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Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner

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ABSTRACT

Four transgenic *Arabidopsis thaliana* lines carrying different reporter gene constructs based on split glucuronidase genes were used to monitor the frequency of somatic homologous recombination after geminivirus infections. *Euphorbia mosaic virus* and *Cleome leaf crumple virus* were chosen as examples, because they induce only mild symptoms and are expected to induce less general stress responses than other geminiviruses. After comparing the different plant lines and viruses as well as optimizing the infection procedure, *Euphorbia mosaic virus* enhanced recombination rates significantly in the transgenic reporter line 1445. The effect was tissue-specific in cells of the leaf veins as expected for this phloem-limited virus. The advantage for geminiviruses to activate a general recombination pathway is discussed with reference to an increased fitness by generating virus recombinants which have been observed frequently as an epidemiologic driving force.

Introduction

DNA damage in plants can be repaired by various eukaryotic protein systems (Bray and West, 2005; Kimura and Sakaguchi, 2006), including somatic homologous recombination (SHR) with high fidelity. Several studies have demonstrated that abiotic and biotic stresses increase the SHR frequency in plants (Boyko et al., 2005; Kovalchuk et al., 2003; Lucht et al., 2002; Molinier et al., 2005; Ries et al., 2000). These stressors trigger either a boost of reactive oxygen species or cause DNA damage directly. For some stress types (*e.g.*, UV-C, elicitor flagellin) the SHR frequency was found to be elevated even within the subsequent, non-stressed plant generation (Molinier et al., 2006). This phenomenon was called "transgenerational stress memory" and is likely an epigenetic effect, because it depends on the functional Dicer-like proteins DCL2 and DCL3 (Boyko et al., 2010; Boyko and Kovalchuk, 2010). However, the transgenerational effect does not occur generally for all stressors (Pecinka et al., 2009).

In the cited studies, transgenic SHR reporter constructs were used to monitor changes of homologous recombination frequencies (HRF). They consist of two non-functional split parts of a reporter gene with partially overlapping sequences of several hundred base pairs of the β-glucuronidase gene (GUS; Fig. 1A). Homologous recombination of the overlapping sequences restores GUS activity which can be detected by histochemical staining. The recombination events monitored as blue spots or sectors in plant tissues allow the quantitative evaluation of SHR. The reporter constructs may be arranged in direct or indirect orientation enabling further insights into the type of the recombination events (Gherbi et al., 2001; Puchta et al., 1995a). They had been integrated into the *Arabidopsis thaliana* genome of two ecotypes at different loci with the help of *Agrobacterium tumefaciens* (Tinland et al., 1994) (Fig. 1A). Consequently, distinct plant lines exhibited different baselines of HRF as well as different responsiveness to stress types which was attributed either to the kind of SHR reporter construction (length or orientation of homologous overlaps), the genomic position or chromatin status of the transgene, the ecotype background, or to a combination of these properties (Pecinka et al., 2009).

Geminiviruses (Jeske, 2009) replicate their circular single-stranded (ss) DNA by three modes of action: complementary strand replication (CSR), rolling-circle replication (RCR) and recombination-dependent replication (RDR) (Alberter et al., 2005; Erdmann et al., 2010; Jeske et al., 2001; Jovel et al., 2007; Preiss and Jeske, 2003). They rely completely on host proteins for replication because they do not encode a DNA polymerase. This is true in particular for the plant homolog of the retinoblastoma protein (pRBR), a cell cycle regulator that blocks replication in differentiated cells (reviewed by Gutierrez et al., 2004; Hanley-Bowdoin et al., 2004). As a consequence host DNA may be

