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Inhibition of systemic onset of post-transcriptional gene silencing by non-toxic concentrations of cadmium

Shoko Ueki and Vitaly Citovsky*
Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794–5215, USA

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*For correspondence (fax +1 631 632 8575; e-mail vitaly.Citovsky@sunysb.edu).

Summary

Post-transcriptional gene silencing (PTGS) is an important mechanism for regulation of plant gene expression and virus–plant interactions. To better understand this process, the heavy metal cadmium was identified as a specific inhibitor in two different PTGS systems, constitutive and inducible. The pattern of cadmium-induced inhibition of PTGS allowed several insights into PTGS development. First, cadmium treatment prevented only systemic but not local onset of PTGS, uncoupling between these two modes of PTGS. Second, non-toxic, but not toxic, levels of cadmium inhibited PTGS, suggesting induction of a pathway that interferes with PTGS. Third, cadmium effects on PTGS closely paralleled those on the movement of tobamoviruses, suggesting that both processes may share common steps in their systemic transport pathways. Interestingly, these effects of cadmium do not represent a general property of toxic metal ions because two other such elements, that is zinc and aluminum, did not interfere with PTGS and viral systemic movement.

Keywords: post-transcriptional gene silencing (PTGS), tobamoviruses, systemic spread, cadmium, heavy metal ions, gene silencing.

Introduction

The vascular system is the major route for systemic transport of protein and nucleic acids in plants (Citovsky and Zambryski, 2000; Oparka and Santa Cruz, 2000). Among the transported molecules, most notable are plant virus genomes (Leisner and Turgeon, 1993), post-transcriptional gene silencing (PTGS) signals (Fagard and Vaucheret, 2000; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998) and specific endogenous RNAs and proteins (Kühn *et al.*, 1997; Lucas *et al.*, 1995; Ruiz-Medrano *et al.*, 1999; Sasaki *et al.*, 1998; Sessions *et al.*, 2000; Xoconostle-Cázares *et al.*, 1999). Although this transport obviously plays an important role in plant–virus interactions as well as in regulation of plant gene expression and development, its underlying molecular mechanisms are still obscure. Here, we demonstrate that systemic spread of PTGS and plant viruses may share common steps in their molecular pathway(s).

Gene silencing is defined as post-transcriptional when RNA of the silenced gene does not accumulate, even though its transcription occurs (Vaucheret *et al.*, 1998). Recent evidence suggests the PTGS signals are RNA molecules (Fagard and Vaucheret, 2000; Hamilton and Baulcombe, 1999; Mourrain *et al.*, 2000; van der Boogaart *et al.*, 1998; Vaucheret *et al.*, 1998; Waterhouse *et al.*, 1998; Di Serio *et al.*, 2001). These signals are presumed to spread between plant cells both locally and systemically (Voinnet *et al.*, 1998), resulting in propagation of PTGS throughout the plant. Similarly, local and systemic transport has been demonstrated for the spread of plant viruses (Lucas and Gilbertson, 1994; Oparka and Santa Cruz, 2000; Rhee *et al.*, 2000).

Following initial infection, plant viruses replicate and spread locally, from cell to cell via plasmodesmata until they reach the vascular system. The invading virions then enter the host vasculature, travel through it with the flow of photoassimilates, and exit to infect non-vascular tissues of systemic organs (Leisner and Howell, 1993; Lucas and Gilbertson, 1994; Oparka and Santa Cruz, 2000; Rhee *et al.*, 2000). Systemic but not local transport of tobamoviruses, such as tobacco mosaic virus (TMV) and turnip vein clearing virus (TVCV), is inhibited by treating the host plants with non-toxic levels of the heavy metal cadmium (Citovsky *et al.*, 1998; Ghoshroy *et al.*, 1998). Thus, it was interesting to determine whether this cadmium treatment also affects transport of PTGS signals. Here, we report that

systemic but not local spread of PTGS is blocked in the presence of low levels of cadmium. The inhibitory effect of cadmium on PTGS paralleled that on viral systemic movement, suggesting that viruses and PTGS signals may share common steps in their systemic transport pathways.

Results

Non-toxic concentrations of cadmium inhibit PTGS and viral movement

We have previously shown that treatment of tobacco plants with-non-toxic concentrations of cadmium, that is 5–20 μM, blocked systemic movement of TVCV, whereas toxic levels of this heavy metal ion, that is 50–100 μM, allowed viral infection (Citovsky *et al.*, 1998; Ghoshroy *et al.*, 1998). Here, we examined how these cadmium treatments affect systemic and local spread of PTGS in a tobacco line 6b5 (Elmayan, T. and Vaucheret, 1996), carrying the bacterial *uidA* gene, which codes for β-glucuronidase (GUS) and whose expression is silenced post-transcriptionally throughout the plant, except for the apical meristem (Béclin *et al.*, 1998). Importantly, the 6b5 plants have been shown to generate and systemically transport PTGS signals that have been proposed to move from the lower to the upper parts of the plant (Palauqui *et al.*, 1997). Thus, expression of the *uidA* transgene in upper, younger and lower, mature leaves was compared between 6b5 plants grown in the absence of cadmium or in the presence of either non-toxic (10 μM) or toxic (100 μM) cadmium concentrations.

