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Systemic *Potato virus* Y^{NTN} infection and levels of salicylic and gentisic acids in different potato genotypes

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Endogenous levels of free and conjugated salicylic (SA) and gentisic (GA) acids, both putative signal molecules in plant defence, were analysed in order to investigate their involvement in the resistance of four potato (*Solanum tuberosum*) genotypes with different susceptibilities to *Potato virus* Y^{NTN} (PVY^{NTN}) infection: the highly susceptible cv. Igor and its extremely resistant transgenic line, the extremely resistant cv. Sante and the tolerant cv. Pentland Squire. The lowest levels of free and conjugated SA were observed in the extremely resistant cv. Sante, while free GA, which was detected in all the other varieties, was absent. The extremely resistant transgenic cv. Igor contained the highest basal total SA level and the lowest level of total GA of all four cultivars. In susceptible cv. Igor, but not in resistant transgenic cv. Igor, a systemic increase of free SA was measured 1 day postinfection (dpi). Even more significant increases of free and conjugated SA and GA were detected 11 dpi when systemic symptoms appeared. In inoculated but not in upper noninoculated leaves of resistant transgenic cv. Igor, significant increase of SA conjugates occurred, but not before 11 dpi. The increase of SA and GA in susceptible cv. Igor could contribute to the general elevated levels of phenolic compounds as a response to stress caused by virus infection. It appears that basal levels of SA and GA do not correlate with resistance to PVY^{NTN} in potato plants.

Keywords: gentisic acid, potato, *Potato virus* Y^{NTN}, salicylic acid, *Solanum tuberosum*

Introduction

Necrotic isolates of *Potato virus Y* (PVY^{NTN}) a member of the *Potyviridae* family that cause potato tuber necrotic ringspot disease have become widespread throughout Europe in recent years (Kus, 1994). The primary symptoms of chlorosis and spot necrosis are observed on infected leaves of susceptible potato cultivars. Later, severe systemic symptoms appear: leaf mosaic and curling, and vein necrosis on younger leaves, accompanied by the loss of older, lower leaves (palm tree symptoms). Infected plants grow more slowly; they become yellow and may die quickly. Infection with PVY^{NTN} not only reduces yield, but also causes necrotic damage to tubers, which makes affected crops unsuitable for sale (Kus, 1994). Potato cultivars differ in their sensitivity to PVY^{NTN} For example, cv. Igor is highly sensitive, with infected plants exhibiting severe primary symptoms several days postinoculation and systemic symptoms after 11 days. In contrast, the tolerant cv. Pentland Squire shows no visible symptoms postinfection, although the virus multiplies in the plant (Ravnikar *et al*., 1996). Sante, a cultivar carrying the Ry_{sto} gene from *Solanum stoloniferum*, shows extreme resistance to PVY^{NTN} (Hinrichs *et al*., 1998); it remains symptomless postinfection and the virus cannot be detected (Ravnikar *et al*., 1996). The same is true for the transgenic $line_{\#35}$ of cv. Igor, transformed with a sequence derived from the coat protein gene of PVY^{NTN} (Stanić-Racman *et al*., 2001).

Potato (*Solanum tuberosum*) plants contain high basal levels of salicylic acid (SA; 2-hydroxybenzoic acid) (Coquoz *et al*., 1995). In plants, SA is found not only as a free molecule, but mostly as a glucose conjugate (Vernooij *et al*., 1994). Many studies have indicated that SA is a key regulatory compound of disease resistance against fungi, bacteria and viruses (reviewed in Dempsey *et al*., 1999). Normally, plants responding to pathogen attack exhibit significantly higher levels of SA than uninfected plants. It was observed, however, that transgenic plants expressing the *nahG* gene did not accumulate SA after exposure to pathogens, and were more susceptible, both to pathogens that normally induce resistance and to those that normally cause disease (Cohn *et al*., 2001). SA has a role in the appearance of hypersensitive (i.e. incompatibility) reactions, induction of systemic acquired resistance and activation of a number of pathogenesis-related (PR) genes (reviewed in Jameson & Clarke, 2002). Some PR gene