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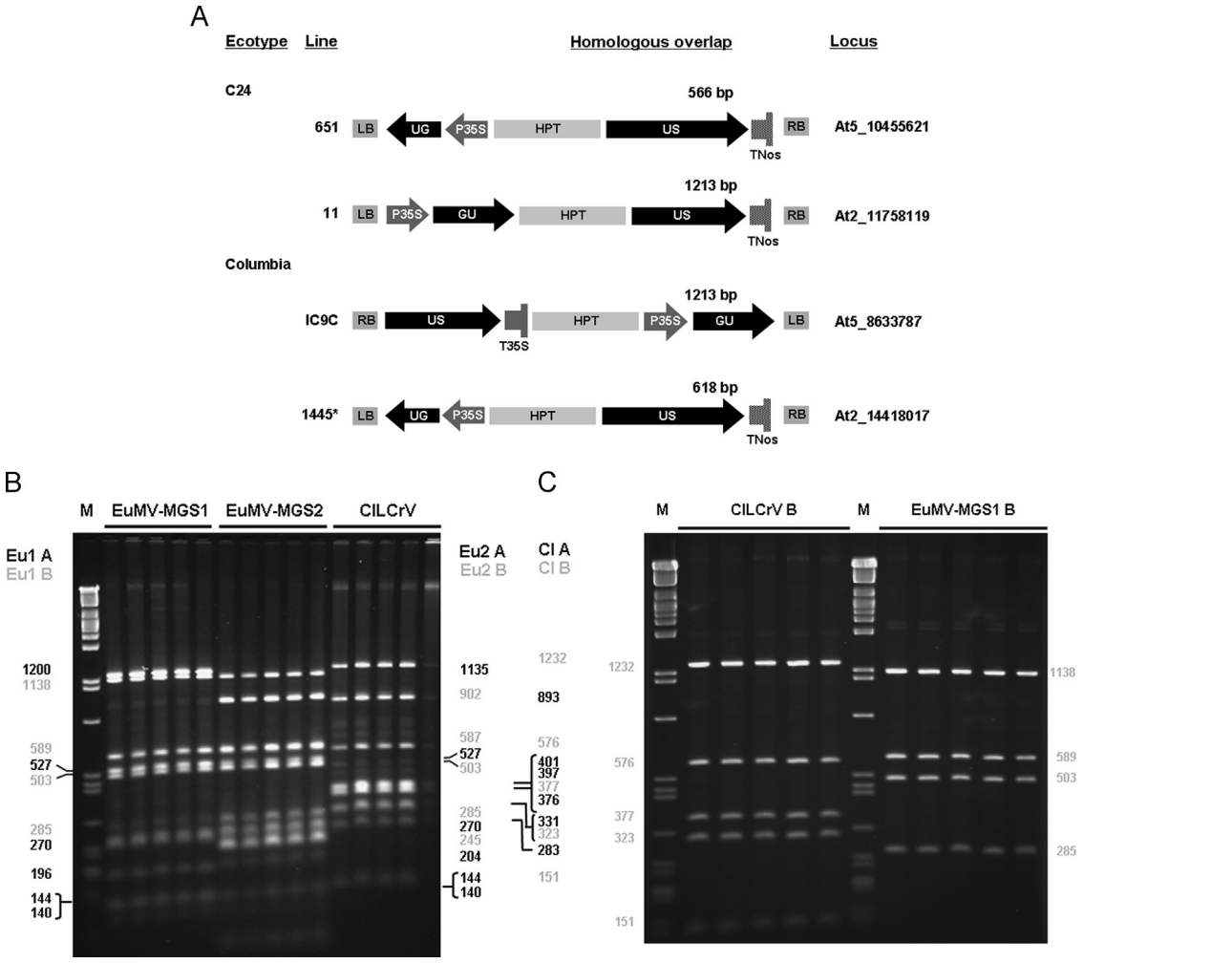

Fig. 1. Prerequisites of the analysis. (A) Schematic representation of SHR reporter constructs present in *A. thaliana* transgenic lines 651 (Puchta et al., 1995b), 11 (Swoboda et al., 1994), IC9C (Molinier et al., 2004) and 1445 (Fritsch et al., 2004; Gherbi et al., 2001; Pecinka et al., 2009). Ecotype background, length (bp) and orientation (direct or inverted) of the GUS overlap construct are indicated for each line. The positions of the genomic integration locus are given according to "The Arabidopsis Information Resource" (TAIR, Apr 02, 2013). LB and RB: left and right border of T-DNA; P35S: cauliflower mosaic virus promoter; T35S: cauliflower mosaic virus terminator; GUS: β-glucuronidase reporter gene; TNos: nopaline synthase terminator; HPT: hygromycin phosphotransferase gene. (B) RFLP analyses to confirm the exclusiveness of full-length DNA A and DNA B from Euphorbia mosaic virus-MGS1 (EuMV-MGS1), Euphorbia mosaic virus-MGS2 (EuMV-MGS2) or Cleome leaf crumple virus (ClLCrV) in the applied inocula. RCA products of viral DNAs from systemically infected wild-type *A. thaliana* plants are shown, which were treated with *Hpa*II (five technical replicates) for each virus. Restriction fragments were separated in 2% agarose gels, with 600 ng of *Pst*I-digested λ DNA as molecular weight marker (M) and staining with ethidium bromide afterwards. Black and gray numbers indicate the expected fragment sizes for DNA As and DNA Bs, respectively. The corresponding undigested RCA products were used for biolistic inoculation. (C) Characterization of the mock-inoculum by RCA/RFLP as in (B). In order to generate RCA products containing only DNA B, restriction enzymes were chosen to linearize only DNA B, and the resulting fragment was gel-purified, recircularized and amplified by RCA. This product was digested with the diagnostic restriction enzyme showing the absence of DNA A or satellite DNA.

re-replicated, as has been shown for plants (Nagar et al., 2002) and yeasts (Kittelmann et al., 2009).

Recombination is an important factor for the evolution and epidemics of geminiviruses (van der Walt et al., 2009, and references therein). At the same time the RDR mode provides an efficient mechanism for early recombination during infection. This motivates our current study on whether host recombination can be influenced by geminiviruses. A transcriptome analysis of *A. thaliana* after geminivirus (cabbage leaf curl virus) infection revealed several changes in the expression of SHR pathway factors (Ascencio-Ibanez et al., 2008). It is therefore plausible that geminivirus infection may influence this host pathway.

Most of the geminiviruses are confined to the phloem tissue (Horns and Jeske, 1991; Wege et al., 2001) allowing us to differentiate between direct effects in the infected tissue and general, stress-induced effects in the whole plant for the first time. The results show that geminiviruses are indeed promoting SHR in phloem tissue under defined experimental conditions.