Figure 1(a) shows that little or no GUS activity was expressed in a leaf disk derived from the upper (third youngest) leaf of an untreated 6b5 plant. In contrast, the *uidA* gene expression was restored in the same leaf of a plant grown in the presence of 10 μM, resulting in a strong GUS staining (Figure 1b). Increasing the cadmium concentration to 100 μM, on the other hand, did not interfere with the silencing of the *uidA* gene (Figure 1c). Thus, non-toxic levels of cadmium blocked PTGS, whereas its toxic levels did not have this suppressing effect.

Unlike PTGS in the upper leaves, the *uidA* gene silencing in the lower, fully expanded leaves (Figure 1d) was not affected by exposure to 10 μM or 100 μM of cadmium (Figure 1e,f, respectively). These results support the idea that low levels of cadmium interfere with the systemic transport of PTGS signals, known to be critical for the onset of silencing (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998), rather than affecting PTGS cell autonomously (Figure 1d–f, see also Figure 5). Thus, we compared cadmium effects on PTGS suppression with those on viral systemic movement. Wild-type tobacco plants were infected with TVCV and viral movement into uninoculated systemic leaves was monitored by *in situ* hybridization using the TVCV cDNA (Lartey *et al.*, 1994; Lartey *et al.*, 1995) as probe. For better comparison with cadmium effects on PTGS, we chose the systemic leaves at the developmental stage equivalent to that used for determination of the *uidA* gene expression (Figure 1a–c). Figure 1(g) shows that TVCV genomic RNA efficiently accumulated in a systemic leaf of an untreated plant. No viral RNA was detected in systemic leaves of plants treated with 10 μM of cadmium (Figure 1h), indicating inhibition of the systemic movement. This inhibition did not occur when the infected plants were grown in the presence of 100 μM cadmium (Figure 1i). These results suggest that low levels of cadmium block a systemic transport step common to both PTGS signals and plant viruses.

Parallels between cadmium effects on PTGS and viral systemic movement in upper leaves were studied in more detail by comparing their dose responses with increasing cadmium concentrations. The *uidA* gene expression, that is suppression of PTGS, was assayed by determination of GUS activity, whereas the TVCV systemic spread was assessed by monitoring the accumulation of the viral coat protein (CP), which represents one of the hallmarks of TVCV infection (Lartey *et al.*, 1997) within the uninoculated leaves. For better comparison between these two processes, we used numerous concentration points over a relatively narrow range, followed by construction of the dose response curves. Figure 2 illustrates the dose response of GUS activity to cadmium concentrations, with the optimal levels of cadmium between 5 μM and 20 μM. Interestingly, this dose response was inversely related to that of the TVCV systemic movement (Figure 2). This very close reciprocity suggests that cadmium-induced suppression of PTGS closely parallels cadmium-induced inhibition in viral systemic transport.

We then investigated whether the effects of cadmium (atomic number 48) on PTGS and TVCV systemic movement could be mimicked by exposure of plants to a wide range of concentrations of ions other heavy and/or toxic metals, such as zinc (atomic number 30) (Williams *et al.*, 2000) and aluminum (atomic number 13) (Delhaize and Ryan, 1995; Kochian, 1995). Plants maintained in ⩾50 μM of zinc or aluminum developed toxicity symptoms similar to those described for cadmium poisoning, for example decrease in chlorophyll content, stunting and reduced root elongation (Barcelo *et al.*, 1988; Baszynski *et al.*, 1980; Ghoshroy *et al.*, 1998; Punz and Sieghardt, 1993) (data not shown). Table 1 shows that, unlike cadmium, neither zinc nor aluminum in the tested concentration range (1–100 μM) affected the *uidA* gene expression and TVCV CP accumulation in the systemic leaves. Thus, not all toxic metal ions are capable of eliciting plant responses that interfere with viral systemic spread and PTGS onset,

