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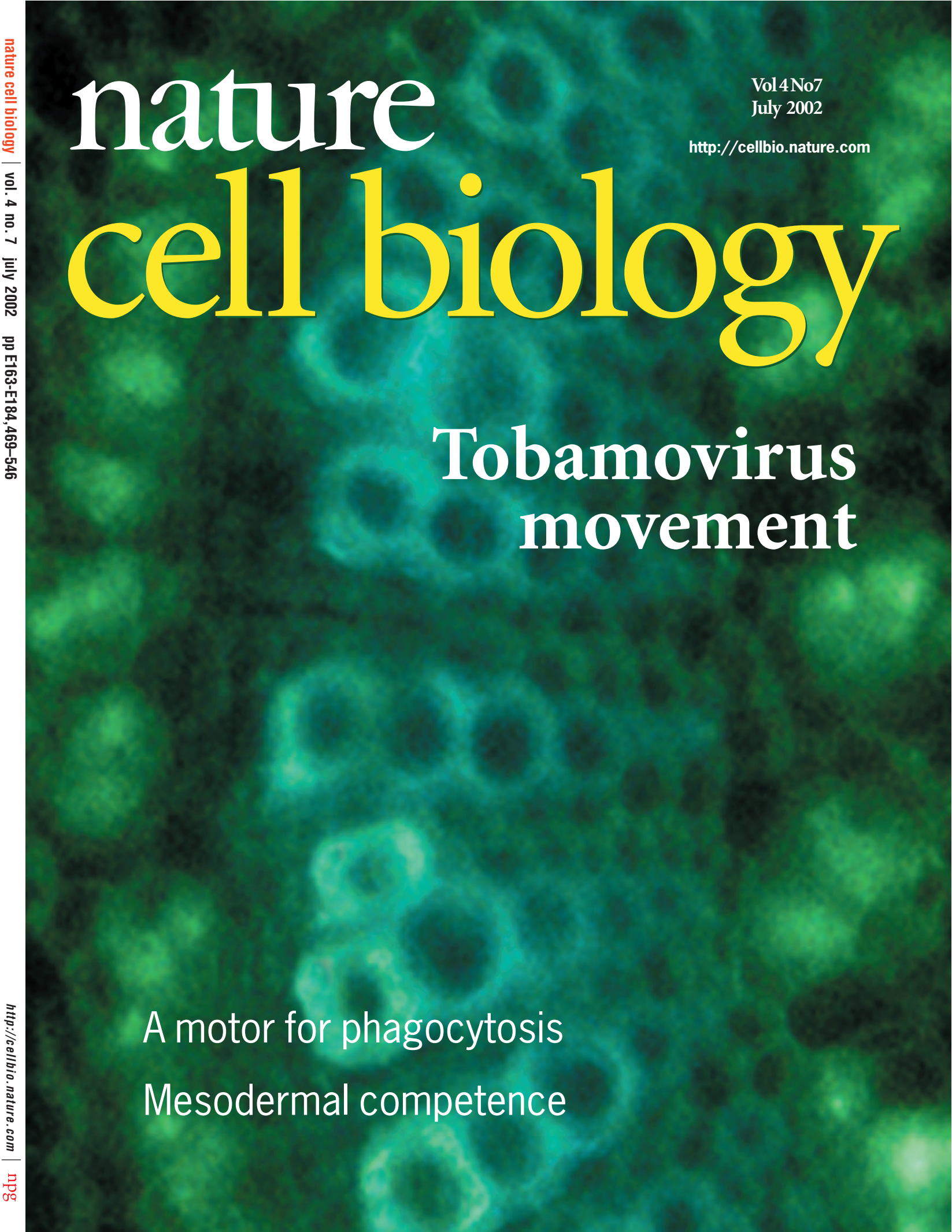

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The systemic movement of a tobamovirus is inhibited by a cadmium-ion-induced glycine-rich protein

Shoko Ueki and Vitaly Citovsky*

**Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794-5215, USA*
e-mail: vitaly.citovsky@stonybrook.edu

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Systemic movement is central to plant viral infection. Exposure of tobacco plants to low levels of cadmium ions blocks the systemic spread of turnip vein-clearing tobamovirus (TVCV). We identified a tobacco glycine-rich protein, cdiGRP, specifically induced by low concentrations of cadmium and expressed in the cell walls of plant vascular tissues. Constitutive cdiGRP expression inhibited systemic transport of TVCV, whereas suppression of cdiGRP production allowed TVCV movement in the presence of cadmium. cdiGRP exerted its inhibitory effect on TVCV transport by enhancing callose deposits in the vasculature. So cdiGRP may function to control plant viral systemic movement.

After initial infection, plant viruses replicate and spread locally, from cell to cell through the 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 nonvascular tissues of uninoculated organs (reviewed in refs 1–3). In the case of tobamoviruses, arguably the best studied family of plant viruses[4], the viral movement protein (MP), coat protein (CP), and a replicase component participate in these transport processes[5–7]. Most of the molecular components of the presumably complex pathways of the viral intercellular transport are, therefore, probably represented by factors in the host cell.

Unlike several cellular proteins, such as tubulins[8,9], protein kinases[10,11] and pectin methylesterases[12,13], implicated in the cell-to-cell movement of tobamoviruses, host factors that control viral systemic transport remain largely unknown. Potentially, such cellular proteins include those responsible for the recently demonstrated specific inhibition of systemic but not local transport of tobamoviruses, for example, tobacco mosaic virus (TMV) and TVCV, in plants treated with non-toxic amounts of ions of the heavy metal cadmium[14,15]. Interestingly, this inhibitory effect of cadmium ions was tissue-specific because, in the uninoculated leaves, the virions accumulated in the host vasculature but were unable to exit to the surrounding non-vascular tissues[15]. Because exposure of plants to higher, toxic cadmium-ion concentrations restored viral systemic movement[14], low levels of cadmium ions probably affect production of host factors that control viral systemic transport whereas cadmium-ion poisoning prevented this response. Here, we report identification and characterization of one such host factor, a glycine-rich protein, that is specifically induced by non-toxic levels of cadmium ions, and expression of which inhibits the systemic movement of TVCV, probably by inducing callose accumulation in the phloem cell walls.