Results

Monitoring SHR by the help of the reporter constructs as presented in Fig. 1A has been shown to be dependent on the physiological condition of the plants. Most reports have used young plants in axenic cultures for optimal differentiation of baseline and stress-induced SHR. Moreover, the kind of the stressor is important for the outcome of the assay. On the other hand, infection of non-transgenic *Arabidopsis* with the geminiviruses used in this study was found to be optimal at later stages of development in potted plants with vigorous vegetative growth (Paprotka et al., 2010). It was therefore necessary to find a compromise between the optimal experimental conditions for monitoring SHR efficiency and for viral infection. A second difference between this study and previous ones is the phloem-limitation of many geminiviruses. If this tissue tropism is true for the investigated geminiviruses, it would allow discrimination of changes in SHR originated by general stresses from those specifically induced by virus infection in phloem cells.

Table 1
Infection rates of EuMV-MGS1, EuMV-MGS2 and ClLCrV on transgenic lines 651, 1445, IC9C and 11.

Late inoculation						
Line	651	1445	IC9C	11		
EuMV-MGS1	10/10	9/10	9/10	9/10		
EuMV-MGS2	6/10	5/10	1/10	0/10		
ClLCrV	10/10	10/10	8/10	3/10		
Early inoculation						
Experiment #	1		2		3	
Line	651	1445	651	1445	651	1445
EuMV-MGS1	5/10	5/10	11/20	15/20	10/20	11/20
EuMV-MGS2	0/10	0/10	–	–	–	–
ClLCrV	0/10	2/10	–	–	–	–

In a first series of experiments, *Euphorbia mosaic virus* (EuMV, isolates EuMV-MGS1 or EuMV-MGS2), or *Cleome leaf crumple virus* (ClLCrV) without their alpha satellite DNAs (Paprotka et al., 2010) were delivered biolistically to the four transgenic lines (Fig. 1) at a later stage of plant development (12–14 leaf stage, see Material and methods for details). Symptoms, presence of viral DNA and SHR rates of three leaves per tested plant were compared to those of mock-inoculated plants of the four lines treated in parallel. Symptoms were generally mild or not visible macroscopically, with some curling in newly emerging leaves in the combination of EuMV-MGS1 and Col line IC9C (data not shown). Using RCA, restriction fragment length polymorphism (RFLP) combined with Southern blot hybridization to detect viral DNA (Fig. 4), EuMV-MGS1 showed the highest infection rate of more than 90% in all transgenic lines (Table 1). However, none of the combinations of viruses and plant lines revealed a statistically significant difference in the SHR frequency compared to mock-inoculated plants in three tested leaves and at this late stage of plant development (data not shown, ten plants per line and inoculation type, thus 40 plants per transgenic line were tested in total).

In a second series of experiments, plants were inoculated at an earlier stage of development (4–6 leaf stage, see Material and methods for details) where they were at a young rosette leaf stage when harvesting at 21 dpi and thus facilitated using the whole plantlets for GUS staining. This procedure reduced the infection rates but provided sufficient infected plants in certain combinations (Table 1). EuMV-MGS1 showed the highest reproducible infection rates in two transgenic lines (651, 1445) and was therefore chosen for the further study.

The punctate signals upon tissue blot hybridization indicated phloem-limitation of EuMV-MGS1 (Fig. 2A), a conclusion confirmed further by microscopic NBT/BCIP-based *in situ* hybridization (Fig. 2B and C; Suppl. Fig. 1 for mock-control) and fluorescence *in situ* hybridization (FISH; Fig. 2D and E; Suppl. Fig. 2). Very few cells, exclusively associated with vascular tissue, showed virus-specific staining or fluorescence. Infected plants showed mild symptoms (Fig. 3) with some leaf curling indicating that only a low level of general stress was elicited by the treatments. Viral DNA was detected with high sensitivity by a combination of RCA/RFLP and Southern blot hybridization as exemplified for the comparison of EuMV-MGS1 and ClLCrV in two plant lines (Fig. 4; for the results of further analyses see Suppl. Fig. 4). This sensitive detection allowed us to unequivocally distinguish infected from uninfected plants for the following comparison.

EuMV-MGS1 infected and mock-inoculated plants of lines 651 and 1445 showed two types of GUS signal in the SHR assay, either in the mesophyll and epidermis (Fig. 5A and B) or associated with veins (Fig. 5C and D), if investigated under the stereomicroscope. The vast majority of these signals represented single cells rather than patches of tissue. In order to enable at least a gross comparison between our environmental condition with those of other authors, a heat stress experiment according to Pecinka et al. (2009) was performed and SHR frequencies determined (Fig. 6A). Numbers of blue spots seen under the stereomicroscope were counted and referred to the fresh weight of the respective plants (see Material and methods for details). Line 1445 showed a significant increase of total SHR signals under long day conditions after the stress (Fig. 6A, total gray box). Although the vein-associated and the non-vein-associated subset of data revealed the same trend when counted individually, the number of data was too small to pass the statistical test.