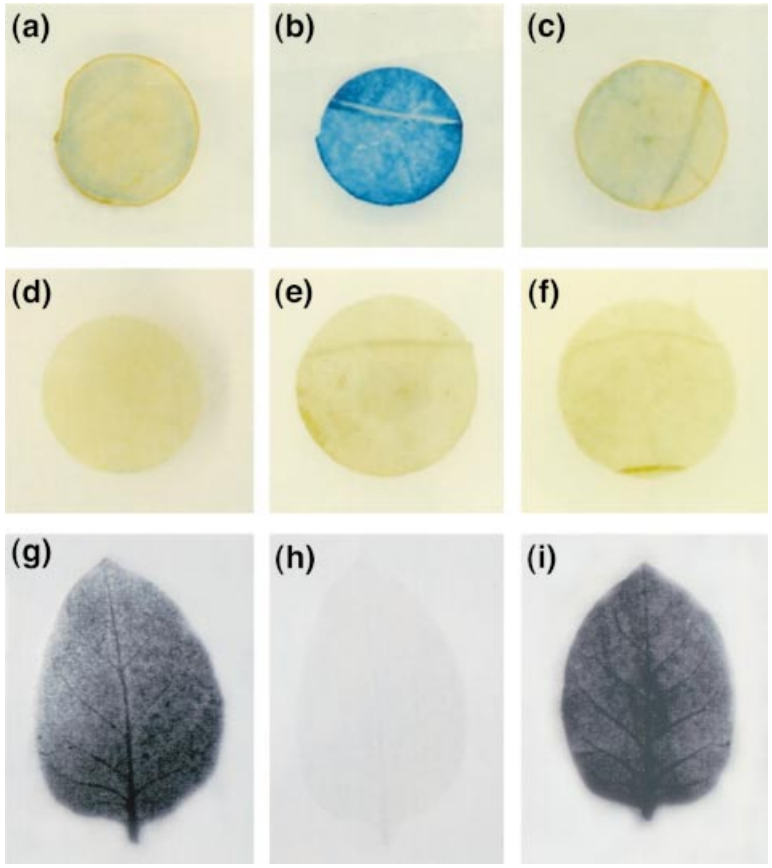

Figure 1. Non-toxic concentrations of cadmium suppress PTGS of the *uidA* transgene and inhibit systemic movement of TVCV.
For detection of PTGS, GUS activity was analyzed in leaf disks harvested from the third youngest leaf (a–c) or the fully expanded, mature leaf (d–f) of untreated plants (a, d), or plants treated with 10 μM (b, e) or 100 μM of cadmium (c, f). For detection of TVCV RNA, uninoculated leaves at the developmental stage equivalent to that used for detection of PTGS in panels a–c were harvested from TVCV-infected plants, which were grown either in the absence of cadmium (g)or in the presence of 10 μM (h)or 100 μM of cadmium (i), and analyzed by *in situ* hybridization and autoradiography.

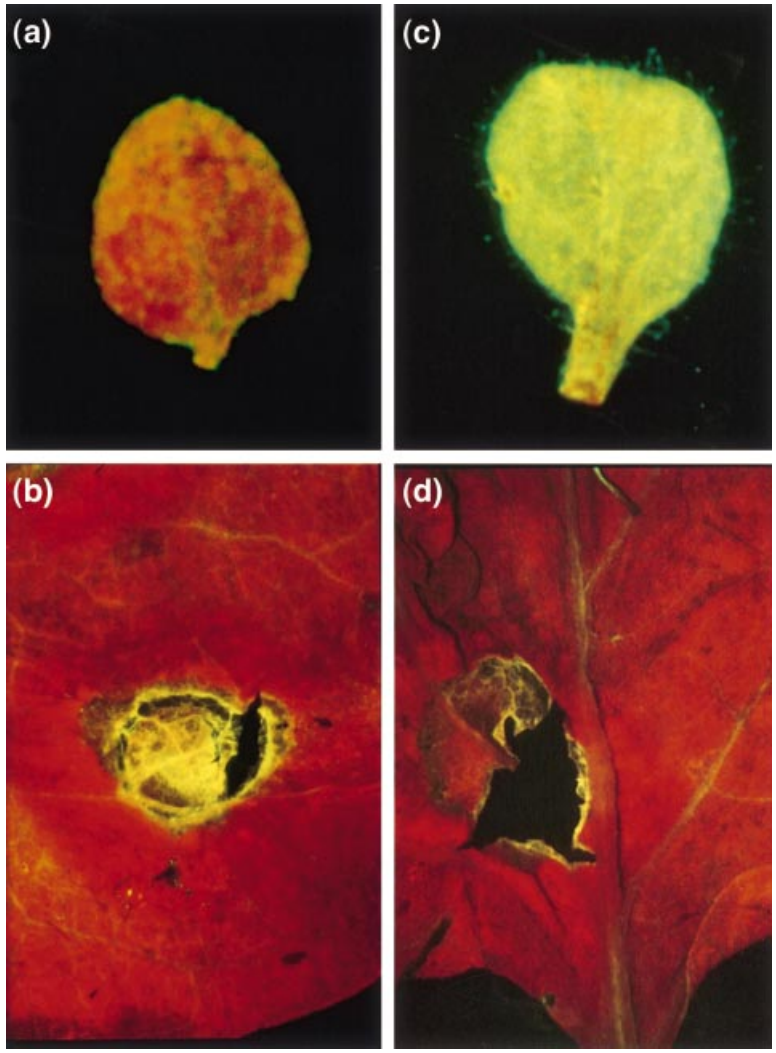

Figure 5. Inhibition of systemic but not local PTGS by non-toxic levels of cadmium.
In GFP-expressing plants grown in the absence of cadmium (a, b) or treated with 10 μM cadmium (c, d), lower leaves were inoculated with *Agrobacterium* to induce PTGS as described in Experimental procedures. Four weeks after inoculation, GFP fluorescence in the upper, systemic leaves (a, c) and in lower, inoculated leaves (b, d) was visualized under a fluorescent binocular microscope. Note that while the full size upper, uninoculated leaves are shown in panels (a) and (c), only parts of the much larger lower, inoculated leaves are presented in panels (b)and (d).The green signal represents the GFP-specific fluorescence, while the red signal represents chlorophyll fluorescence. Perforations on the lower leaves represent the sites of mechanical inoculation; yellow autofluorescence of the damaged cells is seen around these areas.