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products have direct effects on fungi or bacteria, but none has been shown to have a clear role in virus resistance (Murphy *et al*., 1999). Nevertheless, depending upon the virus, SA can induce inhibition of the three main possible stages in virus infection, replication and/or cell-to-cell movement at the site of inoculation, and inhibition of long distance viral movement (reviewed in Singh *et al*., 2004). It was established that SA-inducible RNA-dependent RNA polymerase has an important role in antiviral defence (reviewed in Rovere *et al*., 2002).

Gentisic acid (GA; 2,5-dihydroxybenzoic acid), a metabolic derivative of SA, is one of the most commonly occurring aromatic acids in green plants (Griffiths, 1959). In contrast to SA, GA has been reported as a component of the inducible response of plants to pathogens, either as a mediator or as an induced antimicrobial defence molecule, but only for compatible tomato–pathogen [*Citrus exocortis viroid* (CEVd) and *Tomato mosaic virus* (ToMV)] interactions (Belles *et al*., 1999). Nevertheless, little is known about GA being involved in any other plant–pathogen interactions.

There are several studies showing that some cultivars of rice (Silverman *et al*., 1995) and potato (Coquoz *et al*., 1995) resistant to eukaryotic pathogens contain more endogenous SA than do susceptible ones; no connection between high basal levels of SA and virus resistance has been reported.

This study reports the relationship between basal levels of free and conjugated SA and GA and resistance of four potato cultivars with different susceptibilities to PVY^{NTN}. In addition, the involvement of SA and GA in the defence response to PVY^{NTN} of two potato varieties, the very susceptible nontransgenic and the resistant transgenic forms of cv. Igor, was investigated at different time points after infection.

Materials and methods

Plant material, growth conditions and inoculation

Potato cvs Igor, Pentland Squire and Sante were obtained from the Laboratory for Physiology and Potato Virus Disease, M-KŽK Unit, Kranj, Slovenia. The resistant transgenic $line_{\#35}$ of cv. Igor, transformed with a sequence derived from the coat protein (CP) gene of PVY^{NTN}, was obtained from the laboratory of the present study (Stanić-Racman *et al*., 2001). Plants were multiplied by a stem-node segmentation procedure and grown in modified Murashige-Skoog medium (Murashige & Skoog, 1962). After 2 weeks of cultivation, the plants were transferred into quartz sand (MP–1G/S, Termit Moravće, Slovenia) in a growth chamber, with 70–90 μmol m^{-2} s^{-1} radiation (Osram L36W/77 lamp), a 16-h photoperiod and relative humidity of 75 ± 2%. The temperature was 20 ± 2°C in the light and 18 ± 1°C in the dark. Plants were watered every second day with 30 mL of tap water and once a week with the nutrient solution adapted by Johnson *et al*. (1994) (Milavec *et al*., 2001).

Basal SA and GA levels were determined in three lower (old) and three to four upper (young) leaves of 4-week-old plants. Inoculation with the sap of PVY^{NTN}-infected potato plants was carried out according to Milavec *et al*. (2001). Plants inoculated with a buffered suspension of healthy plant sap were used as controls. Samples of lower inoculated and upper intact leaves of control and virus-inoculated plants of the same age were collected at different time points after infection. They were frozen immediately in liquid nitrogen and stored at –80°C for SA and GA analysis.