Experiment to experiment variation has to be considered for geminivirus infections. Three independent experiments, each with parallel treatments of two plant lines 651 and 1445, revealed different infection rates (Table 1) and varying absolute counts for SHR signals (Fig. 6A and B). The numbers of counts were generally lower for line 651 than for line 1445. In experiment #1 the overall response was similar to that of heat stress but significantly more vein-associated signals were observed. Both lines showed the same trend for more vein-associated signals after infection, but sampling sizes were only large enough to pass the significance test in all three experiments for line 1445 (Fig. 6 asterisks; $p < 0.001$; *t*-test or Mann–Whitney rank sum test if data was not distributed normally). Although absolute numbers varied between experiments, the relative increase after infection was five times higher for vein-associated SHR signals throughout all experiments with line 1445.

In summary, the phloem-limited EuMV-MGS1 is able to increase significantly SHR in individual vein cells in at least one reporter plant line. This is probably due to a specific impact on the SHR machinery of infected cells by the virus.

Discussion

In accordance with the recombination-dependent replication of geminiviruses (Jeske, 2007), the activation of cellular DNA synthesis (Kittelmann et al., 2009; Nagar et al., 2002), and the up-regulation of SHR pathway genes (Ascencio-Ibanez et al., 2008), the results show for the first time that transgenic reporter genes can recombine more frequently after a geminivirus infection. Moreover, this study has distinguished between different tissues in contrast to previous reports which is particularly important for the identification of a specific effect induced by a phloem-limited geminivirus.

The test assay has proven to be sensitive to the experimental conditions as discussed by other authors before (Pecinka et al., 2009), and the proper parameters for geminivirus infection had to be determined here. At least for one combination of virus and transgenic reporter line (EuMV-MGS1 and line 1445), the enhancement of recombination frequencies was significant in three independent experimental sets. The same trend is visible for the other combination of EuMV-MGS1 with line 651 but the difference was too low to pass the statistic tests. Therefore, increasing sample sizes may lead to a similar conclusion. Since both lines (1445 and 651) contain an inverted GUS with approximately the same length of overlap (618 bp and 566 bp), it is likely that the ecotype background and/or the chromatin status at the respective integration sites were the cause of the different SHR baseline level and responsiveness to EuMV-MGS1 infection.

RNA viruses of the genus *Tobamovirus* increased recombination frequencies of their hosts as well. However, the results of the