Results

Identification of a tobacco glycine-rich protein induced by non-toxic but not toxic concentrations of cadmium ions. To better understand the effect of cadmium ions on viral movement, it would be useful to identify cellular proteins induced or repressed by low (10 μM) but not high concentrations (100 μM) of cadmium ions. To this end, we performed a polymerase chain reaction (PCR)-based subtraction cloning between cDNAs obtained from tobacco plants treated with 10 μM cadmium ions and control, untreated plants. For induced genes, the cDNA from the untreated plants was subtracted from that of the treated plants whereas, for repressed genes, the reciprocal subtraction was performed. This latter experiment did not identify any cDNA clones (data not shown), suggesting that this treatment — exposure of plants to 10 μM cadmium ions — does not cause strong repression of genes. In contrast, the subtraction experiments for gene induction yielded 96 candidate cDNA clones, expression of which was then examined by two sequential procedures to identify clones preferentially induced by treatment with 10 μM but not 100 μM cadmium ions. First, these clones were subjected to reverse northern hybridization[16]. Figure 1a illustrates the analysis of seven of these clones with three separate probes comprising labelled cDNA synthesized from the RNA obtained from untreated plants (column 1), plants treated with 10 μM cadmium ions (column 2), or plants treated with 100 μM cadmium ions (column 3). Some of the subtracted cDNA clones were not significantly induced by either concentration of cadmium (for example, clones a, b, and e), others were induced to similar levels by both 10 μM and 100 μM of cadmium ions (for example, clone g), whereas thirteen cDNAs (for example, clones c, d, and f) showed enhanced induction at 10 μM cadmium ions. These positive clones were then used as probes in a conventional northern-blot analysis, and two cDNAs were identified, expression of which were significantly enhanced (10–20-fold as quantified by densitometry of northern blots) in plants treated with 10 μM cadmium ions (Fig. 1b, lane 2) as compared with untreated plants or plants treated with 100 μM cadmium ions (Fig. 1b, lanes 1 and 3, respectively). The nucleotide sequence analysis revealed that both clones represent the same gene, which encodes a protein product with a glycine-rich (GRP) motif that is present in several other proteins in different tobacco cultivars[17–19]. So we called this protein cdiGRP (cadmium-ion-induced GRP). Figure 1c shows that the predicted amino-acid sequence of cdiGRP contains three known functional motifs: an amino-terminal secretion signal (residues 1–20), an internal glycine-rich domain (residues 47–75), and a carboxy-proximal cysteine-rich domain (residues 76–95) that may target proteins to the cell wall[18].

cdiGRP is expressed in vasculature and localized to the cell walls. Next, we examined the expression patterns of cdiGRP in tobacco

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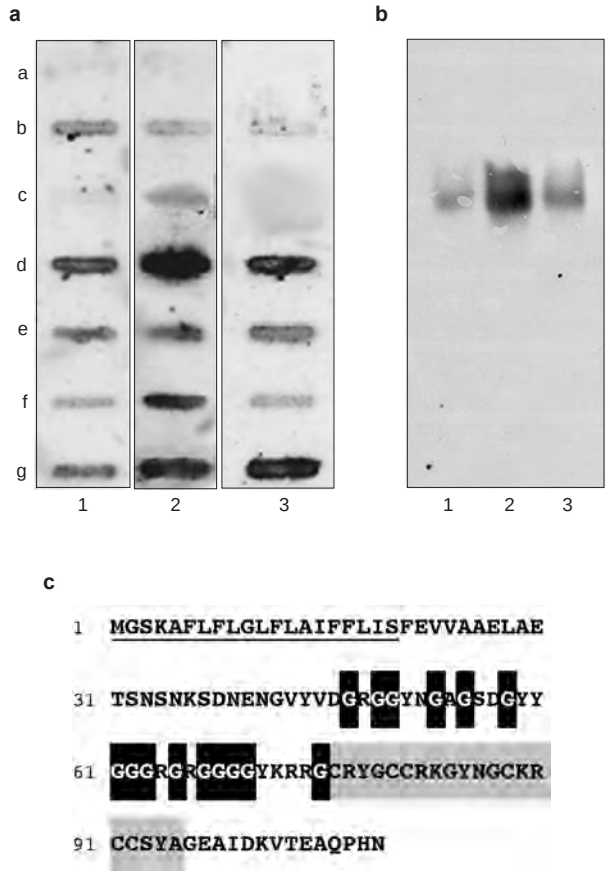

Figure 1 **Identification of *cdiGRP*. a,** A representative result of reverse northern screening of a subtraction library for cellular transcripts induced by low but not by high concentrations of cadmium ions. Letters on the left indicate individual subtraction cDNA clones. Note that both d and f clones are the cdiGRP sequence. Columns 1, 2, and 3 indicate the subtraction library clones probed with cDNA from untreated plants, and plants treated with 10 μM and 100 μM of cadmium ions, respectively. **b,** Northern blot analysis of *cdiGRP* gene expression in untreated (lane 1), and 10 μM (lane 2) and 100 μM cadmium-ion-treated plants (lane 3). Each lane contained 15 μg of total RNA. The size of the detected *cdiGRP* transcript is 700 base pairs. **c,** The amino acid sequence of cdiGRP. The N-terminal secretion signal sequence is underlined, the glycine residues of the glycine-rich domain are indicated by black boxes, and the C-proximal cysteine-rich cell-wall-targeting domain is indicated by a gray box.

leaves by immunostaining with anti-cdiGRP antibodies followed by a inodicarbocyanine (Cy5)-conjugated secondary antibody and confocal fluorescence microscopy. Because Cy5 is excited near 650 nm and fluoresces near 670 nm, this method substantially circumvents the autofluorescence usually associated with the plant cell cytoplasm. In confocal optical sections of young, upper leaves of untreated plants, only very low levels of cdiGRP were detected in the vascular bundles of the leaf and none at all in the non-vascular cells (Fig. 2a,b). Exposure to 10 μM cadmium ions substantially increased the cdiGRP expression in the vascular bundles but not in the surrounding non-vascular cell layers (Fig. 2c,d). These elevated levels of cdiGRP were found in all three classes of leaf veins, that is, class I (Fig. 2c,d), and classes II and III (data not shown), known to be involved in viral systemic spread[20,21]. Higher magnification images showed that cdiGRP predominantly accumulated at the cell periphery of the expressing phloem cells (Fig. 2g,h). Identical patterns of cdiGRP accumulation were found in mature, lower leaves (data not shown). In control experiments using preimmune serum, no fluorescent signal was detected (Fig. 2e,f). Although we cannot rule out that the enhanced immunofluorescence staining in cadmium-ion-treated tissues may also derive from other, as yet unidentified cdiGRPs, this possibility seems unlikely because our subtraction cloning experiments isolated the same cdiGRP cDNA twice (see Fig. 1a), and did not identify any other GRP clones, suggesting that our cdiGRP is the only or, at least, the most abundant species of this cadmium-ion-induced protein.

