Activation of geminivirus V-sense promoters in roots is restricted to nematode feeding sites

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SUMMARY

Obligate sedentary endoparasitic nematodes, such as the root-knot and cyst nematodes, elicit the differentiation of specialized nematode nurse or feeding cells [nematode feeding sites (NFS), giant cells and syncytia, respectively]. During NFS differentiation, marked changes in cell cycle progression occur, partly similar to those induced by some geminiviruses. In this work, we describe the activation of V-sense promoters from the *Maize streak virus* (MSV) and *Wheat dwarf virus* (WDV) in NFS formed by root-knot and cyst nematodes. Both promoters were transiently active in microinjection experiments. In tobacco and *Arabidopsis* transgenic lines carrying promoter- β -glucuronidase fusions, the MSV V-sense promoter was activated in the vascular tissues of aerial plant parts, primarily leaf and cotyledon phloem tissue and some floral structures. Interestingly, in roots, promoter activation was restricted to syncytia and giant cells tested with four different nematode populations, but undetectable in the rest of the root system. As the activity of the promoter in transgenic rootstocks should be restricted to NFS only, the MSV promoter may have utility in engineering grafted crops for nematode control. Therefore, this study represents a step in the provision of some of the much needed additional data on promoters with restricted activation in NFS useful in biotechnological nematode control strategies.

INTRODUCTION

Obligate sedentary endoparasitic nematodes, such as the root-knot and cyst nematodes, induce the differentiation of special-

ized feeding cells used in feeding at nematode feeding sites (NFS) throughout the life cycle. Several data have suggested that NFS differentiation is elicited by nematode secretions and involves deep changes in gene expression, often through transcriptional regulation (reviewed by Caillaud *et al.*, 2008). This type of regulation has been addressed by studying changes in promoter activity at NFS (e.g. Barcala *et al.*, 2008; Gheysen and Fenoll, 2002). It is known that nematode-induced reprogramming of cellular gene expression facilitates DNA amplification at NFS through endoreduplication (reviewed by Gheysen and Mitchum, 2009).

Similar to many animal viruses, geminiviruses (GVs) replicate by exploiting the plant's cellular DNA synthesis machinery (Hanley-Bowdoin *et al.*, 2004). Phloem-restricted GV replicates and transcribe only in phloem cells of primarily young organs, and coat protein promoter-reporter gene fusions in transgenic plants show phloem-specific expression (Dinant *et al.*, 2004; Dry *et al.*, 2000; Lacatus and Sunter, 2008; Mazithulela *et al.*, 2000; Nikovics *et al.*, 2001; Sunter and Bisaro, 1997). Interestingly, many GV leave the vasculature and reproduce in mesophyll quiescent cells that are not engaged in active DNA synthesis. This apparent contradiction is explained by the fact that these GV can reprogram plant cell gene expression, allowing specific plant cell DNA replication and transcription factors to be made available (Gutierrez, 2000). Therefore, GV are viable candidates to share transcriptional regulatory mechanisms with those that must be activated in NFS.

Maize streak virus (MSV) is a member of the GV group with a very small genome, with approximately 2700 bases and the coding capacity of only four proteins (Hanley-Bowdoin *et al.*, 2000; Palmer and Rybicki, 1998). Transient expression experiments in maize and rice have shown that the MSV coat protein promoter (termed 'virion-sense' or 'V-sense' promoter) is active in the absence of virus-encoded proteins, and, in transgenic

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plants, is expressed in phloem-associated cells (Fenoll *et al.*, 1988, 1990; Mazithulela *et al.*, 2000; Nikovics *et al.*, 2001). MSV V-sense promoter transient expression in mesophyll cells is enhanced in the presence of the viral RepA protein (Munoz-Martin *et al.*, 2003), whose interaction with the cellular retinoblastoma (RB) protein may be necessary for cells to sustain DNA replication (McGivern *et al.*, 2005). RepA also enhances transient V-sense expression in suspension cultures in an RB-binding domain-dependent manner (Munoz-Martin *et al.*, 2003). Finally, in partially synchronized transgenic maize cell cultures, and in the absence of RepA, the V-sense promoter is active in S and early G2 phases. NFS (in particular, giant cells formed by root-knot nematodes) progress through repeated mitotic G2 phase cycles with incomplete cytokinesis, and also undergo endoreduplication cycles (reviewed by Gheysen and Mitchum, 2009). Therefore, GVs and nematodes might share a similar strategy, involving the transcriptional regulation of key replication factors, to make plant cells competent for DNA synthesis.