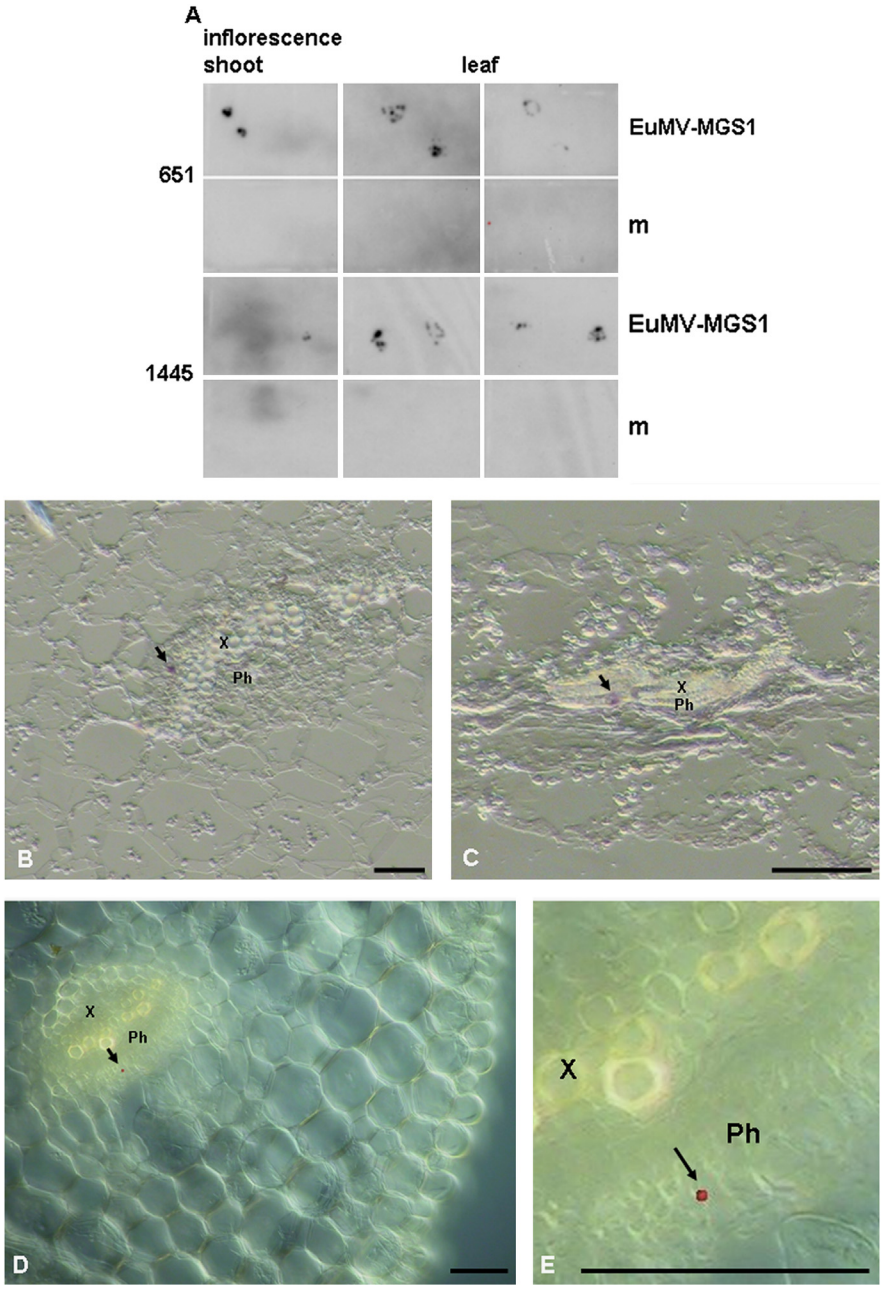

Fig. 2. Phloem-limitation of EuMV-MGS1 in *A. thaliana* plants. (A) Tissue blots of infected or mock-inoculated (m) lines 651 and 1445 were probed at 33 dpi. Cross-sections of inflorescence shoots or furled rosette leaves from one plant per line and infection type were dappled onto nylon membranes and hybridized with a full-length DNA A probe (3 h exposition time). Punctuate hybridization signals indicate a restriction to vein cells. (B and C) Microscopic images of infected rosette leaves of lines 1445 (B) and 651 (C) harvested at 33 dpi after *in situ* hybridization with full-length DNA A probes using NBT/BCIP for staining. Mock-controls are provided in Suppl. Fig. 1 (D and E) FISH detection of EuMV-MGS1 in infected (21 dpi) plants of line 1445 with a 5'-Cy3-labeled primer hybridizing within the AV1 ORF. Merged DIC and fluorescent image from Suppl. Fig. 2C and D with two magnifications to show the localization of the hybridization signal within the phloem of the petiole. Corresponding mock controls in Suppl. Fig. 2A and B.Virus-specific signals are indicated by arrows. X: xylem, Ph: phloem; bar = 50 μm.

experiments are difficult to compare directly to our data due to differences in the experimental design. Oilseed rape mosaic virus (ORMV) and tobacco mosaic virus infection (TMV) enhanced SHR two- to threefold in *Nicotiana tabacum* (Kovalchuk et al., 2003). The luciferase-based system in this study was nine- to twelve-fold more sensitive compared to the GUS-based system (Ilnytskyy et al., 2004). Moreover, the SHR increase was not limited to infected tissues, but was transmittable to healthy plants via a "systemic recombination signal" as shown by grafting experiments. A further study revealed that ORMV infection promoted HRF (1.6-fold) and genome rearrangements in general (mutation frequency, microsatellite instability) in *A. thaliana* using GUS-based detection systems (Yao et al., 2011). HRF increase was variable depending on the concentration of the inoculated virus, plant age and the time or speed of virus replication (Yao et al., 2013). A boost of reactive oxygen species, salicylic acid-dependent systemic acquired resistance signaling or transport of viral small RNAs were discussed to be responsible for the activation of SHR in uninfected tissue.

Recently, the unambiguity of the results with the SHR reporter lines used in the current study was questioned (Ülker et al., 2012), and the authors postulated alternative explanations (post-transcriptional

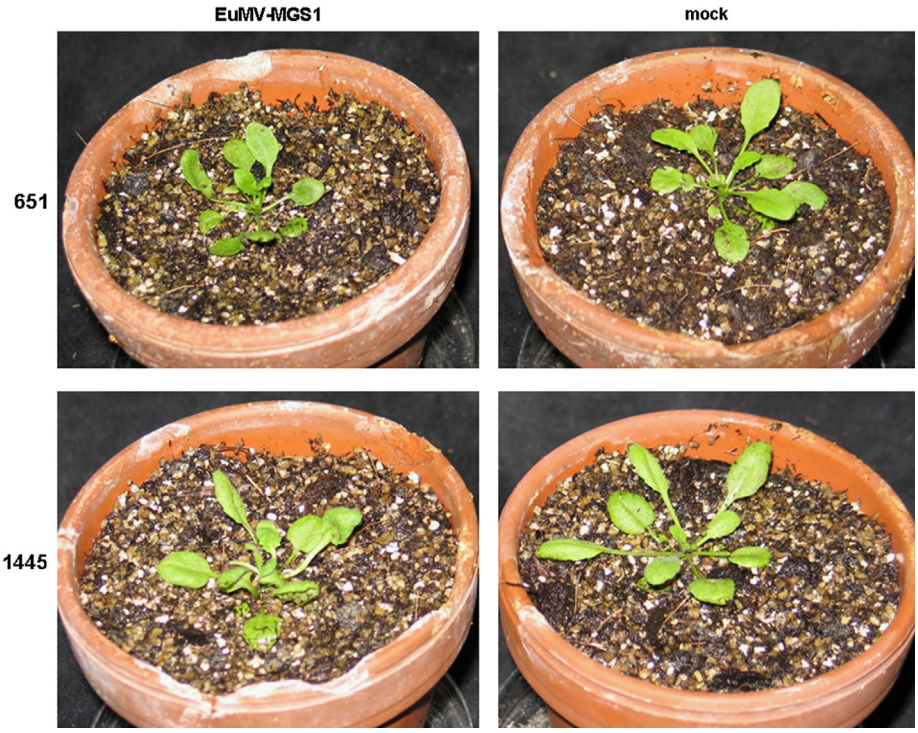

Fig. 3. Symptoms after early inoculation at the 4–6 leaf stage. EuMV-MGS1 infected and mock-inoculated *A. thaliana* plants of the C24 line 651 and the Col line 1445 at 16 dpi. Infected and mock-inoculated plants at higher magnification show mild but distinct leaf rolling in line 1445, which was barely visible in line 651.

