	32.3 ± 8.4	31.2 ± 0.2

[a] Measurements represent the mean ± SE (n in parentheses). *: Statistical significance is based on Student's t-test. Significantly different from healthy control at (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.
[b] ND, not detectable.
[c] The quantitation is based on the integration of HPLC peaks areas at 265 nm (Dermastia et al. 1994).

the increased concentrations of their riboside forms, especially noticeable in the case of DHZR (Table 2, LS).

Four days after virus inoculation, there were no visible symptoms of disease on the susceptible plants of cv. Igor. However, it is likely that the stimulus of the infection-induced systemic metabolic changes that may affect the balance of cytokinin metabolites in the very early stage of virus replication. The cytokinin concentrations in the shoots of primarily infected plants of the cultivar Igor were not significantly different from the healthy ones (Table 1, LS). However, in roots there was reduction of cytokinin bases Z and iP and their corresponding ribosides, including DHZR. Simultaneously the concentration of total 9-glucosides increased from 48% in virus-free to 58% in primarily infected potatoes, with the highest rise in the amount of DHZ-9-G (Table 1, R). The process of cytokinin-9-glucosylation was even more evident in secondarily infected plants where 9-glucosides accounted

for up to 72% of the total root cytokinins (Fig. 1A). The main contribution to the increased level of 9-glucosides was due to three times higher concentration of DHZ-9-G in comparison to healthy plants. A smaller, but still significant increase in Z-9-G concentration was also observed (Table 1, R). It seems that the virus infection had rather little influence on the iP-9-G, the cytokinin with the highest yield in potato roots.

Cytokinin free bases and their ribosides are considered as the biologically active form of the hormone within the plant (Letham and Palni 1983). Although the phytohormone conjugates are abundant in plant tissues, their normal biological functions remain obscure. Cytokinin glucosylation on N7 and N9 is considered to be one of the mechanisms of irreversible cytokinin inactivation in plant cells (Horgan 1992; van Staden and Drewes 1992), while O-glucosides are the major mobilizable conjugated cytokinin forms (Letham and Palni 1983) from which active cytokinins can be released by endogenous
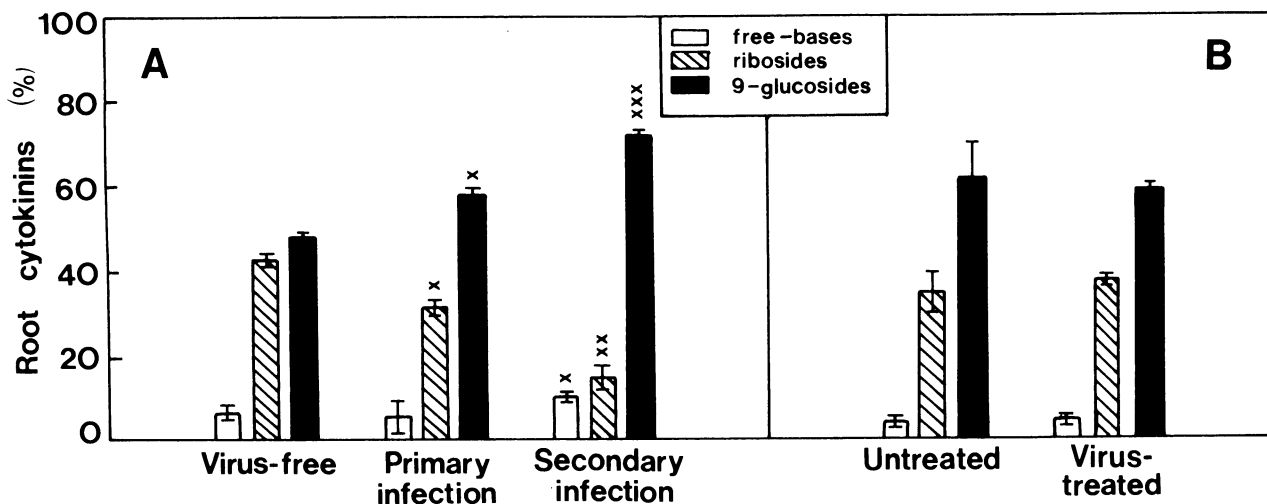
Fig. 1. Total detected cytokinin free bases, their ribosides and 9-glucosides (%) in PVY^{NTN}-susceptible potato cultivar Igor (A) and in PVY^{NTN}-resistant cultivar Sante (B). The Student's t-test was used for evaluating the levels of significant differences between the healthy control and infected plants. Symbols used in the figure are: (^{X}) $P < 0.05$; (^{XX}) $P < 0.002$; (^{XXX}) $P < 0.0001$; bars mean ± SE.

hydrolases, such as β-glucosidase, Zm-p60 (Brzobohatý *et al.* 1993). Generally high levels of 9-glucosides probably reflect the lack of enzyme systems in plants to convert them back to the free bases. It was shown that only the β-glucosidase from *Agrobacterium rhizogenes* encoded by the *rolC* oncogene is able to hydrolyze zeatin-7- and -9-glucosides (Estruch *et al.* 1991). The rolC protein was proposed as a part of the mechanism by which the bacterial pathogen subverts normal plant development to its own advantage (Brzobohatý *et al.* 1993). The metabolic stability and biological inactivity of N-C glucosides including 9-glucosides was demonstrated recently by Van Staden and Drewes (1991, 1992) using cytokinin-dependent soybean callus. Their findings were confirmed by our study on *Amaranthus* seedlings where in the case of Z-9-G, DHZ-9-G, and iP-9-G there were no responses to bioassay.

Since root tips are reported to be sites for cytokinin biosynthesis (Chen *et al.* 1985; Bollmark *et al.* 1988), the terminal inactivation of metabolically active cytokinins in roots could prevent their reutilization in active forms or their transport to the upper parts of the plant. It is possible, therefore, that the observed virus-induced reduction of active cytokinins in roots may contribute to the premature leaf senescence and a leaf drop as evident symptoms of PTRND in the cultivar Igor.

It is not clear whether the 9-glucosylation of cytokinin bases and ribosides is a component of pathogenesis or a consequence of long-term effect of virus infection on overall plant metabolism and development. However, the general presence of irreversible 9-glucosylation process from the very short time after virus inoculation and its persistence in secondarily infected stock supports the involvement of the terminal cytokinin deactivation in pathogenesis. Moreover, an unchanged root cytokinin metabolism of the virus treated PVY^{NTN}-resistant cultivar Sante is an additional confirmation of the hypothesis.

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