Extraction and analyses of SA and GA

Free and conjugated salicylic and gentisic acids were extracted using existing protocols (Raskin *et al*., 1989; Meuwly & Métraux, 1993) with modifications. Leaf tissue (0·5 g) was ground in liquid nitrogen to a fine powder using a mortar and pestle, resuspended in 3 mL 90% methanol and sonicated for 20 min (Sonis 4 sonicator, 30 kHz, 400VF, Iskra, Slovenia). Ten microlitres of orthoanisic acid (1 mg mL^{-1}) (Fluka, Neu-Ulm, Germany) were added as an internal standard. The extract was centrifuged at 10 000 ***g*** for 15 min. The pellet was resuspended in 2·5 mL 100% methanol and re-centrifuged as above. The two supernatants were combined and centrifuged a third time at 4500 ***g*** for 10 min. The methanol was evaporated at 40°C under vacuum, 1 mL of 5% (w/v) trichloroacetic acid was added to the residual aqueous phase and the mixture centrifuged at 3000 ***g*** for 10 min. The supernatant was partitioned twice (10 min each time) against 3 mL of a 1:1 (v/v) mixture of ethylacetate and cyclopentane. The top organic layers containing the free phenolic portion were evaporated at 40°C under vacuum and then resuspended in 400 μL 100% methanol. After filtration, 10 μL of the sample was used for HPLC analysis.

The aqueous phases containing the methanol-soluble conjugated phenolics were diluted with equal volumes of 8 N HCl and hydrolysed for 1 h at 80°C. The mixture was then centrifuged at 3000 ***g*** for 10 min. The supernatant was partitioned twice and the top organic layers evaporated, as above. The sample was resuspended in 400 μL 100% methanol and, after filtration, 10 μL was used for HPLC analysis.

SA and GA were separated by HPLC (Waters™ system, Milford, USA) and 10 μL were injected onto a Pelliguard LC–18 Supelcosil precolumn (50 mm long × 4 mm wide with 40 μm packing) linked to a C-18 Eurospher 100 column (250 long × 4·6 mm wide with 5 μm packing) equilibrated with a mixture of 90% 20 mM sodium acetate buffer (pH 5) and 10% methanol, and maintained at 35°C. The flow rate was 1 mL min^{-1}. A gradient of methanol (10–100%) was applied over 20 min. Phenolics were measured spectrophotometrically at 290 nm (Photodiode Array Detector, Waters 996 A) and quantified fluorimetrically using authentic standards (Scanning Fluorescent Detector, Waters 474, excitation at 305 nm, emission at 407 nm).

Statistical analyses

Basal SA and GA levels quoted are mean values from six extracts (three from each of two experiments). Levels

quoted after inoculation are mean values from nine extracts (three from each of three experiments). The Student *t*-test was used to calculate significant differences between cultivars (Fig. 1) and between inoculated and noninoculated plants. Recoveries calculated from the internal standard were 69–75% for SA and 63–72% for GA. No correction was made for the losses.

Results

Basal levels of SA and GA in potato leaves

In potato cultivars with different sensitivities to virus infection, free and conjugated forms of SA and GA were measured in upper young and lower old leaves of 4-week-old plants. In all samples, the basal SA level comprised two- to 8·4-fold more conjugated form than free (Fig. 1a and b). More SA was found in young leaves than in old ones in all cultivars except cv. Sante. The basal levels of free SA did not differ greatly between cultivars. However, the highest content of conjugated SA was found in both old and young leaves of the resistant transgenic Igor, while the statistically significant lowest levels were found in both types of leaves in resistant cv. Sante (Fig. 1a and b).

Although the basal SA levels in lower (old) and upper (young) leaves were found to differ, the percentages of free and conjugated SA were closely similar in old and young leaves in all cultivars (Fig. 2a). In susceptible cv. Igor and tolerant cv. Pentland Squire, the levels of free SA, as percentages of total SA, were similar (17%). In cv. Sante, the average level of free SA was 32%. In contrast, in transgenic Igor the percentage of free SA was lower than in all other cultivars (12% on average).

Unlike SA, no difference in endogenous GA content was observed between young and old leaves in any of the four cultivars. The basal level of conjugated GA was 5·5- to 16·4-fold greater than that of the free form (Fig. 1c and d). The highest GA level was observed in the tolerant cv. Pentland Squire and the lowest in transgenic Igor. Interestingly, no free GA was detected in any leaves in resistant cv. Sante.