The presence of the cysteine-rich cell-wall targeting domain within cdiGRP (see Fig. 1c) and the location of this protein along the periphery of the vascular tissue cells (Fig. 2d,h) suggest that cdiGRP may reside in the cell wall. To test this idea, we used immunoelectron microscopy and polyclonal antibodies generated against the purified cdiGRP to examine the subcellular localization of this protein in the leaf phloem of tobacco plants treated with 10 μM cadmium ions. We observed cdiGRP-specific staining in the cell wall (CW) but not in the cytoplasm, chloroplasts (CHL), plastids (P), vacuole (V), nucleus, or mitochondria (Fig. 2i and data not shown) in sieve elements (SE) and companion cells, the two major type of phloem cells. No cell wall-specific staining was observed using preimmune antiserum (data not shown).

Effects of constitutive antisense and sense expression of cdiGRP on viral systemic movement. To study the biological role of cdiGRP in viral systemic infection *in planta*, we generated transgenic tobacco plants constitutively expressing the *cdiGRP* cDNA in the antisense or sense orientation. We wanted to study the effects of the lack of cdiGRP in the presence of cadmium ions with the antisense plants, whereas constitutive sense expression allowed us to elucidate the effects of elevated levels of this protein in the absence of cadmium ions. We produced 20 and eight independently transformed antisense and sense lines, respectively, and two lines of each type were analysed in detail.

Northern-blot analysis of total RNA obtained from both lines of the antisense plants, designated cdiGRP AS1 and cdiGRP AS2, detected only very low levels of the *cdiGRP* transcript, irrespective of the presence of cadmium ions (Fig. 3). As expected, the wild-type plants showed a significant increase in expression of *cdiGRP* in the presence of 10 μM cadmium ions (Fig. 3a). That samples were equally loaded was confirmed by assessing the amount of ribosomal RNA (rRNA) in all lanes (Fig. 3a). So antisense expression of the *cdiGRP* cDNA in transgenic tobacco substantially reduced cadmium-ion-induced transcription of the endogenous *cdiGRP* gene. Note that the constitutive, residual levels of the *cdiGRP* transcript detected in the antisense lines probably represented the antisense transcripts because these experiments used a double-stranded *cdiGRP*-specific probe to detect both sense and antisense transcripts in a single hybridization.

Next, the cdiGRP antisense plants were tested for their ability to support TVCV systemic movement in the presence or absence of cadmium ions. Untreated and cadmium-ion-treated AS1 and AS2 plants were inoculated with TVCV, and viral systemic movement was assessed by detecting TVCV CP, a known hallmark of TVCV infection[22], in uninoculated leaves[14,15]. CP was identified by western-blot analysis using anti-TVCV CP antibodies (Fig. 3b). Figure 3b shows that the wild-type plants, which supported systemic movement of TVCV in the absence of cadmium ions, became largely resistant to the viral systemic spread when treated with 10 μM cadmium ions, resulting in negligible levels of CP in their uninoculated leaves. In contrast, the uninoculated leaves of the AS1 and AS2 lines accumulated high amounts of CP both in the absence and in the presence of cadmium ions (Fig. 3b). This CP accumulation was comparable, that is, 98–110%, to that found in the untreated wild-type plants, indicating efficient systemic movement of TVCV in the cdiGRP antisense plants in the presence of 10 μM cadmium ions. These results imply that cdiGRP is a cellular factor responsible, at least partly, for the cadmium-ion-induced inhibition of TVCV systemic movement.

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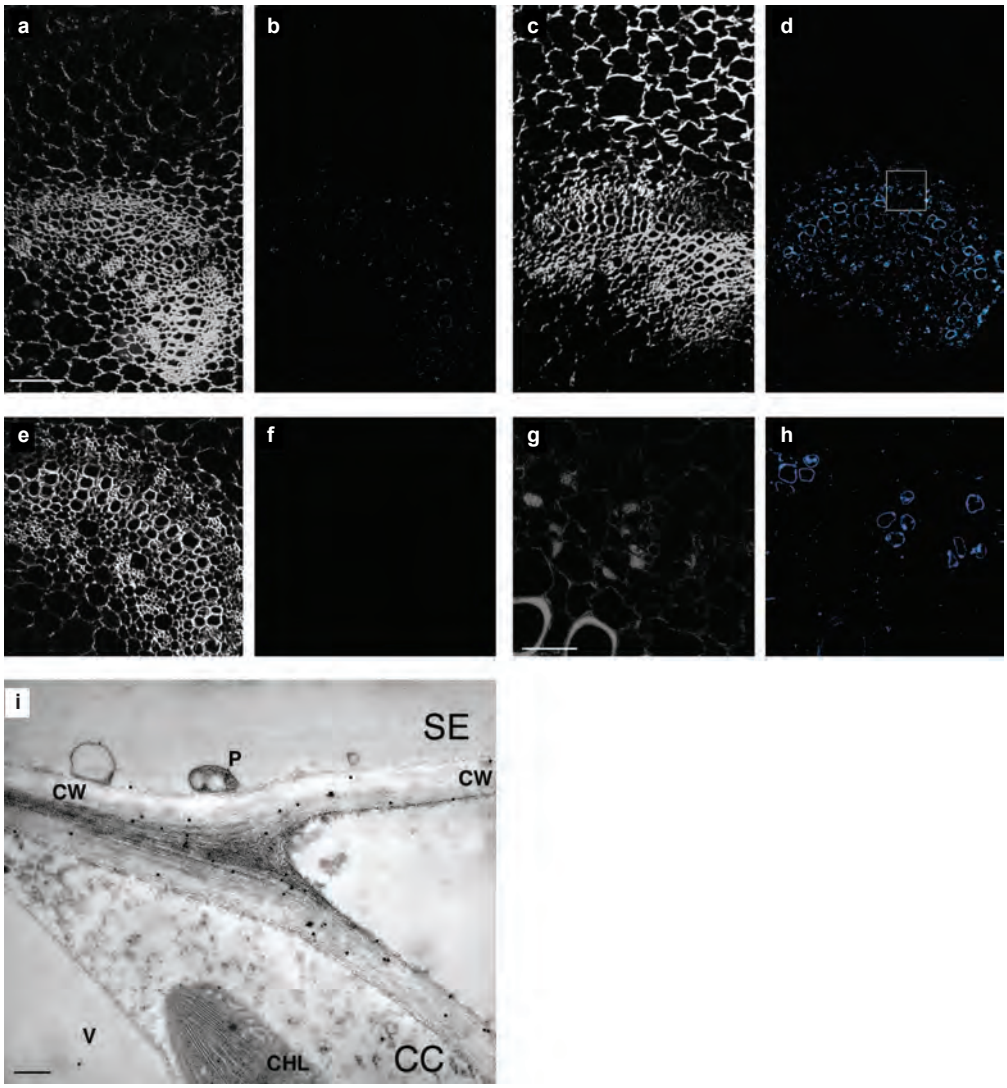