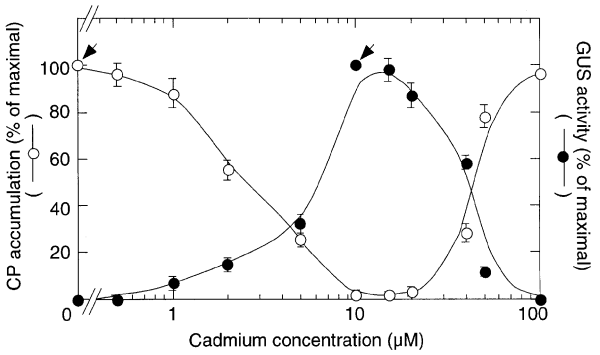

Figure 2. Dose effect of cadmium on GUS activity and accumulation of TVCV CP in the systemic tobacco leaves.
TVCV Cp accumulation (open circles) is expressed as a percentage of the maximal amount of CP within uninoculated leaves of cadmium-free plants (indicated by arrowhead). GUS activity (filled circles) is expressed as a percentage of the maximal activity measured in plants treated with 10 μM cadmium (indicated by arrowhead). Cadmium concentrations were plotted on a logarithmic scale, and dose response curves were constructed using the Smooth Curve Fit function of the KaleidaGraph™ (Abelbeck Software, Wellesley, MA, USA) software. According to the manufacturer's description, the basis of this statistical analysis is fitting of a curve to the data (i.e. cadmium concentrations versus the respective CP accumulation and GUS activity values) by application of a Stineman Function; the output of this function then has a geometric weight applied to each point ± 10% of the data to arrive at the smooth curve. All data represent average values of three independent measurements with indicated standard deviation values.

Table 1. Effects of zinc and aluminum on GUS activity and accumulation of TVCV CP in the systemic tobacco leaves

		(%)	
Metal ion	μM added[a]	CP accumulation[b]	GUS activity[c]
Zn^{2+}	0	100[b]	3 ± 1
Al^{3+}		100[b]	2 ± 1
Zn^{2+}	1	102 ± 3	2 ± 1
Al^{3+}		98 ± 8	5 ± 2
Zn^{2+}	2	102 ± 10[d]	5 ± 1
Al^{3+}		95 ± 8	8 ± 8
Zn^{2+}	5	101 ± 8	5 ± 6
Al^{3+}		96 ± 5	12 ± 8
Zn^{2+}	10	98 ± 11	5 ± 3
Al^{3+}		102 ± 3	8 ± 5
Cd^{2+}		2 ± 1	100[c]
Zn^{2+}	20	98 ± 4	6 ± 3
Al^{3+}		92 ± 8	8 ± 5
Zn^{2+}	50	109 ± 12	12 ± 10
Al^{3+}		95 ± 13	8 ± 4
Zn^{2+}	100	98 ± 6	4 ± 2
Al^{3+}		102 ± 5	4 ± 3

[a]Concentrations added to the Hoagland's solution, which already contains 1 μM $ZnSO_4$ as an essential microelement.
[b]TVCV Cp accumulation is expressed as percent of the maximal amount of CP within uninoculated leaves of untreated plants.
[c]GUS activity is expressed as percent of the maximal activity measured in unsilenced plants, i.e. plants treated with 10 μM cadmium.
[d]All data represent average values of three independent measurements with indicated standard deviation; yellow autofluorescence of the damaged cells is seen around these areas.

suggesting that this effect of cadmium may be relatively specific and/or unique.

Next, it was important to determine the fate of cadmium ions during treatments. Specifically we examined cadmium accumulation in roots, and young and mature leaves of *N. tabacum* plants treated with increasing concentrations of cadmium. These measurements were performed at two time points: 2 days and 2 weeks; the first time point corresponds to the length of cadmium treatment at the time of virus inoculation and the second time point corresponds to the length of cadmium treatment after which the levels of TVCV CP and GUS activity were determined (see Experimental procedures). The amounts of cadmium were quantified in dried tissues using atomic absorption spectroscopy. Figure 3 shows that, among the tobacco tissues tested, roots absorbed the highest amounts of cadmium and that these amounts correlated with the external concentrations of cadmium in the hydroponic solution. In contrast, cadmium accumulation in leaf tissues was relatively low. These results are consistent with previous observations (Ghoshroy *et al.*, 1998; Leita *et al.*, 1991) that most of the absorbed cadmium is retained in the plant roots and only low levels of this heavy metal ion are detected in leaf tissues. Cadmium levels accumulated within leaf tissues also appeared to be relatively invariable, showing only a slight increase over the tested range, that is toxic and non-toxic, of external cadmium concentrations. Furthermore, only small differences in cadmium accumulation were found between upper, young and lower, mature leaves. Both roots and leaves exhibited slightly but consistently higher accumulations of cadmium after prolonged (2 weeks) exposure to this heavy metal ion (Figure 3). Collectively, these results suggest that cadmium toxicity likely derives from its accumulation in the root system, and that leaves may have only a limited capacity to accumulate cadmium.