The percentages of free and conjugated GA (Fig. 2b) were about the same in both types of leaves in all cultivars, as shown above for SA. In susceptible cv. Igor and tolerant cv. Pentland Squire, the percentages of free GA were similar (6%). In contrast to free SA, the 16% free GA found in transgenic cv. Igor was the highest of all the cultivars.

SA and GA levels following inoculation with PVY^{NTN} in potato leaves

Samples were collected 1, 2, 5 and 11 days postinoculation (dpi). The levels of free SA in leaves of susceptible cv. Igor were significantly higher 1 day after infection (Fig. 3a and b). Interestingly, at 5 dpi, when primary symptoms appeared, there was no further significant change in SA level. The difference in levels of free and conjugated SA between control and virus-inoculated leaves was most

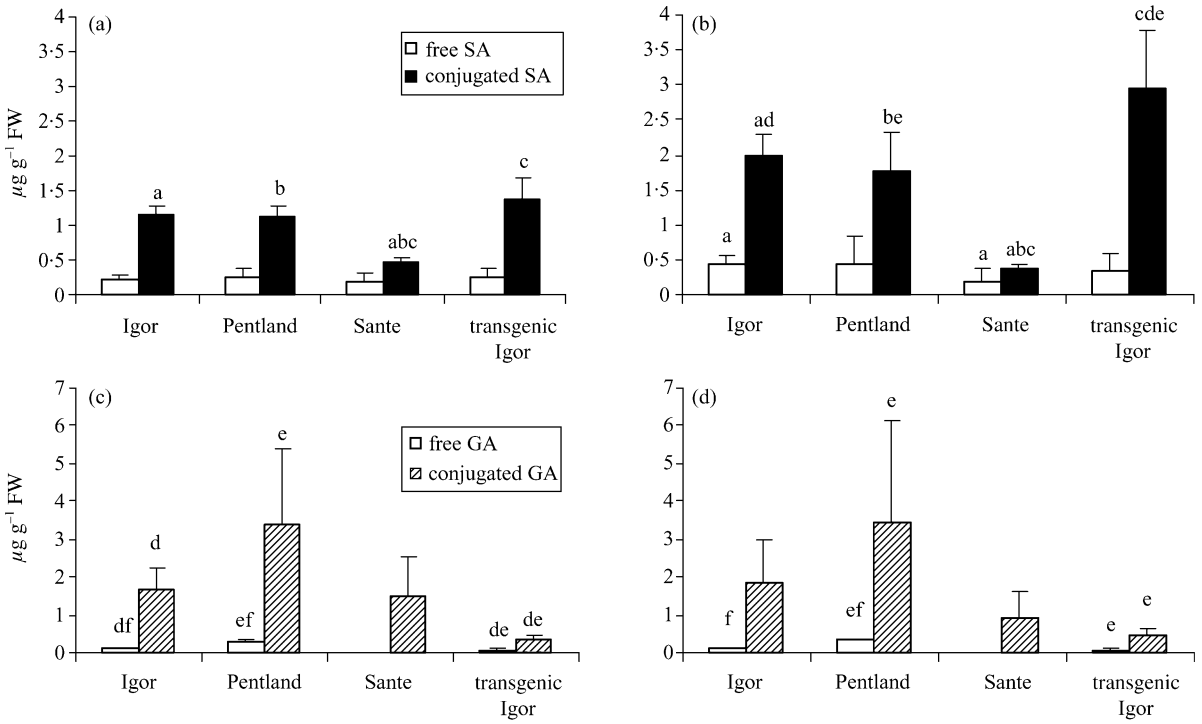

Figure 1 Total amounts of basal free and conjugated salicyclic acid (SA) (a, b) and conjugated gentisic acid (GA) (c, d) in old and young leaves, respectively, of 4-week-old potato plants of various cultivars. SA and GA are expressed as μg g^{-1} fresh weight (FW). Significant differences (student's *t*-test, $P < 0.05$) of SA and GA between pairs of cultivars are indicated by lower case letters: (a) Sante vs. Igor; (b) Sante vs. Pentland Squire; (c) Sante vs. transgenic Igor; (d) Igor vs. transgenic Igor; and (e) transgenic Igor vs. Pentland Squire.