Figure 2 **Cadmium-ion-induced tissue-specific expression of cdiGRP in the cell walls of vascular tissues. a,b** Leaf sections of untreated plants probed with anti-cdiGRP antibody. **c,d** Leaf sections of 10 μM cadmium-ion-treated plants probed with anti-cdiGRP antibody. **e,f** Leaf sections of 10 μM cadmium-ion-treated plants probed with preimmune antibody. **g,h,** Higher magnification images of the area indicated by the white rectangle in **d**. **a**, **c**, **e** and **g** are phase-contrast images, and **b**, **d**, **f** and **h** are Cy5 confocal fluorescence images. Note that vascular tissue is seen as smaller cells arranged in bundles, whereas non-vascular tissue is represented by larger cells surrounding the vascular bundles. Scale bars, 100 μm (**a–f**) and 20 μm (**g,h**). **i,** Immunohistochemical detection of cdiGRP in cell walls of the tobacco leaf phloem. SE, sieve elements; CC, companion cells. Note that SE are identified by the presence of P-plastids (P)[50], characteristic round-shaped organelles associated with the cell wall (CW), whereas CCs contain a vacuole (V) and chloroplasts (CHL). Scale bar, 0.5 μm

To further explore the potential involvement of cdiGRP in regulating viral systemic movement, we analysed the transgenic plant lines, designated cdiGRP S1 and cdiGRP S2, that constitutively express the *cdiGRP* cDNA in the sense orientation. Figure 4a shows that the *cdiGRP* message in these plants accumulated to high levels that were comparable to those induced in 10 μM cadmium-ion-treated wild-type plants. (Assessing rRNA levels confirmed that the RNA samples were equally loaded; Fig. 4a). Then, TVCV systemic movement in these plants was examined using the CP western-blot assay. When inoculated with TVCV, both sense plant lines were remarkably resistant to viral systemic infection. Even long after inoculation — 14 and 35 days — CP accumulation in the uninoculated leaves of the cdiGRP S1 and S2 plants was significantly reduced, reaching only 5–20% of that observed in the infected wild-type plants (Fig. 4b). This degree of inhibition of TVCV systemic infection was confirmed by western-blot analysis of the CP bands and by northern-blot analysis with the viral genomic cDNA as probe[23] (data not shown). Because in all cdiGRP S1 and S2 plants TVCV systemic spread was substantially reduced, but not entirely blocked, suggests that the sense expression of cdiGRP did not generate or sustain protein concentrations required to completely inhibit viral movement. Alternatively, in cadmium-ion-treated plants, which consistently showed somewhat higher degrees of inhibition of TVCV movement (0–2% infection, see Fig. 3b and refs 14,15), cdiGRP may be augmented by other cadmium-ion-induced cellular factors.

Inhibition of TVCV systemic movement in cdiGRP S1 and S2 plants correlated with the lack of TVCV disease symptoms, that is, wrinkled and slightly deformed leaves with dark patches[14]. In wild-type plants, TVCV infection caused wrinkling and mosaic sectors in uninoculated tobacco leaves (compare Fig. 5a with b). These symptoms usually appeared on the sixth day after inoculation (data

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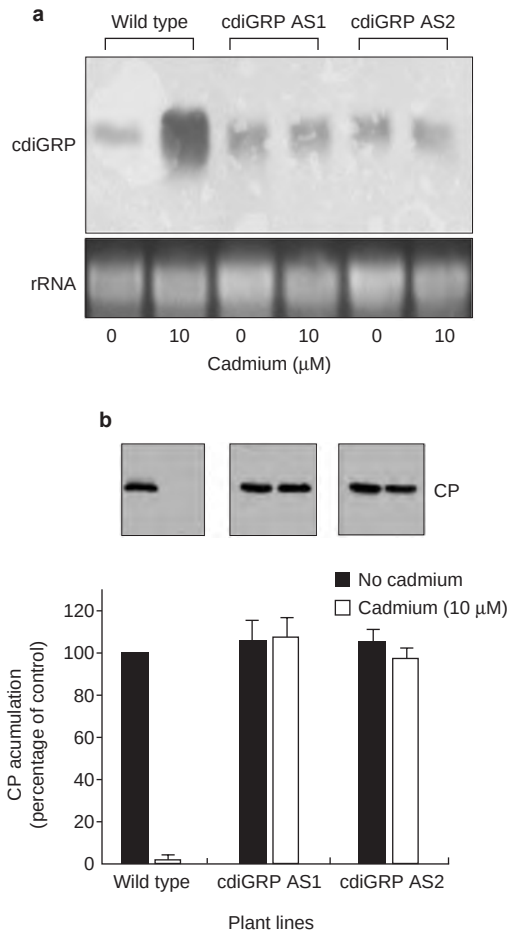

Figure 3 **TVCV systemic movement in cdiGRP antisense plants is not inhibited by cadmium ions. a,** Northern-blot analysis of cdiGRP expression in untreated and cadmium-ion-treated wild-type and cdiGRP antisense plants. cdiGRP AS1 and cdiGRP AS2 represent two independent antisense plant lines. The upper panel shows the cdiGRP expression as detected by northern-blot hybridization, and the lower panel shows the amounts of rRNA in each lane as detected by the ethidium bromide staining. **b,** CP accumulation in uninoculated leaves of TVCV-infected cdiGRP antisense lines AS1 and AS2. CP amounts were determined in the uninoculated leaves 14 days after inoculation with TVCV. Filled and open bars indicate CP levels in untreated plants and plants treated with 10 μM cadmium ions, respectively. CP accumulation is expressed as a percentage of the maximal amount of CP in uninoculated leaves of the wild-type cadmium-ion-free control plants. All data represent average values of three independent measurements with indicated standard deviation values. Blots above graphs show representative results of the western analysis of CP content in each plant line.