Cadmium treatment affects systemic but not local spread of Ptgs

PTGS propagates by two mechanisms: cell-to-cell (local) and systemic (Palauqui *et al.*, 1997; Vaucheret *et al.*, 1998; Voinnet *et al.*, 1998). By analogy to plant viruses, the local transport of PTGS signals, which are presumed to be RNA molecules (Fagard and Vaucheret, 2000; Hamilton and Baulcombe, 1999; Mourrain *et al.*, 2000; van der Boogaart *et al.*, 1998; Vaucheret *et al.*, 1998; Waterhouse *et al.*, 1998; Di Serio *et al.*, 2001), should occur much slower than their

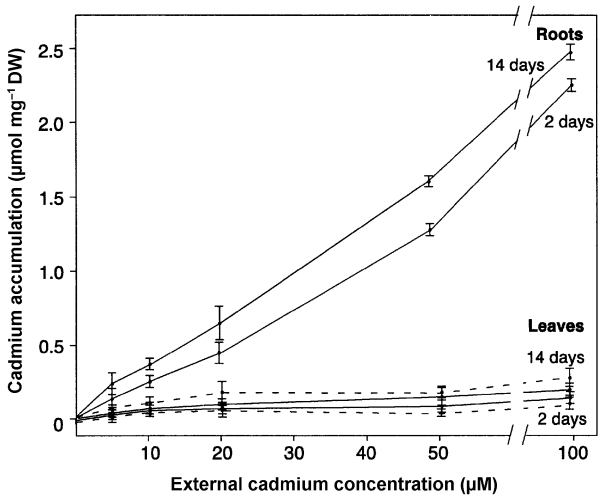

Figure 3. Cadmium accumulation in tobacco roots and leaves as a function of its external concentration in the growth medium.
The cadmium content within roots and leaves of the treated plants was determined by atomic absorption spectroscopy as described in Experimental procedures. All experiments were done in triplicate. Bars indicate standard deviations between samples. For cadmium content in the leaves, solid lines indicate samples taken from mature leaves and dashed lines indicate samples taken from young, upper leaves.

systemic transport through the vascular system (Gibbs, 1976). Also, viral systemic movement, which occurs with the flow of photoassimilates, first distributes the virions from the inoculated mature leaves to the sink tissues, such as the smallest upper leaves (Leisner and Howell, 1993). Thus, the onset of PTGS in the young leaves of 6b5 plants is likely due to the systemic movement of the signal molecules from the older leaves. In this case, removal of all leaves that function as sources of PTGS signals is expected to delay the *uidA* gene silencing in the first newly developed leaf; later, as this leaf grows, it will develop PTGS due to the local movement of the signals. These signals will then traffic systemically to the subsequently developing leaves, silencing their *uidA* gene expression. This systemically induced *uidA* gene silencing is expected to be blocked by treatment with 10 μM of cadmium.

To test this hypothesis, we ablated all leaves from 6b5 plants (Figure 4a, step I), leaving the apical meristem intact (step II), harvested the first-to-develop leaf for determination of GUS activity (step III, leaf 1), allowed the second leaf to grow (step IV, leaf 2) and quantitatively analyzed GUS expression in the expanded second and newly developed third leaf (step V, leaves 2 and 3). Figure 4(b) demonstrates that, in the absence of cadmium, the first leaf (1) to develop after all leaf ablation expressed GUS activity, while a similar size leaf in intact, non-ablated 6b5 plants was already completely silenced (Figures 4b, 4). The second leaf (2), which was allowed to grow further, developed PTGS, resulting in a much weaker GUS activity (Figure 4b). Importantly, the *uidA* gene in the third leaf (3) was largely silenced already early in the leaf development (Figure 4b). Treatment of plants with 10 μM cadmium did not affect GUS expression in the first (1) and second leaves (2) but efficiently blocked PTGS in the third leaf (3) (Figure 4b), suggesting the specific arrest in PTGS systemic transport in these plants. Statistical evaluation of the results in Figure 4(b) by the unpaired two tailed Student's *t*-test confirmed that GUS activity values in leaves (3) were significantly different between untreated and cadmium-treated plants, whereas the GUS activity in leaves (1) and (2) did not exhibit a statistically significant difference between these plants. In control experiments, quantifying GUS activity in the intact plants, treatment with 10 μM cadmium blocked PTGS in the young leaf (4) but had little effect on the mature leaves (5) (Figure 4b), arguing against its cell autonomous action (Figure 1).

The leaf ablation approach was then used to examine whether the effects of cadmium on transport of PTGS signals from leaf (2) to leaf (3) correlate with its effects on viral systemic movement between the equivalent leaves. To this end, leaf (2) at step IV of the experiment (Figure 4a) was inoculated with TVCV and the amount of viral CP was quantified at the same time point when GUS activity was determined in the equivalent leaves [i.e. leaves (2) and (3), see Figure 4(a), step V]. Figure 4(c) shows that TVCV CP accumulated in the inoculated leaf (2), probably as a result of the local, cell-to-cell spread of the virus within this leaf. In the absence of cadmium, TVCV then spread systemically, into the leaf (3). Exposure to 10 μM cadmium, however, prevented this systemic movement, leaving leaf (3) almost free of TVCV CP in cadmium-treated plants (Figure 4c). As expected, in control experiments using non-ablated plants, TVCV moved locally, within the inoculated leaf (5) in both cadmium-treated and untreated plants, but spread systemically, into the upper leaf (4) only in cadmium-treated plants (Figure 4c). These observations therefore further support correlation between cadmium effects on PTGS and viral systemic spread.