RESULTS AND EXPERIMENTAL DETAILS

To explore this hypothesis, plasmids carrying β -glucuronidase (GUS) fusions to GV promoters were tested for transient expression through microinjection in *Arabidopsis thaliana* syncytia induced by the cyst nematode *Heterodera schachtii*, as described previously by Bockenhoff and Grundler (1994). The constructs used were *pG208::GUS* from MSV (Munoz-Martin *et al.*, 2003), which contained the MSV V-sense promoter involved in the expression of the late viral genes (Fenoll *et al.*, 1988; Munoz-Martin *et al.*, 2003), and *pWDV4i::GUS* from the *Wheat dwarf virus* (WDV), containing the large intergenic region (LIR) and an intronless C1:C2 cDNA coding sequence, as described in Collin *et al.* (1996). Syncytia (10–30) were microinjected with each construct or with sterile water as controls. Microinjection of *pWDV4i::GUS* or *p208::GUS* in *Arabidopsis* syncytia showed a clear blue precipitate, 8–10 days post-inoculation (Fig. 1A), when assayed for GUS activity as described by Jefferson *et al.* (1987). The MSV construct exhibited approximately 28% positive syncytia, whereas WDV sustained 13% positive syncytia. Negative controls lacked GUS staining (Fig. 1A), suggesting that the observed blue precipitate corresponded to the transcriptional activation of both geminiviral promoters in the microinjected syncytia.

Next, *Arabidopsis thaliana* and *Nicotiana tabacum* transgenic plants carrying two different MSV V-sense promoter constructs driving the *GUS* reporter gene were obtained by *Agrobacterium tumefaciens*-mediated transformation (Guerineau *et al.*, 1990; Valvekens *et al.*, 1988). The transgenic plants contained *pG208::GUS* or *pG208DGC::GUS* (described in Fenoll *et al.*, 1990; Fenoll and Munoz-Martin, 1999). The transgenic plant lines were designated *G208* or *G208DGC*, followed by the line

number. Six tobacco lines were selected, including *G208DGC24*, *G208DGC25*, *G208DGC29* and *G208.15* with one insertion, *G208.14* with four and *G208.18* with an undetermined insertion number. In *Arabidopsis*, only homozygous lines with one insertion were tested: *G208.1.3*, *G208.1.5*, *G208DGC.4.5*, *G208DGC.5.5* and *G208DGC.5.4*.

To analyse promoter activation during plant development, tobacco and *Arabidopsis* plants were grown in soil, and 10 plants per transgenic line in *Arabidopsis* and four in tobacco were checked in each of three independent experiments. All tobacco *G208* lines exhibited a similar expression pattern restricted to the vascular tissue of above-ground organs, particularly in cotyledons, leaves and stems, whereas no GUS activity was detected in roots (Fig. 2A; Fig. S1A, see Supporting Information; Table 1). GUS activity was not detected in plants sampled prior to 3 weeks post-germination (data not shown). In flowers, some GUS staining was observed in sepal vascular tissue, and a very faint signal was detected in petals; there was intense GUS activity in the apex of both sepals and petals, and in glandular trichomes. The style and anthers were stained in all lines (Fig. 2E; Table 1), the activity being higher in young flowers (data not shown). In fruits, the vascular and glandular trichomes were stained, but no activity was detected in seeds (Fig. 2C; data not shown). In *Arabidopsis*, the MSV V-sense promoter in *G208* lines was also active in the vascular tissue of above-ground organs, but not in roots (Fig. 2B; Fig. S1C, see Supporting Information). Promoter activity in flowers was detected only in the sepals and style of fully developed flowers, but not in petals or anthers (Fig. 2D,F); as in tobacco, the construct was silent in seeds (Fig. 2D). Stem semi-thin sections of both tobacco and *Arabidopsis* transgenic plants were obtained and stained for GUS activity as described in Escobar *et al.* (2003). GUS activity was primarily localized in phloem, with some activity associated with xylem parenchyma cells in tobacco (Fig. 2G,H). The MSV V-sense promoter-deleted version in *G208DGC* was tested in several tobacco and *Arabidopsis* lines. Only one tobacco line showed consistent GUS expression, which was restricted to the vascular tissues of aerial organs and was lower than in *G208* (Table 1).