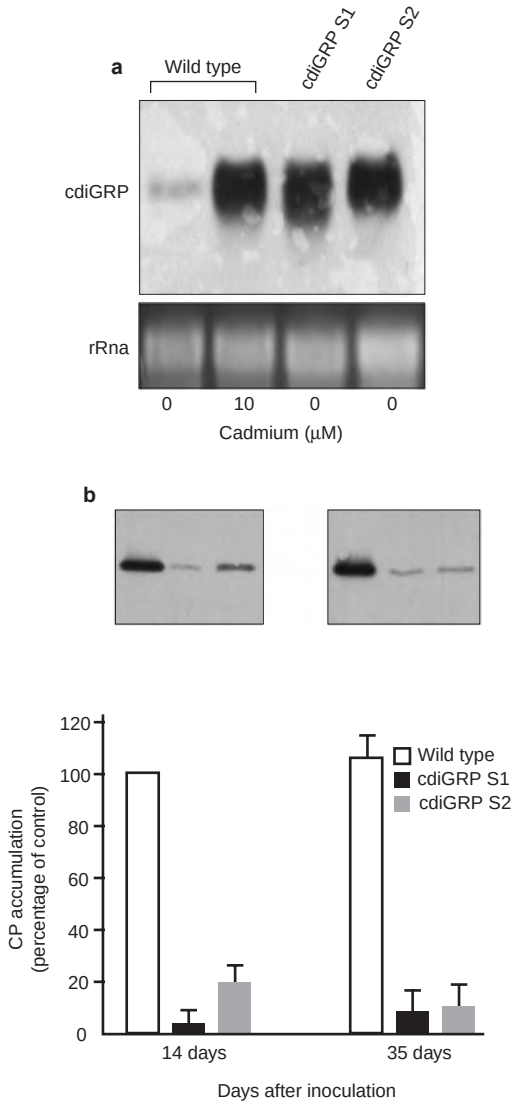

Figure 4 **Reduced TVCV systemic movement in cdiGRP sense plants. a,** Northern-blot analysis of cdiGRP expression in wild-type and cdiGRP sense plants. cdiGRP S1 and cdiGRP S2 represent two independent sense plant lines. Upper panel shows the cdiGRP expression as detected by northern blot hybridization, and lower panel shows the amounts of rRNA in each lane as detected by the ethidium bromide staining. **b,** CP accumulation in uninoculated leaves of TVCV-infected cdiGRP sense lines S1 and S2 14 and 35 days after inoculation. All experiments were performed in the absence of cadmium ions. Open, filled and hatched bars indicate CP levels in wild-type plants and in cdiGRP sense lines S1 and S2, respectively. CP accumulation is expressed as percent of the maximal amount of CP within uninoculated leaves of the wild-type control plants. All data represent average values of four independent measurements with indicated standard deviation values. Blots above graphs show representative results of the western analysis of CP content in each plant line.

not shown). But even 35 days after TVCV inoculation, virtually no (Fig. 5c) or only very mild signs of viral disease (Fig. 5d) were detected in the cdiGRP S1 and S2 plants, respectively. As expected, no TCVC disease symptoms developed in the infected wild-type plants treated with 10 μM of cadmium ions (Fig. 5e). So constitutive cdiGRP expression seemed to protect plants from developing symptoms of TVCV disease.

TVCV systemic movement arrests in vascular tissues of both cadmium-ion-treated and cdiGRP sense plants. We previously showed that, in cadmium-ion-treated TVCV-infected plants, the viral systemic movement is arrested in vascular bundles[15]. Here, we used immunofluorescence confocal microscopy to examine whether the constitutive expression of cdiGRP also confines the TVCV virions to the vascular tissue. Fourteen days after inoculation with TVCV, sections were prepared from the uninoculated leaves from control plants, plants treated with 10 μM cadmium ions, and cdiGRP S2 plants; the sections were stained with anti-TVCV CP antibody[23] followed by a Cy5-conjugated secondary antibody.

In TVCV-infected control plants, virus spread throughout the entire uninoculated leaf, with the fluorescently-stained virus particles detected both in the vascular bundle and in the surrounding non-vascular, mesophyll cells (Fig. 6a,b). Note that in these confocal

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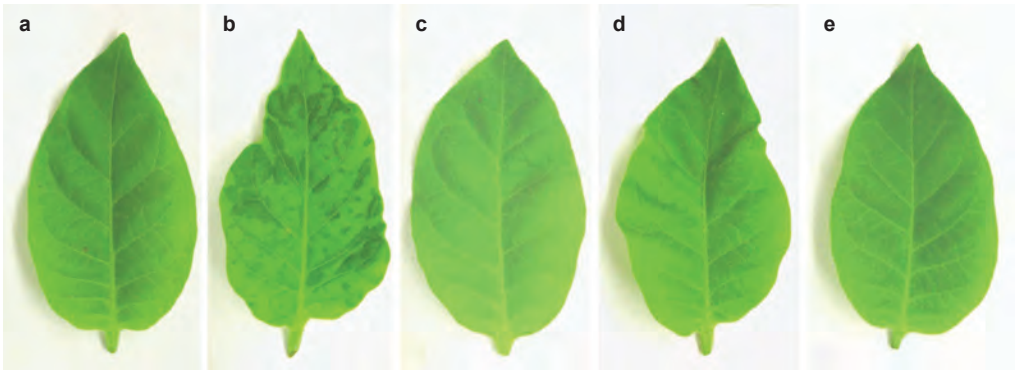