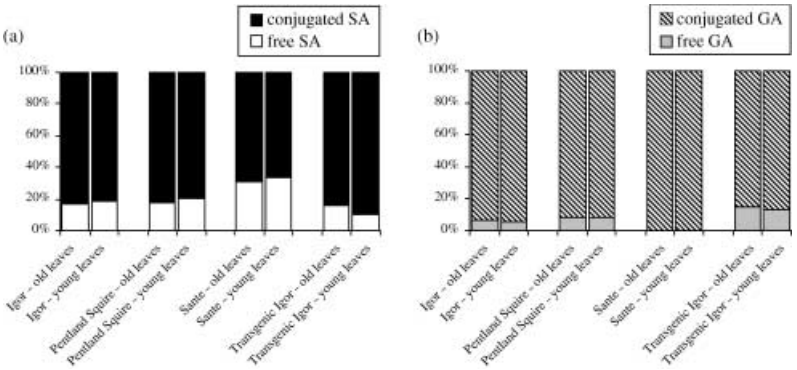

Figure 2 Percentages of free basal and conjugated salicyclic (SA) (a) and gentisic acid (GA) (b) in old and young leaves of 4-week-old potato plants of various cultivars.

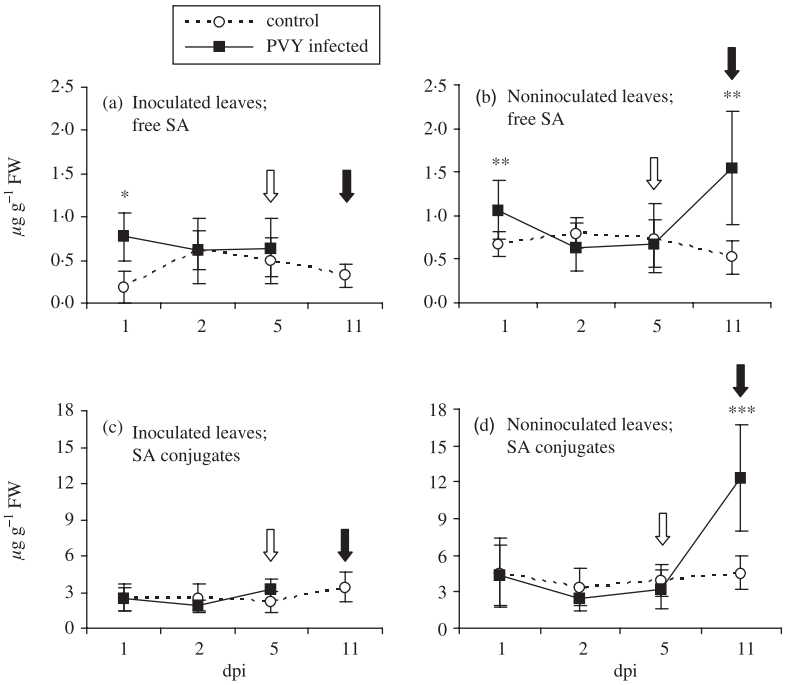

Figure 3 Free (a, b) and conjugated (c, d) salicyclic acid (SA) in inoculated (a, c) and noninoculated (b, d) leaves of 4-week-old susceptible potato cv. Igor plants 1, 2, 5 and 11 days postinoculation (dpi) with PVY^{NTN}. SA is expressed as μg g^{-1} fresh weight (FW). Student's *t*-test revealed differences between control and PVY^{NTN}-infected plants ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, $n = 9$, bars indicate SD). The white arrow indicates appearance of primary symptoms, while the black arrow indicates appearance of systemic symptoms. At 11 dpi plants were without lower PVY^{NTN}-inoculated leaves (a, c).

pronounced at 11 dpi, when the highest amounts of both types of SA were observed in intact but noninoculated leaves of infected plants (Fig. 3b and d). At that time, systemic symptoms (leaf mosaic and curling on upper intact leaves, but absent from infected lower leaves) appeared in infected plants of susceptible cv. Igor.