The transgenic plants were then infected with three species of root-knot nematode as described previously by Escobar *et al.* (2003): *Meloidogyne javanica* (MIK) (Portillo *et al.*, 2009), *M. incognita* and *M. arenaria* (obtained from Dr Andrés, CSIC, Spain). *In vitro*-grown, 18-day-old *Arabidopsis* and 25-day-old tobacco plants were inoculated with 8–10 sterile preparasitic *Meloidogyne* spp. J2 per root tip. Gall formation was monitored every 24–48 h and labelled to precisely record the infection times. A minimum of 26 galls was scored in each of at least three independent experiments per line tested. The cyst nematode *H. schachtii* was used to infect *Arabidopsis* seedlings as described by Sijmons *et al.* (1991), inoculating five to eight juveniles per plate and following syncytia formation in a similar manner as for galls.

Fig. 1 (A) Microinjected *Arabidopsis* syncytia, 8–10 days after inoculation with *Heterodera schachtii*. Plants were microinjected with *pWDV4i::GUS* from *Wheat dwarf virus* (WDV) (left) or *p208::GUS* from *Maize streak virus* (MSV) (right); negative control injected with water (middle). The blue precipitate depicts promoter region activation. Arrows indicate the positions of syncytia. (B) Histochemical β -glucuronidase (GUS) assay of transgenic *Arabidopsis* plants carrying a *p208::GUS* fusion infected with *H. schachtii*, 14–20 days post-inoculation. GUS staining in syncytia (left); semi-thin sections (7 μ m) indicating GUS precipitate localization in syncytial cells (right). Scale bars represent: (A) and (B) left, 1 mm; (B) right, 100 μ m.

Although the roots of all *Arabidopsis* and tobacco *G208* lines showed no GUS staining (Fig. 2A,B; Fig. S1, see Supporting Information), promoter activity was clearly evident in *M. javanica*-induced galls (root-knot NFS), 12–14 days post-infection (dpi), in both tobacco and *Arabidopsis* (Fig. 3A,E, respectively). In tobacco, the proportion of blue galls increased with the time of infection (Fig. 3G), with a maximum between 8 and 12 dpi, and decreased at the latest infection stages assayed (30 dpi). The lines with the lowest and highest GUS staining in vascular aerial tissues, *G208.15* and *G208.14*, respectively, showed the same relative gall staining intensities (Table 1). In *Arabidopsis*, the pattern was similar (Fig. 3G), but variation between the staining intensity in both galls and above-ground tissues in the different lines was lower (Table 1).

A very low percentage (3%) of blue galls was detected at early infection times (3 ± 2 dpi; data not shown). Semi-thin sections showed that the GUS signal was centred in giant cells and adjacent cell layers from the vascular cylinder in both tobacco and *Arabidopsis* (Fig. 3B,F). A low percentage (10%) of syncytia induced in *Arabidopsis* by the cyst nematode *H. schachtii*, 14–20 days post-inoculation, also showed GUS staining (Fig. 1B). Semi-thin sections revealed that the signal was centred in the syncytia nurturing cells, similar to NSF induced by root-knot nematodes (Fig. 1B).

The activity of the MSV promoter was also tested with *M. arenaria* and *M. incognita*. The tobacco *G208.14* line was selected, as it showed high GUS activity in *M. javanica*-induced galls (Fig. 3G, Table 1). The promoter was active in galls induced

Fig. 2 Histochemical β -glucuronidase (GUS) assay of transgenic tobacco (A, C, E, G) and *Arabidopsis* (B, D, F, H) plants carrying the *Maize streak virus* (MSV) *p208::GUS* fusion. (A, B) GUS staining in vascular tissue of above-ground organs: cotyledons, leaves and stem. (C) Flower with GUS staining in sepals. (D, F) GUS precipitate in sepals and a faint signal in the style of fully developed flowers; note the absence of a signal in siliques and seeds. (E) Intense GUS activity in the apex of sepals and in glandular trichomes (left) and in the style (right). (G, H) Semi-thin sections (7 μ m) of tobacco and *Arabidopsis* stems showing GUS localization in phloem tissue. Some activity was detected in tobacco parenchyma cells associated with xylem tissue. Scale bars represent: (A–F) 1 mm; (G, H) 100 μ m.