Figure 5 **Effect of constitutive cdiGRP expression on TVCV disease symptoms.** Wild-type and cdiGRP S1 and S2 plants were inoculated with TVCV, and uninoculated leaves of the same age were harvested from each plant 35 days after inoculation and photographed. **a,** Uninfected wild-type plant. **b,** Infected wild-type plant. **c,** Infected cdiGRP S1 plant. **d,** Infected cdiGRP S2 plant. **e,** Infected wild-type plant treated with 10 μM cadmium ions. All images were recorded under the same conditions from plants grown in a single experiment.

optical sections, which had a plane of focus through the centre of the cell layer, the black, signal-free cell areas represent large central vacuoles, whereas the TVCV-specific signal accumulates in the cytoplasm between the vacuole and the cell membrane. In contrast, in cadmium-ion-treated plants, TVCV accumulated in the vascular bundles but was not found in the adjacent mesophyll cells (Fig. 6c,d). Similarly, in cdiGRP S2 plants, non-vascular cells were mostly virus-free, with TVCV detected predominantly in the vasculature (Fig. 6e,f). The residual TVCV-specific staining in some of the mesophyll cells of the cdiGRP S2 plants (Fig. 6f) may reflect the incomplete inhibition of the viral systemic movement in this plant line (see Fig. 4b). In control experiments, no TVCV-specific signal was detected in uninfected plant tissues probed with anti-TVCV CP antibody or in infected plants probed with preimmune antibody (data not shown). So constitutive cdiGRP expression blocks TVCV movement in the same tissues, that is, the vascular bundles, in which the virus is arrested by cadmium-ion treatment.

Cadmium-ion treatment and constitutive expression of cdiGRP enhance callose deposition in the plant vasculature. It is possible that the cdiGRP in the cell walls of the plant vascular tissue exerts its effect on TVCV systemic movement by altering the properties and/or composition of the cell wall itself. Because callose is one of the cell-wall components known to restrict intercellular transport and prevent viral[24–26], we used callose-specific staining with aniline blue fluorochrome[27] to examine whether or not cdiGRP S2 plants have increased callose deposition in their vasculature. The phloem callose levels of cdiGRP S2 plants were significantly higher than those of the control plants (compare Fig. 7c with a).

These enhanced callose depositions were also found in the phloem of cadmium-ion-treated plants (Fig. 7b), confirming that cdiGRP expression, at least in part, underlies the effect of cadmium ions on plant cell walls and, consequently, on viral systemic movement. Similar to cadmium-ion-induced cdiGRP expression (see Fig. 2), increased callose deposits were observed both in plants expressing cdiGRP and those treated with cadmium ions. Deposits were observed in the class I (Fig. 7c and b), and class II and class III veins (data not shown). Also, in both types of plants, young, upper leaves (Fig. 7) and mature, lower leaves (data not shown) developed identical levels of callose deposits. These results suggest that elevated levels of cdiGRP in cell walls of the phloem cells function to induce callose depositions that probably block viral egress from the phloem into the surrounding tissues.

The notion that inhibition of TVCV systemic movement in cadmium-ion-treated and cdiGRP S1 and S2 plants resulted from induction of callose depositions was further examined by inducing callose formation by an unrelated mechanism. To this end, we used a transgenic tobacco TAG4.4 line, in which class I β-1,3-glucanase is specifically blocked by antisense suppression[25,27]. Because β-1,3-glucanase degrades callose, its deficiency results in enhanced callose formation[25,27]. The vascular bundles of TAG4.4 plants had more callose depositions as compared with wild-type plants (Fig. 8a). Importantly, when inoculated with TVCV, these plants were remarkably resistant to viral systemic spread — that is, only 10–20% of the extent of TVCV systemic movement observed in the infected wild-type plants (Fig. 8b), which was comparable to that observed with cdiGRP S1 and S2 plants (see Fig. 4b).

Discussion

In contrast to the local transport of plant viruses, studies of which have gained momentum in recent years (reviewed in refs 2,28,29), little is known about molecular mechanisms governing viral systemic transport. Here, we took advantage of specific inhibition of systemic movement of tobamoviruses by cadmium ions[14,15] to identify a cadmium-ion-induced tobacco cDNA that encodes a cell-wall protein, cdiGRP, that is a cellular factor that can negatively regulate tobamoviral systemic spread. Inactivating cdiGRP by antisense suppression blocked the ability of tobacco plants to inhibit TVCV systemic movement after cadmium-ion treatment. It is possible that cdiGRP antisense expression silenced cdiGRP and any closely related homologs (reviewed in ref. 30). This possibility was ruled out by using transgenic plants in which constitutive expression of the *cdiGRP* cDNA conferred resistance to TVCV systemic movement and prevented formation of the symptoms of the viral disease. Our results indicate, therefore, that cdiGRP is likely to underlie the effect of non-toxic concentrations of cadmium ions on tobamoviral systemic movement. That TVCV systemic movement was arrested in the same vascular tissues both in cadmium-ion-treated plants and in plants constitutively expressing cdiGRP lends further support to this idea. Consistent with the specific effect of cdiGRP on the viral systemic movement, TVCV local movement in the inoculated leaves of both antisense and sense cdiGRP plants remained virtually unchanged (data not shown).

That cdiGRP is induced after cadmium-ion treatment suggests that the synthesis of this protein may represent a plant-defense response to environmental stress. In fact, some other GRPs have been implicated in the hypersensitive response, being induced by salicylic acid treatment or viral infection[17,31]. cdiGRP, however, was only slightly induced by TVCV infection (<20% of the induction levels achieved the presence of cadmium ions, data not shown); furthermore, cadmium-ion-induced inhibition of TVCV systemic movement has been shown to occur independently of salicylic acid[15].