No significant difference was observed in GA level between control and virus-inoculated potato plants of cv. Igor, except in noninoculated leaves at 11 dpi, when systemic symptoms appeared. Because of the more pronounced variation in GA level between experiments, the data in Table 1 are given as means of three separate experiments. Compared with control plants, significantly higher levels of free GA were observed in upper noninoculated leaves of infected plants. The same trend was observed for GA conjugates.

Surprisingly, in transgenic cv. Igor almost no statistically significant differences in either SA or GA levels were found. The only significant change occurred in conjugated SA in inoculated leaves at 11 dpi (Fig. 4c).

Discussion

It is believed that certain natural products synthesized by plants contribute to their resistance to pests and pathogens. Moreover, it has been shown that phenylpropanoid products (precursors for phenolic compounds) contribute to disease limitation (Maher, 1994). In comparison to several other plants, potato contains high basal levels of SA (40- to 100-fold higher than those found in tobacco and *Arabidopsis*) (Yu *et al*., 1997). It was also found that potato cultivars showing field resistance to late blight (*Phytophthora infestans*) contained higher amounts of conjugated SA than susceptible ones (Coquoz *et al*., 1995). As reported here, these authors found the highest levels of SA in young leaves. However, Yu *et al*. (1997) demonstrated that the high constitutive level of total SA in potato does not lead to constitutive resistance to *P. infestans* in healthy potato plants. In the present study it was shown that the extremely resistant cv. Sante has the lowest levels of SA conjugates of all the cultivars studied,

Table 1 Levels of free and conjugated gentisic acid (GA) in noninoculated leaves of control and PVY^{NTN}-infected potato cv. Igor plants at 11 dpi in three separate experiments

	Free GA (μg g^{-1} FW)		Conjugated GA (μg g^{-1} FW)	
Experiment no.	Control	PVY^{NTN}	Control	PVY^{NTN}
1	0·63 ± 0·070[a]	0·94 ± 0·050[a,b]	24·67 ± 1·68[a]	30·61 ± 0·04[a,b]
2	0·26 ± 0·630[a]	0·63 ± 0·090[a,b]	5·18 ± 4·40[a]	11·91 ± 0·34[a]
3	0·07 ± 0·004[a]	0·21 ± 0·020[a,b]	1·38 ± 0·27[a]	1·45 ± 0·36[a]

[a]Mean (± SD) of three replicates.
[b]Significant difference (P = 0·05).