Finally, we directly assayed the effects of non-toxic concentrations of cadmium on local and systemic spread of PTGS signals using an inducible PTGS system (Voinnet and Baulcombe, 1997; Voinnet *et al.*, 1998). In this approach, a green fluorescent protein (GFP) transgene reporter is expressed in a transgenic line 16c but it becomes silenced following induction of PTGS by inoculating one of the lower plant leaves with *Agrobacterium*, introducing an additional copy of the GFP gene, which, in turn, silences the GFP expression throughout the plant (Voinnet and Baulcombe, 1997; Voinnet *et al.*, 1998). Thus, this inducible system allowed us to distinguish between local and systemic movement of PTGS signals by monitoring GFP silencing within the induced (*Agrobacterium*-inoculated) leaf and uninduced, systemic leaves, respect-

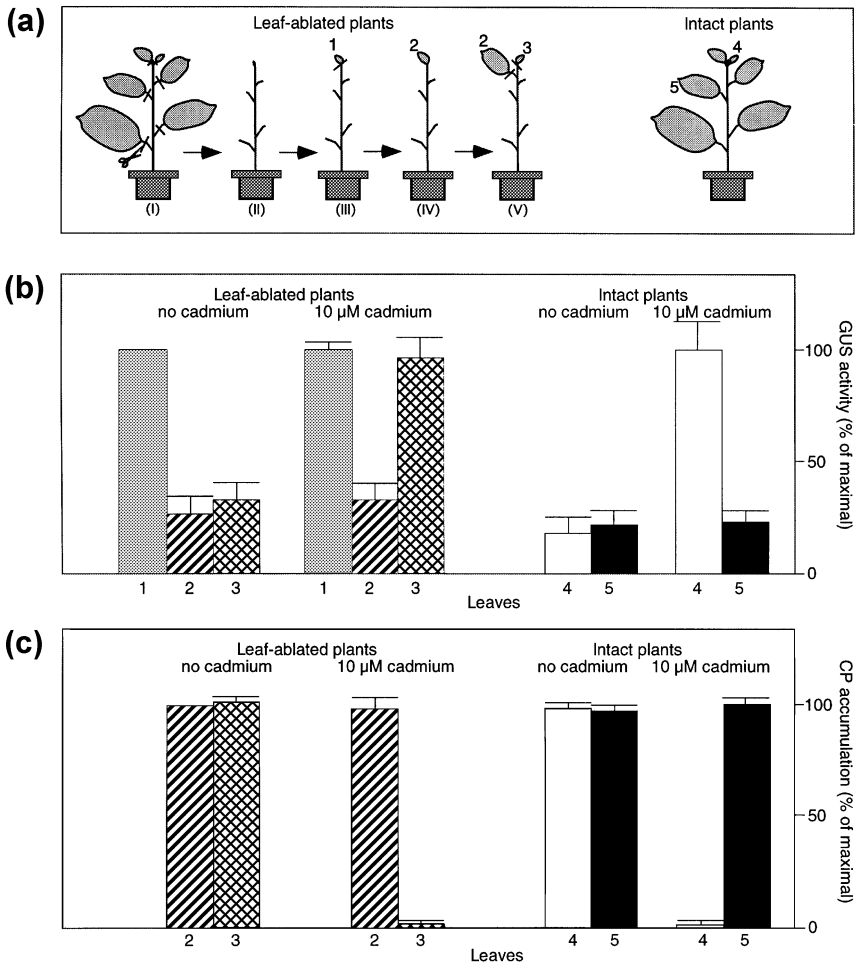

Figure 4. Effect of leaf ablation on expression of GUS activity and TVCV CP accumulation in untreated and cadmium-treated plants.
(a) A schematic representation of the experimental design. For details, see text.
(b) Levels of GUS activity. GUS activity is expressed as a percentage of the maximal activity measured in the first new leaf of cadmium-free plants.
(c) TVCV CP accumulation. CP amounts are expressed as a percentage of the maximal amount of CP within uninoculated leaves of cadmium-free plants. All data represent average values of three independent measurements. Bars 1, 2, 3, 4, and 5 correspond to leaves 1, 2, 3, 4, and 5 indicated in panel (a).

ively. Figure 5 shows that, in the absence of cadmium, inoculation of the larger, lower leaf with *Agrobacterium* carrying the GFP gene in its T-DNA region resulted in efficient GFP gene silencing both in the inoculated leaf (panel b) and in the upper, systemic leaf (panel a). Treatment with 10 μM of cadmium completely suppressed the GFP gene silencing in the upper leaf, resulting in GFP fluorescence (Figure 5c) virtually identical to that observed in non-silenced, uninfected plants (data not shown). Importantly, however, cadmium had no effect on GFP silencing within the inoculated leaf (Figure 5d), which developed PTGS indistinguishable from that observed in the absence of cadmium (Figure 5b). These results strongly suggest that cadmium treatment does not affect the local spread of PTGS within the induced (inoculated) leaf, but blocks the systemic onset of PTGS in the uninduced leaves.