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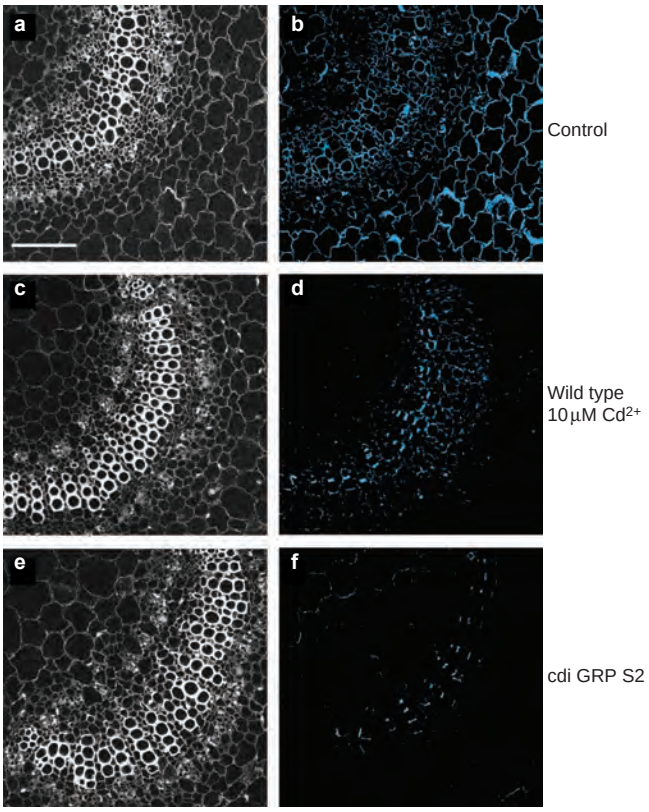

Figure 6 **TVCV accumulation in vascular tissues of uninoculated leaves of cadmium-ion-treated and cdiGRP S1 plants. a,b,** Control plants. **c,d,** Plants treated with 10 μM cadmium ions. **e,f,** cdiGRP S2 plants. **a**, **b** and **e** are phase-contrast images, and **b**, **d** and **f** are Cy5 confocal images of immunofluorescence detection of TVCV virions. Note that vascular tissue is seen as smaller cells arranged in bundles, whereas non-vascular tissue is represented by larger cells surrounding the vascular bundles. Scale bar, 100 μm.

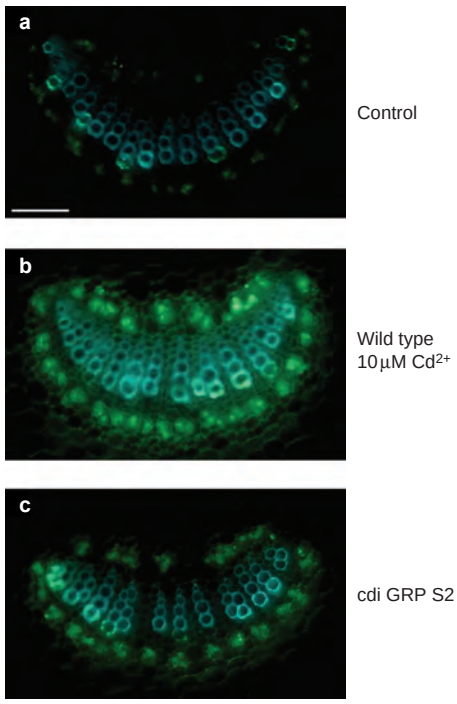

Figure 7 **Callose accumulation in the phloem of cadmium-ion-treated and cdiGRP S2 plants. a,** Control plants. **b,** Plants treated with 10 μM cadmium ions. **c,** cdiGRP S2 plants. Callose deposits stained with aniline blue show bluish green-white fluorescence[25]. Scale bar, 100 μm.

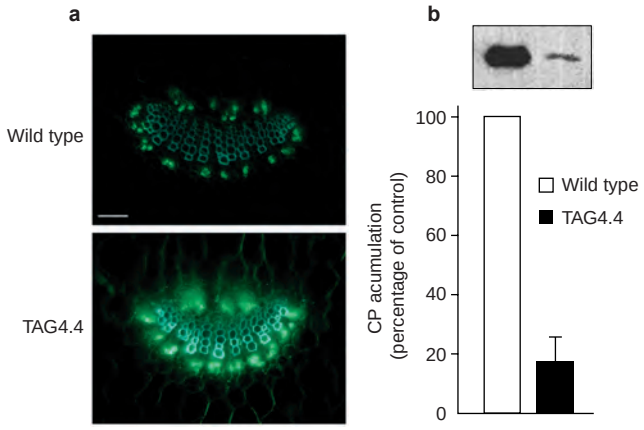

Figure 8 **Callose accumulation and TVCV systemic movement in class I β-1,3-glucanase-deficient TAG4.4 plants.** All experimental details were as described in refs 25,27. **a,** Callose-specific staining. Scale bar, 100 μm. **b,** CP accumulation in uninoculated leaves. Open and filled bars indicate CP levels in wild-type plants and in the TAG4.4 line, respectively. CP accumulation is expressed as percentage of the maximal amount of CP in uninoculated leaves of the wild-type control plants. All data represent average values of four independent measurements; standard deviation values are indicated. The blot above the graph is representative of the western analysis of CP content in each plant line.

Tissue-specific expression of cdiGRP in the plant vasculature suggests that the effects of cdiGRP on viral systemic movement should occur in the vascular bundles; indeed, in both cadmium-ion-treated plants and plants that constitutively express cdiGRP, the systemic transport of TVCV is arrested within the vascular bundles. How does cdiGRP inhibit TVCV systemic movement? As cdiGRP resides in the cell wall, elevated levels of this protein may affect properties of the cell wall, for example, rigidity, thereby interfering with the ability of plasmodesmata to dilate to allow passage of the virus. Indeed, plants that constitutively express cdiGRP developed increased deposits of callose in their phloem. Because callose, a (1→3)-β-glucan[32] deposited between the plasma membrane and the cell wall, is believed to restrict intercellular transport[24–26], it may be directly responsible for inhibiting TVCV movement in cadmium-ion-treated as well as in cdiGRP-overexpressing plants. cdiGRP, therefore, functions to induce callose formation in the cell walls of the phloem. These callose deposits, in turn, probably reduce TVCV transport from the phloem into the surrounding non-vascular cells. Indeed, induction of callose deposits by an unrelated mechanism, that is, antisense suppression of a callose-degrading enzyme, also inhibited TVCV systemic transport. Interestingly, in cadmium-ion-treated and in cdiGRP transgenic plants, only viral exit from the vasculature of uninoculated, upper leaves was blocked — viral entry into the vascular tissues of the inoculated, lower leaves was not affected. That cdiGRP and callose accumulated to the same levels both in the upper and lower leaves of these plants suggests that TVCV may use different strategies for its entry into and exit out of the host vasculature, and that cdiGRP expression and subsequent callose deposition interfere with the exit but not the entry step of TVCV systemic movement.