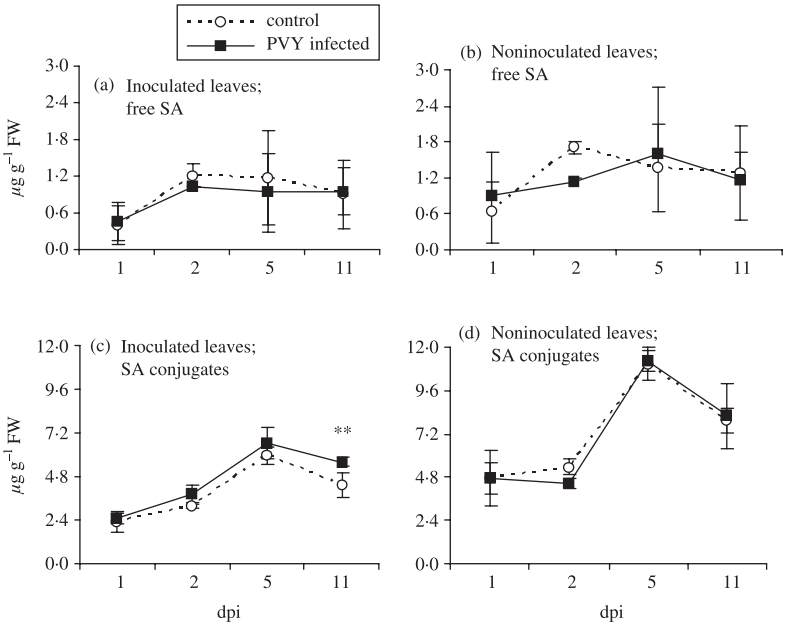

Figure 4 Free (a, b) and conjugated (c, d) salicyclic acid (SA) in inoculated (a, c) and noninoculated (b, d) leaves of 4-week-old resistant potato plants of the transgenic line of cv. Igor 1, 2, 5 and 11 days postinoculation (dpi) with PVY^{NTN}. SA is expressed as μg g^{-1} fresh weight (FW). Student's t-test revealed differences between control and PVY^{NTN}-infected plants (*P < 0·05, **P < 0·01, ***P < 0·001, n = 6, bars indicate SD).

while no free GA in cv. Sante could be detected, indicating that the resistance of cv. Sante to PVY^{NTN} does not correlate with high endogenous level of SA or GA. However, the highest total SA and the lowest total GA levels were detected in the resistant transgenic cv. Igor. SA and GA conjugates predominated over the free forms in all four potato cultivars. SA is usually conjugated with glucose to form β-*O*-D-glucosylsalicylic acid, and GA is thought to conjugate similarly (Yalpani *et al*., 1993; Belles *et al*., 1999). The significance of this conjugation is not fully understood. The SA glucosides may serve as a storage form for SA or, alternatively, the conjugation may detoxify high levels of SA. Since SA and GA glucosides are hydrolysable, the two phenolic compounds, SA and GA, can be released when needed (reviewed in Vernooij *et al*., 1994).

With regard to percentages of free vs. bound SA and GA in potato leaves, the extremely sensitive cv. Igor had almost the same values as the tolerant cv. Pentland Squire. In both these cultivars, the virus can be detected with ELISA and it can spread through the plant (Ravnikar *et al*., 1996). Of the four cultivars, the resistant cv. Sante (with the Ry_{sto} gene) had the highest and the resistant transgenic cv. Igor (with inserted CP transgene) the lowest percentage of free SA (Fig. 2a); the opposite was true for GA (Fig. 2b). Thus, if the ratio of free to conjugated forms is more important than the overall free SA content, then the high percentage of free relative to conjugated SA could be connected to the extreme virus resistance of cv. Sante. Nevertheless, this was not the case for the resistant transgenic Igor, possibly because of a different resistance mechanism. Although the overall amounts of each SA form were higher in younger than in older leaves of all the cultivars examined, the ratio between free and conjugated SA remained more or less the same, indicating its importance for the plant. The results indicate, apparently for the first time, that the basal levels of SA and GA do not correlate with the constitutive defence of potato against the virus.

Salicylic acid has been shown to mediate resistance in many plant–virus interactions. It has been proposed that it acts through inhibition of virus replication and/or cell-to-cell movement at the site of inoculation, or through inhibition of viral long distance movement (Murphy *et al*., 2001). The literature on the involvement of GA in the resistance response of plants to pathogens is very limited compared with the literature on SA. Nevertheless, it was demonstrated that GA acts as a pathogen-inducible chemical in tomato–CEVd and in tomato–ToMV interactions, which are classified as compatible, non-necrotizing