or posttranslational event; read-through transcription, alternative splicing, trans-splicing or split protein complementation). In particular, line 1445 raised skepticism as the gene locus was not characterized sufficiently. Puchta and Hohn (2012) have already responded to these claims in general and in some details which we follow. Especially the inverted orientation of the GUS overlap in the tested lines 651 and 1445 rules out that read-through transcription or alternative splicing restore the enzyme activity. Although the fusion of two separately transcribed mRNAs by trans-splicing or transcriptional slippage may occur (reviewed by Dubrovina et al., 2013), we consider this mechanism to be highly unlikely for the lines 651 and 1445, because no promoter is present in these constructs for transcription of the second half (US, Fig. 1). For the same reason, protein complementation seems to be implausible.

The line 1445 has been genotyped in the course of our experiments. The transgene integration of reporter line 1445 is located on chromosome 2 at position 14424870 (corresponding to pos. 14418017 in the latest release of the *A. thaliana* genome; The Arabidopsis Information Resource (TAIR 10), ftp://ftp.arabidopsis.org/home/tair/Sequences/ on www.arabidopsis.org, Apr 02, 2013) according to several reports (Fritsch et al., 2004; Gherbi et al., 2001; Pecinka et al., 2009). One publication (Sun et al., 2008) deviated from this statement mentioning a position on chromosome 5 (Pos. 8633790, corresponding to pos. 8633787 in TAIR10), which was possibly an erroneous assignment only, since this is the integration locus of line IC9C (Molinier et al., 2004) (see Fig. 1). After having tested all plants of each transgenic line by PCR-based genotyping (Supplementary material for line 651 and 1445, 11 and IC9C not shown), no doubt remains about the real integration locus.

Although the comparative heat stress test showed similar trend values under our conditions for enhanced vein-associated signal enhancement, the geminiviral infection should exert a more specific effect on phloem cells. All geminiviruses replicate in nuclei-containing cells of the phloem (companion cells, phloem parenchyma cells) as reviewed by Wege (2007) and most of them are confined to this tissue, as shown for EuMV-MGS1 in this study. This limitation can be overcome partially by co-infection with other viruses upon systemic infection (Pohl and Wege, 2007; Sardo et al., 2011; Wege, 2009) or during agroinfiltration in locally infected cells (Zhang et al., 2001). Therefore it is believed that the restriction is caused by host defense mechanisms rather than the inability of geminiviruses to multiply in other tissues.

All geminiviruses need to activate DNA metabolism in differentiated cells, in the first instance in the phloem and some of them in palisade, spongy parenchyma and epidermal cells thereafter (reviewed by Gutierrez et al., 2004; Hanley-Bowdoin et al., 1999; Hanley-Bowdoin et al., 2004). This task is mainly realized by the interaction of the geminiviral replication-initiator protein Rep and the plant retinoblastoma related cell cycle regulator pRBR which promotes DNA synthesis, but is not absolutely necessary for the replication in the phloem as discussed in detail recently (Ruschhaupt et al., 2013). Interestingly, Rep was also found to interact with key enzymes (Rad54 and Rad51) of the recombination pathway for mungbean yellow mosaic India virus (Kaliappan et al., 2012). The combination of host cell re-replication and recruitment of recombination enzymes would explain convincingly the enhanced recombination-rate observed in the phloem in this study. Alternatively, viral ssDNA could be perceived as DNA damage or a genotoxic stressor by its host (Weitzman et al., 2004). This would trigger an increase in SHR similar to other stressors mentioned before and could in turn be exploited for virus replication. However, geminivirus infection does probably not provoke a general genotoxic stress response as shown by comparing transcriptome profiles of infected and genotoxically stressed *A. thaliana* plants (Ascencio-Ibanez et al., 2008). Together with our data, this lends support to the conclusion that SHR increase is a specific effect of the geminivirus infection rather than a general stress consequence differing thus from the tobamovirus infections mentioned above.

A general enhancement of the recombination frequency upon geminivirus infection could explain the frequent appearance of