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It is tempting to speculate that the regulatory effect of cdiGRP extends beyond that on viral movement; for example, cdiGRP may help control systemic transport of endogenous macromolecules, such as phloem-transported proteins[33] or small-RNA elicitors of post-transcriptional gene silencing[34–37]. Besides these general biological implications, our results may be useful in designing new virus-protection strategies, which, unlike most genetic engineering approaches based on overexpression of viral proteins[38–40], would use an endogenous host factor, cdiGRP. □

Methods

PCR-based subtraction cloning

Total RNA, isolated as described previously[41] from 10 g of fresh leaf tissue derived from untreated and 10 μM cadmium-ion-treated tobacco plants, was processed using the Clontech PCR-Select™ cDNA subtraction kit according to the manufacturer's instructions. The subtracted cDNA fragments were cloned into the AdvanTAge™ PCR cloning vector (Clontech, Palo Alto, California), and the resulting subtraction library was screened using reverse northern-blot analysis[16] and first-strand digoxigenin (DIG)-labelled DNA probes reverse transcribed from total RNA purified from untreated plants and from plants treated with 10 μM or 100 μM cadmium ions. cDNA clones specifically induced by 10 μM but not by 100 μM of cadmium ions were selected and used as DIG-labelled probes in northern analysis[42] of total RNA from untreated and cadmium-ion-treated plants.

Plant growth and cadmium-ion treatment

Tobacco (*Nicotiana tabacum* cv. Turk) seeds were germinated and grown in soil. The 3–4 week old seedlings of similar height and with equal number of leaves were removed from Vermiculite and grown hydroponically in a modified Hoagland's solution[43] for 4–5 days in growth-chamber conditions with controlled humidity (50%), temperature (24–28 °C), and a photoperiod of16 h of light (photon flux density of 150 μE m^{-1} sec^{-1}) before cadmium ions were added. 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 TVCV.

For cdiGRP-independent induction of callose, the wild-type Havana 425 tobacco and its line TAG4.4 deficient for class I β-1,3-glucanase due to antisense suppression[25,27] were obtained from F. Meins, Jr. (Friedrich Miescher Institute, Basel, Switzerland), grown in soil at 32 °C, virus-infected and analysed exactly as described previously[25,27].

Antibody production, immunofluorescence confocal microscopy and immunoelectron microscopy

Anti-TVCV CP antibodies have been described previously[22]. Anti-cdiGRP antibodies were produced in rabbits against recombinant cdiGRP overexpressed in *Escherichia coli* from the *cdiGRP* cDNA in the pET28a(+) vector (Novagen, Madison, Wisconsin) and purified from a polyacrylamide gel. Preimmune serum was used in all immunolocalization experiments as a negative control. For immunofluorescence microscopy, leaf samples (0.5–1.0 cm) were harvested, fixed with a mixture of formaldehyde (4%) and glutaraldehyde (0.5%) in a sodium phosphate buffer, pH 7.2, and dehydrated in a series of diluted ethanol. The samples were then embedded in Paraplast Plus™ (Fisher Scientific, Pittsburgh, Pennsylvania), sectioned (8 μm), and reacted with polyclonal anti-cdiGRP antiserum or anti-TVCV CP antibodies as described previously[15]. The sections were then stained with Cy5-conjugated secondary antibody (Jackson ImmunoResearch, Inc., West Grove, Pennsylvania) and examined using a Bio-Rad MRC 600 krypton/argon laser scanning confocal attachment and a Nikon Diaphot inverted microscope.

For immunoelectron microscopy, leaf samples (see above) were processed for using the Durcupan ACM embedding protocol[44]. Ultra-thin sections (70–80 nm) were reacted with polyclonal antibody raised against the purified cdiGRP, followed by anti-rabbit IgG conjugated to 15 nm colloidal gold as described previously[44], and examined under a JEOL 100C transmission electron microscope.

Generation of cdiGRP sense and antisense plants

The cdiGRP cDNA was first inserted in either positive or reverse orientation, respectively, as a PCR-amplified *Eco*RI fragment into a plant-expression vector, pCd, containing the 35S promoter of cauliflower mosaic virus, tobacco mosaic virus translational enhancer[45], and the nopaline synthase polyA signal. Then, the entire sense and antisense expression cassettes were subcloned as *BamH*I-*Xba*I fragments into the binary vector pBIN19, carrying a kanamycin selection marker, and introduced into the disarmed *Agrobacterium* strain C1C58, which was then used to transform tobacco plants as described previously[46]. The resulting transgenic plants were selected on a kanamycin-containing medium and maintained for one month under sterile conditions on an MS basal medium[47] with no exogenous growth regulators. Plants were then transferred to soil in a greenhouse, allowed to set seed, and the transgenic progeny were selected by germinating the seeds on MS agar in the presence of kanamycin. All PCR reactions were performed using a high-fidelity proofreading *Pfu* DNA polymerase (Stratagene, La Jolla, California), and their products were verified by dideoxynucleotide sequencing[48].

Preparation of virus and inoculation of plants

TVCV was purified as described previously[49]. Two mature, lower leaves 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 allowed to grow for 2–4 weeks in the presence or absence of cadmium ions, and the viral systemic movement was assessed using the CP assay.

TVCV CP assay

For detection of TVCV CP, tissue samples (0.2 g fresh weight) from upper, uninoculated leaves were harvested, extracted, and analysed for the presence of CP by SDS polyacrylamide gel electrophoresis (PAGE) as described previously[14,15] followed by western-blot analysis using anti-TVCV CP antibodies and the ECL Western Blotting kit (Amersham Biosciences, Piscataway, New Jersey). Although our data (see Figs 3, 4, and 8) show only those blot areas that correspond to the CP band, no other, non-specific bands were detected in these analyses (data not shown). The amounts of CP were quantified by scanning densitometry (BioRad model GS-670) of the western-blot bands.

Callose staining

Callose was stained in leaf samples by the aniline blue method as described previously[27]. Briefly, tissue was hand-sectioned, fixed in 1% glutaraldehyde in 90 mM Na_2HPO_4, 5 mM sodium citrate, pH 7.4, transferred to distilled water, boiled for 3 min, transferred into 95% ethanol and incubated for 30 min to remove chlorophyll. The tissue sections were then stained in 0.1% water-soluble aniline blue in 67 mM K_2HPO_4, pH 12, and examined by epifluorescence microscopy.

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Correspondence and requests for materials should be addressed to V. C.

COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.

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