Discussion

To better understand the mechanism by which PTGS signals spread within plants, it would be useful to develop an inhibitor of this process. Here, we report that non-toxic concentrations of cadmium act as such a specific inhibitor in two independent PTGS systems, a constitutive and an inducible system. Development of PTGS often depends on the size of the plants, with larger and older plants showing better silencing (Elmayan and Vaucheret, 1996). Such developmental effects, however, could not account for cadmium-induced PTGS suppression because both untreated and cadmium-treated plants developed the same number of new leaves during the same growth period (data not shown, but see Figure 4). This observation that low levels of cadmium are indeed non-toxic and have no or little effect on plant growth is consistent with our previous studies, which demonstrated that 10 μM cadmium treatment does not affect the ultrastructure of cellular organelles, plant growth and leaf chlorophyll content (Ghoshroy *et al.*, 1998). Also, it is unlikely that cadmium inhibits PTGS simply by inactivating cellular enzymes, for example RNA-dependent RNA polymerases, thought to be involved in PTGS (Hamilton and Baulcombe, 1999; Mourrain *et al.*, 2000). Such putative inhibition should have been even more effective following treatment with higher amounts of cadmium which, in reality, had no effect on PTGS. Thus, cadmium treatment most likely

induces an active plant response to this toxic metal ion, which in turn suppresses the spread of PTGS.

The pattern of cadmium-induced inhibition of PTGS allowed several insights into PTGS development. First, cadmium treatment prevented PTGS in young, systemic leaves, probably due to the block in the systemic transport of PTGS signals. Alternatively, exposure of plants to cadmium may have interfered with the perception of the systemic PTGS signals in the target leaves. This latter possibility assumes that systemic PTGS signals are transported into the young leaves of the cadmium-treated plants but are unable to elicit gene silencing, whereas perception of local PTGS signals, which are generated and transported within the same leaves as they develop and grow, is not affected. In any case, cadmium treatment did not impair the slower onset of PTGS as a result of cell-to-cell spread or cell autonomous generation of PTGS signals within larger leaves. Thus, cadmium treatment uncoupled between these two modes of PTGS. In this regard, cadmium likely suppressed PTGS by a mechanism different from that recently reported for PTGS suppression following TVCV infection, which affected gene silencing in a cell autonomous fashion (Mourrain *et al.*, 2000).

Second, only non-toxic levels of cadmium inhibited PTGS, whereas higher, toxic concentrations had no effect. One possible scenario is that cadmium at low concentration induces a plant response, altering the cell walls and interfering with transport of viruses and PTGS signals, whereas at high concentration the metal ion toxicity interferes with additional cellular components, for example transcription of key genes, and represses this active response. This toxic effect of cadmium most likely originated in the plant roots because they absorbed cadmium proportionally to its external concentration, whereas cadmium levels in the leaves remained low and relatively unchanged both under toxic and non-toxic conditions. On the other hand, the inhibitory effect of cadmium on PTGS and TVCV systemic spread may either also initiate in the root or be elicited by the low levels of accumulation in the leaf tissue; in the latter case, under toxic conditions, cadmium-induced PTGS and TVCV movement inhibition originating from the leaves would be negated by the cadmium-induced toxicity originating from the roots.

Third, cadmium effects on PTGS closely paralleled those on the movement of tobamoviruses. Specifically, only systemic but not local viral spread was inhibited, and only low but not toxic concentrations of cadmium showed this inhibitory action. Dose response curves of PTGS and viral transport to cadmium concentrations also coincided, suggesting that a common step in both processes may have been affected by cadmium treatment. Interestingly, the effect of cadmium on transport of TVCV and onset of PTGS did not represent general property of toxic metal ions because a wide range of concentrations of ions of two other such elements, that is zinc and aluminum, did not interfere with TVCV infection or PTGS. Note, that these experiments employed two different cultivars of tobacco; specifically, PTGS was monitored in the Paraguay cultivar, which harbors the N gene (H. Vaucheret, pers. comm.) that confers resistance to tobamoviruses (Erickson *et al.*, 1999) and thus precludes studies of TVCV systemic movement, whereas the viral systemic spread was examined in the Turk cultivar, which is susceptible to TVCV systemic infection (Ghoshroy *et al.*, 1998).

The availability of cadmium as a specific suppressor of viral and PTGS systemic transport may provide a unique tool to define the molecular mechanisms of these processes and identify cellular proteins comprising the transport pathway(s). Also, further elucidation of the cadmium effect on PTGS and viral transport may allow the design of new strategies for suppression of PTGS of highly expressed transgenes and for production of virus-resistant plants; these approaches will target the host factors required for the systemic transport of PTGS signals and virus genomes.