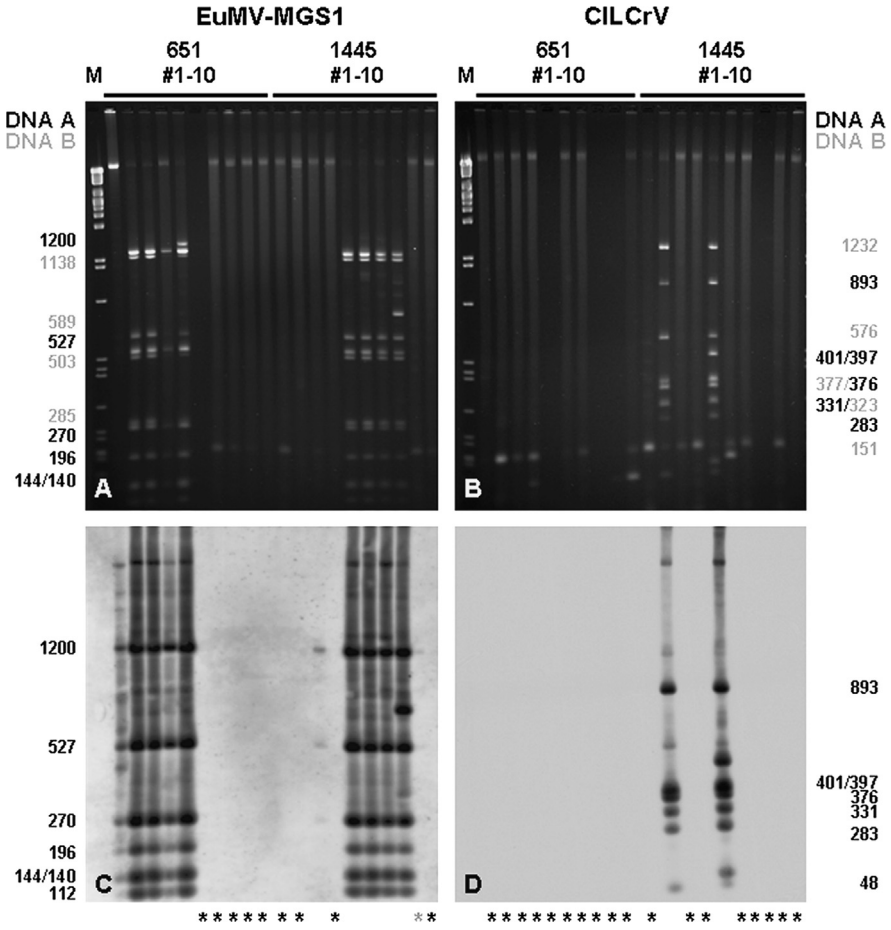

Fig. 4. Detection of EuMV-MGS1 and ClLCrV DNA in reporter lines 651 and 1445 at 14 dpi. (A and B) RCA/*Hpa*II RFLP analyses as described in Fig. 1B, (C and D) Southern blot hybridization with full-length EuMV-MGS1 or ClLCrV DNA A probes. Total nucleic acids of rosette leaves from ten plants (#1–10) per virus and plant line revealed either the expected RFLP patterns (bp; black and gray numbers for DNA A and B, respectively) or proved to be uninfected (marked with black asterisks) and were considered similar to mock-inoculated for the GUS assay evaluation. Specimens marked with gray asterisks were not used for GUS experiments. The figure shows the representative result of one infection experiment.

recombinant gemini- and related viruses in the field (Stainton et al., 2012 and references therein; van der Walt et al., 2009) and thus increase the fitness of these viruses.

Materials and methods

Plants and viruses

Transgenic *A. thaliana* plants of two ecotypes (Columbia and C24) were kindly provided by Drs. O. Mittelsten Scheid (Gregor Mendel Institute, Vienna) and H. Puchta (Karlsruhe Institute of Technology, Karlsruhe): Ecotype C24 transgenic lines 651 and 11 according to (Puchta et al., 1995b; Swoboda et al., 1994) and Columbia lines 1445 (Fritsch et al., 2004; Gherbi et al., 2001; Pecinka et al., 2009) and IC9C (Molinier et al., 2004). They contained the GUS reporter constructs described in Fig. 1A.

Rolling circle amplification (RCA) products of Euphorbia mosaic virus (EuMV; isolates -MGS1 and -MGS2) and Cleome leaf crumple virus (ClLCrV) DNA without satellite DNA were used to inoculate the plants (Paprotka et al., 2010). For mock inoculation DNAs B alone of EuMV-MGS1 or ClLCrV were engineered in the following manner: RCA products of viral DNAs were generated from total nucleic acid extracts from infected *A. thaliana* plants and linearized with singly cutting restriction enzymes (for EuMV-MGS1 *Xma*I; for ClLCrV *Nsi*I). The resulting fragments were gel-purified and re-circularised by T4 DNA ligase, and used as templates for a second RCA to provide the inoculum. The resulting DNAs B alone were unable to infect the plants.

Biolistic inoculation

RCA products of the respective viral DNAs were amplified from total nucleic acids of previously infected *A. thaliana* (Col) plants (Paprotka et al., 2010) and inoculated biolistically (Biolistic Particle Delivery System, PDS-1000/He; rupture discs for 450 psi or 900 psi, macrocarriers, 1.1 μm tungsten microcarriers or 1 μm gold microcarriers; all from Bio-Rad, Munich, Germany) as described (Zhang et al., 2001). Construct integrity was checked by *Hpa*II digestion and gel electrophoresis beforehand (Fig. 1B and C). Per plant, 1.5 μl of the viral RCA product was diluted with 2 μl of H_2O and mixed with 10 μl of gold or tungsten suspension. 2 μl of 0.1 M spermidine and 5 μl of 2.5 M $CaCl_2$ were added, briefly mixed and centrifuged at 14,000 rpm for 10 s. The precipitate was washed twice with 20 μl of 70% ethanol and re-suspended in 10 μl of $\geq$ 99.8% ethanol.