systemic infections, but not in the tomato–*Pseudomonas syringae* pv. *syringae* incompatible, necrotizing reaction (Belles *et al*., 1999). The present results indicate that, in the very sensitive cultivar Igor, the systemic increase of free SA, but not GA, observed at 1 dpi in infected plants is part of the early response to virus infection, but not to the extent that they can seriously affect virus replication and/or movement. At that time, PVY^{NTN} was not detected in the upper noninoculated leaves of cv. Igor by any of the methods used for PVY^{NTN} detection described by Mehle *et al*. (2004). The increase of SA in upper intact leaves at 1 dpi indicates that some systemic signal is moving faster than the virus. However, PVY^{NTN} cDNA was detected in intact leaves of infected susceptible cv. Igor at 5 dpi by real-time PCR (Mehle *et al*., 2004). In the recent study of Whitham *et al*. (2003) it was demonstrated that at 1 dpi diverse RNA viruses induced several defence-related genes of susceptible *Arabidopsis thaliana*, some of which have been found to be involved in signal transduction pathways regulated by SA. The threefold higher levels of total SA and twofold higher levels of total GA in infected potato compared with control plants at 11 dpi (when systemic symptoms appeared) can be associated with the general elevated levels of phenolic compounds as a response to stress in plants caused by the virus (Dixon & Paiva, 1995; Whitham *et al*., 2003), since it was found that PVY^{NTN} multiplies vigorously in upper noninoculated leaves of cv. Igor from 5 to 11 dpi (Mehle *et al*., 2004). When the infection was suppressed in transgenic cv. Igor, there was no induction of free SA in inoculated or intact leaves over the time-frame examined. This result is perhaps not surprising since Pruss *et al*. (2004) reported that the reduced number of *Tobacco mosaic virus* lesions per unit leaf area and enhanced resistance to *Tomato black ring nepovirus* in inoculated leaves of tobacco plants expressing the potyviral helper-component protease (HC-Pro) do not appear to be SA-dependent. HC-Pro is a plant viral suppressor of RNA-silencing, enhancing resistance to several viral pathogens via both SA-dependent and -independent mechanisms (Pruss *et al*., 2004). On the other hand, Ji & Ding (2001) demonstrated that SA induces virus resistance by potentiating a RNA-silencing antiviral defence that is targeted by the *Cucumber mosaic virus* (CMV)-encoded 2b protein (Cmv2b). Cmv2b is a viral counterdefence factor that interferes with the establishment of virus-induced gene silencing in plants (Mayers *et al*., 2000), like HC-Pro.

The mechanism of resistance against pathogens varies in different plant species. The role of SA in activating defence responses in tobacco and *Arabidopsis* following infection by many different bacteria, fungi and viruses has been demonstrated unequivocally. In these plants, which have low basal levels of SA, this compound appears to be one of the limiting factors for defence responses. It is only synthesized or made available upon attack by pathogens (reviewed in Shah & Klessig, 1999). Plants containing high endogenous SA levels (e.g. rice, potato and tomato) are more resistant to certain pathogens (Silverman *et al*., 1995). In the case of such plants, it has been proposed that the limiting factor for defence responses might be some component(s) of the signal transduction pathway downstream of SA and not the increase in SA level. This component(s) could be synthesized or made available only upon pathogen infection, at which time the plant would become sensitive to the high basal levels of SA and activate systemic acquired resistance (Yu *et al*., 1997). Achuo *et al*. (2004) concluded that the plant defence responses activated by the SA-dependent pathway depend on the particular host–pathogen system, so that results of one system may not be extrapolated, even to another closely related system.

The work presented here is the first demonstration of SA induction in susceptible plant–potyviral interactions. If SA and GA are not the limiting factors in the signalling pathways of potato leading to activation of resistant defence responses to PVY^{NTN} infection, then the systemic increase of SA observed at 1 dpi and the increases of total SA and GA at the time of appearance of systemic symptoms in the very susceptible cv. Igor may contribute to the elevated levels of phenolics in response to stress caused by virus infection. These increases, however, were probably insufficient or too late to prevent the development of disease.

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