This study may also help to understand the physiological effects of plant exposure to cadmium better. Unlike animals, plants cannot physically escape from changes in their environment; instead, they have developed a wide variety of complex responses aimed at alleviating the stress conditions. One such stress conditions is caused by heavy metal ions, such as cadmium, which have become major environmental pollutants that affect plants from the advent of industrial development. Many studies have documented cadmium toxicity to plants (Prasad, 1995). Here, we show that exposure to low levels of cadmium may also benefit plants, boosting their defense against the systemic spread of foreign molecules, such as transgene-silencing signals and viral genomes.

Experimental procedures

Plant material, metal ion treatments, and induction of PTGS

Seeds of wild-type tobacco plants (*Nicotiana tabacum* cv. Turk), constitutively silenced GUS transgenic tobacco line 6b5 (*N. tabacum* cv. Paraguay) (Elmayan and Vaucheret, 1996), and inducibly silenced GFP transgenic line 16c (*N. benthamiana*) (Voinnet *et al.*, 1998), were germinated and grown in vermiculite. In the case of wild-type tobacco and the line 6b5 plants, the 3–4-wk-old seedlings of similar height and with equal number of leaves were removed from vermiculite and grown hydroponically in a modified Hoagland's solution (Salt *et al.*, 1995) under controlled humidity (50%), temperature (24–28°C) and photoperiod (16 h of light) for 4–5 days before addition of cadmium. The nutrient solution was continuously aerated with an air pump and changed every 3 days. Cadmium ions were added in the form of $CdCl_2$ and the plants were maintained for 3–4 days before inoculation with virus, or for 2 wk for determination of β-

glucuronidase (GUS) activity. For investigating the effects of zinc and aluminum, cadmium in the hydroponic medium was substituted with these metal ions in the form of $ZnSO_4$ and $AlCl_3$, respectively, as described (Larsen *et al.*, 1996; Pence *et al.*, 2000). In the case of $AlCl_3$, the hydroponic solution was maintained at pH < 5.0 to preserve the trivalent aluminum species (Delhaize and Ryan, 1995); this slight lowering of pH in itself had no effect on PTGS or on TVCV movement (data not shown).

In the case of the line 16c plants, the seedlings were transferred to hydroponic solution when they were at the 3–4 leaf stage and then maintained and treated with cadmium as described above for 3–4 days. For induction of PTGS, a lower, fully expanded leaf of the line 16c plant was infiltrated with the *Agrobacterium tumefaciens* strain harboring the GFP sequence under the control of the 35S CaMV promoter (Voinnet *et al.*, 1998) and the plants were maintained for an additional 4 wk; this time period is required to detect local and systemic propagation of GFP PTGS in these plants (Voinnet *et al.*, 1998). GFP fluorescence was monitored using a fluorescence binocular microscope equipped with a long-wave ultraviolet light.

GUS assays

For histochemical detection of GUS activity, the tobacco leaves were removed, stained with the chromogenic substrate X-Gluc, and depleted of chlorophyll by ethanol extraction as described (Nam *et al.*, 1999). The intensity of indigo dye formed during the GUS assay in the stained leaves was quantified by photodensitometry as described (Citovsky *et al.*, 1992; Howard *et al.*, 1992). For statistical evaluation, mean values and standard deviations were calculated based on five independent measurements, that is $n = 5$. To determine whether any two sets of measured values were statistically different from each other, data were subjected to the unpaired two tailed Student's *t*-test. For this test, a combined $n = 10$ (derived from the two sets of GUS activity measurements to be compared) was used. 2P values less then 0.01, corresponding to the statistical probability of greater than 99%, were considered statistically significant.

Preparation of virus and inoculation of plants

TVCV was purified as described (Lartey *et al.*, 1993). Two mature, lower leaves of *N. tabacum* cv. Turk plants located at the same level in each plant were mechanically inoculated by rubbing 20 μl of TVCV suspension (5 μg ml^{-1} of viral protein) on each leaf. The infected plants were then allowed to grow for 2 wk in the presence or absence of cadmium.

TVCV CP assay and in situ *hybridization*

For detection of TVCV CP, leaf samples (0.2 g FW) were harvested, extracted and analyzed for the presence of CP by SDS polyacrylamide gel electrophoresis as described (Citovsky *et al.*, 1998; Ghoshroy *et al.*, 1998). Following electrophoresis, TVCV CP was visualized by Coomassie blue staining and quantified by scanning densitometry of the stained gels (Citovsky *et al.*, 1998).

For detection of TVCV RNA, tobacco leaves were analyzed by a modified leaf skeleton hybridization procedure (Lartey *et al.*, 1997; Melcher *et al.*, 1981) using a ^{33}P-labeled TVCV cDNA (Lartey *et al.*, 1994; Lartey *et al.*, 1995) as probe as described previously (Ghoshroy *et al.*, 1998). After hybridization, the leaves were air dried and autoradiographed.

Cadmium determination

Roots and young (second and third upper) and mature, fully expanded leaves from cadmium-treated plants were dried and ground to a fine powder. The dried samples were weighed and dissolved in 50 ml of acidified water for several hours at room temperature. The resulting liquid was filtered through two layers of cheese cloth and brought to 100 ml with distilled water. The amount of cadmium present in the samples was determined using a Perkin Elmer atomic absorption spectrophotometer (Synergy Software, Reading, PA, USA).

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