Table 1 Expression patterns of the different *Maize streak virus* (MSV) promoter lines, p208::GUS, in transgenic tobacco and *Arabidopsis* plants.

Line	Plant	Aerial vascular tissue	Roots	Petals	Sepals	Pistils	Galls and GC
<i>G208.14</i>	Tobacco	*	No detection	‡	†	†	*
<i>G208.15</i>	Tobacco	No detection	No detection				‡
<i>G208.18</i>	Tobacco	‡	No detection	‡	‡	‡	No detection
Lines of <i>Arabidopsis</i> (208.1.3 and 208.1.5)		*	No detection	No detection	‡	No detection	*
Promoter pG208							
<i>GDGC24</i>	Tobacco	†	No detection	‡	†	†	*
<i>GDGC25</i>	Tobacco	No detection	No detection	Sometimes‡	Sometimes‡	Sometimes‡	No detection
<i>GDGC29</i>	Tobacco	No detection	No detection	No detection	No detection	No detection	No detection
Lines of <i>Arabidopsis</i> (DGC5.5, 5.4 and 4.5)		No detection	No detection	No detection	No detection	No detection	No detection
Promoter pG208DGC							

*High expression.

†Medium expression.

‡Weak expression. Empty box, not tested. GC, giant cells.

by all three root-knot nematode species (Fig. 3D) and localized to the same cells (compare Fig. 3A and Fig. 3C; and data not shown).

Of all the *G208DGC* lines (which carry an MSV V-sense promoter with an internal deletion that reduces expression in transient transformation assays; Fenoll *et al.*, 1990; Fenoll and Munoz-Martin, 1999) tested, GUS activity was only found in the galls of the *G208DGC24* tobacco line and in its aerial and floral tissues. This expression pattern could be a result of integration on an enhancer region, as none of the remaining tobacco lines, nor the three equivalent *Arabidopsis* lines, exhibited staining in infected or uninfected plant structures, with the exception of sporadic staining in flowers from the *G208DGC25* and *G208DGC29* tobacco lines (Table 1). All of these observations suggest that the deletion of the GC boxes could reduce MSV V-sense promoter activity in plants, as reported in transient expression experiments in maize seedlings bombarded with a similar construct (Munoz-Martin *et al.*, 2003).

The effect of wounding on promoter activation was tested in *G208* tobacco leaves by three independent treatments: puncture and tweezer crushing (in 3-week-old *in vitro*-grown plants), and scalpel cutting (for soil-grown mature plants), followed by GUS staining 30 min or 2 h after treatment; nonwounded plants were used as controls. None of the treatments induced substantial GUS accumulation; the signal level and expression patterns were similar in all lines tested, and concentrated in above-ground plant vascular tissue, with no observable differences between wounded and nonwounded plants (data not shown). Similarly, roots wounded following the protocol described in Nishiuchi *et al.* (1997) did not show any GUS signal in either *Arabidopsis* or tobacco, or in their respective unwounded controls (Fig. S1, see Supporting Information).

To analyse the presence of putative *cis* elements, the MSV region used as the V-sense promoter for expression studies (coordinates 1838–292 in the viral genome) was submitted to the Signal Scan Program in PLACE (Higo *et al.*, 1999). *Cis* elements that may interact with transcription factors were identified as

sequence cores with more than four nucleotides and repeated at least once in the positive strand (Table 2). Among the most interesting *cis* elements was the binding site of ARR1, a cytokinin-regulated factor that activates the promoter of a non-symbiotic haemoglobin in rice (OsNSHB2). This element is also present in its *Arabidopsis* homologue (At2G16060; Table 2). The MSV promoter region studied contains a dehydration-responsive element (*DRE*) represented three times in the positive strand, together with eight repeats of the binding site for AtMyc2, both involved in responses to dehydration and cold. Interestingly, both *cis* elements were represented several times in the promoters of genes encoding two late embryogenesis abundant (LEA) proteins (LEA14-A and LEMMI9), which are activated after nematode infection in potato and *Arabidopsis* (Escobar *et al.*, 1999; Gheysen *et al.*, 1996; de Meutter *et al.*, 2005).

DISCUSSION

The results indicate that the MSV V-sense promoter in *Arabidopsis* and tobacco *G208* transgenic lines shows a similar activation pattern restricted to the vascular tissues of aerial plant parts, suggesting that the transcription factors involved in this promoter activation may be similar in both taxa. The expression pattern is congruent with that of a similar construct in rice (Mazithulela *et al.*, 2000). However, root expression was clearly detected in rice, but absent in *Arabidopsis* and tobacco (Mazithulela *et al.*, 2000; this work). The MSV V-sense promoter expression in tobacco was located in the phloem and some parenchyma cells associated with the xylem of above-ground parts (Fig. 2). These data are concordant with LIR GUS activity in the MSV V-sense promoter in rice and that of WDV in *Nicotiana benthamiana*, *N. tabacum*, *A. thaliana* and *Cucumis melo* (Dinant *et al.*, 2004; Mazithulela *et al.*, 2000).

Interestingly, the MSV (pG208::GUS) promoter was highly active in two types of NFS: giant cells induced by three root-knot nematode species (Fig. 3) and cyst nematode-induced syncytia

Fig. 3 Histochemical β -glucuronidase (GUS) assay of transgenic plants carrying a *p208::GUS* fusion in galls, 12–14 days after infection: tobacco (A–C) and *Arabidopsis* (E, F). *Meloidogyne javanica* galls (A, E) and *M. incognita* galls (C). (B, F) Sections (7 μ m) of tobacco and *Arabidopsis M. javanica* galls, respectively. (D) Table representing the percentage of blue galls obtained with different *Meloidogyne* species. (G) Graph showing the percentage of blue galls after infection of tobacco and *Arabidopsis* lines with *M. javanica* at different infection points. Giant cells (GCs) are shown. Scale bars represent: (A, C, E) 1 mm; (B, F) 100 μ m.

Table 2 Putative *cis*-elements present in the *HindIII*-*XbaI* (1168) promoter fragment of pG208 (Munoz-Martin *et al.*, 2003). Its location in the *Maize streak virus* (MSV) promoter (relative to the *HindIII* restriction site) and in different plant genes is indicated, as well as the putative binding transcription factors. *AtNSHB1* (At2g16060), *Arabidopsis* homologue to a rice gene (*OsNSHB2*) coding a nonsymbiotic haemoglobin.

<i>Cis</i> -element.	Nucleotide sequence	Number of repetitions and localization in the promoter	Binding site	Involved	<i>cis</i> -AtNSHB1 At2g16060*	Number of <i>cis</i> -elements in GhLEA14 A (7), LeLEMMI9 (8) and position*
ARR1AT	NGATT	7: 162, 446, 454, 755, 787, 853, 978	(1,2)ARR1	Citoquinin	227, 273, 927, 975, 1026, 1030, 1546, 1568, 1654, 1916, 2019, 2055, 2166, 2744	LEA14 A: 635, 807 LEMMI9: 353, 624, 753, 783, 802, 1459, 1586, 1887, 2090
CBFHV	RYCGAC	2: 538, 743	(3)HvCBF1* and (4)HvCBF2 (DRE)	Cold responses		LEA14 A: 127 LEMMI9: 934
MYCCONSUSAT	CANNTG	4: 435, 655, 847, 1146	(5)AtMyc2, (6)AtICE1	Dehydration	66, 201, 267, 483, 790, 1045, 2277, 2733, 2902, 2939, 2963, 2980, 2988	LEA14 A: 9, 170, 540, 571, 1286, 1846, 1977 LEMMI9 58, 250, 977, 1098, 1162, 1193, 1233, 1390.

*Positions are given relative to the transcription starting point.

(Fig. 1). Plant injury did not activate the promoter in either aerial parts or roots (data not shown; Fig. S1, see Supporting Information), which suggests that the activation in NFS is not associated with a defence response. A systemic effect on promoter activation after infection was not detected, as aerial tissues showed the same staining pattern in infected and uninfected plants. Therefore, MSV V-sense promoter activation by nematodes is an NSF-specific effect, perhaps triggered by the nematode secretions that are believed to induce NSF differentiation. This finding raises the interesting possibility that the mechanisms involved in GV promoter activation during viral infection might share common features with NSF differentiation. GVs use mechanisms similar to animal oncoviruses to drive infected cells to G1/S phases of the cell cycle. The interaction of RepA proteins with RB-related proteins is at least partly responsible for this process (Collin *et al.*, 1996; McGivern *et al.*, 2005; Munoz-Martin *et al.*, 2003; Xie *et al.*, 1996). For example, an intact retinoblastoma related protein (RBR)-binding motif is required for MSV invasion of mesophyll cells in cereals (McGivern *et al.*, 2005), suggesting that these protein–protein interactions may be involved in the colonization of quiescent cells in tissues other than phloem. Microarray analysis of the *Arabidopsis* transcriptome in response to *Cabbage leaf curl virus* (CaLCuV) infection has suggested that GVs modulate the plant cell cycle by differential impacts on the cyclin D (CYCD)/RBR/E2F regulatory network and promote the progression into the endocycle (Ascencio-Ibanez *et al.*, 2008). Similarly, NFS undergo a complex reactivation of the cell cycle from stem cells (reviewed in Gheysen and Fenoll, 2002; Gheysen and Mitchum, 2009). Infective juveniles of the potato cyst nema-

tode secrete a mitogenic low-molecular-weight peptide(s) that costimulates the proliferation of tobacco leaf protoplasts and human peripheral blood mononuclear cells (Goverse *et al.*, 1999). These and other observations suggest that nematode secretions may bring about direct or indirect reprogramming of particular cell cycle pathways that induce cells to re-enter the S phase (Caillaud *et al.*, 2008; Gheysen and Fenoll, 2002; Goverse *et al.*, 2000). This cellular state might mimic, at least in part, the cellular conditions suitable for viral DNA replication induced by GVs in otherwise quiescent cells, i.e. maize mesophyll cells (Gutiérrez, 2000; McGivern *et al.*, 2005). In support of this hypothesis is the finding that the MSV V-sense promoter is active during the S phase and increases at the G2 cell cycle phase in maize cell suspensions (Nikovics *et al.*, 2001). Further support for this comparison comes from the fact that S phase and G2–M phase progression cyclin genes, *CycA2;1* and *CycB1;1* and the cyclin-dependent kinases, *cdc2aAt* and *cdc2bAt*, are activated in nematode feeding cells (de Almeida Engler *et al.*, 1999; Niebel *et al.*, 1996). The induction of the MSV V-sense promoter specifically in nematode feeding cells, and its silence in the rest of the root cells, suggests an intriguing commonality of some molecular processes in these very different biological systems. It also reveals that the V-sense MSV region used in this work could act as a suitable promoter for a biotechnological control approach to prevent nematode establishment, especially at early to mid-infection stages. Such strategies may include the use of these promoters to direct *in planta* RNAi to target plant-parasitic nematodes. To date, the most successful attempts used to confer RNAi-mediated resistance to nematodes (Gheysen and Van-

holme, 2007) have been based on plant promoters, such as the *TOBRB7* (Yamamoto *et al.*, 1991) or CaMV35S promoter driving the expression of dsRNA against nematode genes. The restricted expression (only aerial vascular tissues in addition to NFS) of the MSV V-sense promoter could be particularly useful to engineer grafted crops such as grapevine. It is important to note that, when GUS activity was developed in either uninfected or infected plants for long periods, such as up to 8 days, no different GUS expression pattern to that described in this work, or GUS diffusion, was observed in the tissues. Thus, promoter activity in transgenic rootstocks is expected to be limited to NFS, similar to the results reported here. Further research on important agronomic plants should be performed to elucidate the putative application of the MSV promoter tested in this initial work.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Histochemical β -glucuronidase (GUS) assay of roots from transgenic tobacco 35 days after germination (A, B) and *Arabidopsis* 21 days after germination (C, D) carrying a *p208::GUS* fusion after wounding (B, D) and their respective unwounded controls (A, C). No GUS staining was observed either in the controls or in the wounded roots of both species. Scale bars represent 0.5 mm.

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