Inoculation was carried out with a pressure of 450 psi for younger plants (4–6 leaf stage) or 900 psi for older plants (12–14 leaf stage). Infection was determined by symptom evaluation and RFLP analysis of RCA products after 14 dpi. Plants which have been assigned as non-infected by this way were rated like mock-inoculated for the statistical analysis of GUS assay results.

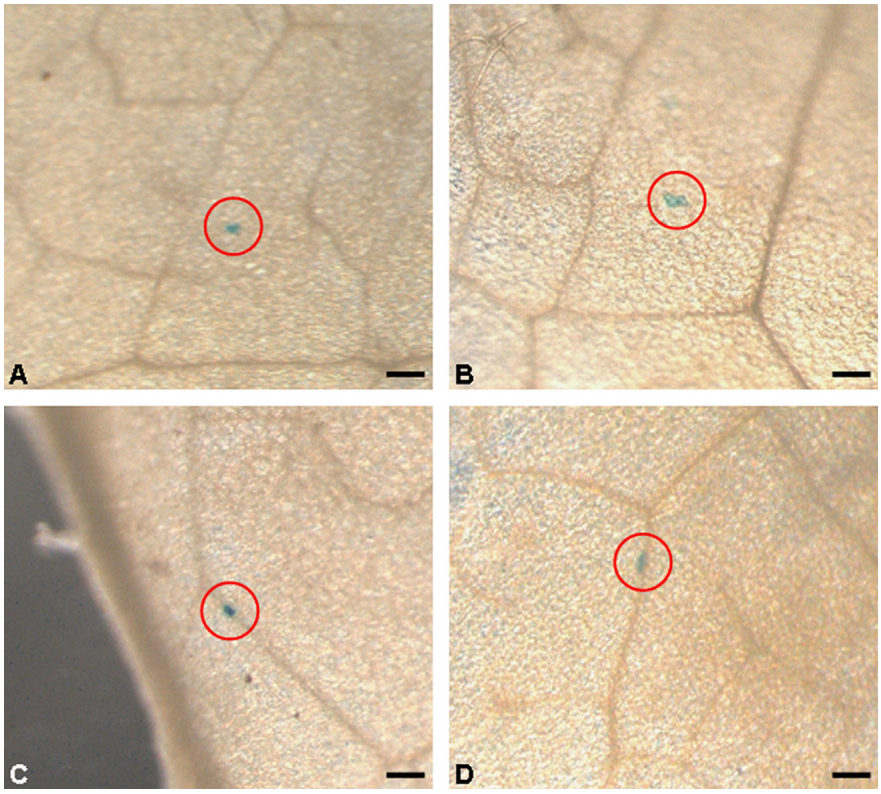

Fig. 5. Recombination events detected by GUS activity. Plants of line 1445 after mock-treatment (A and C) or EuMV-MGS1 (B and D) infection at 21 dpi show GUS signals (blues spots) in single cells. GUS activity (encircled) was observed after histochemical staining with X-Gluc by stereomicroscopy in the mesophyll and epidermal tissues (A and B) or associated to vascular tissues (C and D). Bar = 100 μm.

Plant treatments

Late inoculation

Seeds of lines 651, 11, 1445 and IC9C were kept in standard soil for 7 d under long day conditions (16 h light, 24 °C/8 h dark, 18 °C) and 70% relative humidity for germination. From day 8 on, conditions were changed to a short day cycle (8 h light, 22 °C/16 h dark, 18 °C) and 65% relative humidity. On day 15, plantlets were picked and separately planted into clay pots and kept under short day conditions until day 35. On day 36, plants were transferred to long day conditions (16 h light, 22 °C/8 h dark, 16 °C) and 70% relative humidity. Plants were inoculated from days 40 to 45 (12–14 leaf stage) and were kept further under long day conditions.

Early inoculation

Plants of lines 651 and 1445 were kept until day 21 as described before, but inoculation was performed on day 22 (4–6 leaf stage) under short day conditions. After day 29 (7 dpi), conditions were changed to the long day regime.

Heat stress assay

In a growth chamber (KBW 400, Binder, Tuttlingen, Germany), ten 18 day old plantlets of *A. thaliana* line 1445 were challenged for 24 h at 37 °C, and ten non-stressed plants were kept for control. Both treatments were performed under long day conditions (16 h light/8 h dark). Plants were initially grown as described before, but were acclimatised to long day conditions since day 16. After the heat stress treatment, plants were grown for four days under non-stress conditions for recovery. The GUS assay was performed at the fifth day after stress treatment (day 23).

Total nucleic acid extraction

Leaf samples were ground in liquid nitrogen and nucleic acids were extracted using CTAB or phenol-based techniques as described (Haible et al., 2006; Jeske et al., 2001).

Genotyping of A. thaliana plants by PCR

Genotypes of the plants were determined by PCR using Taq polymerase (Qiagen, Hilden, Germany) and two primer pairs (Metabion, Martinsried, Germany) specific for either the transgene or the insertion locus in wild-type (Col or C24) *A. thaliana* plants (Fig. 1; Suppl. Table 1 and 2). Undiluted, 1:10 or 1:100 diluted plant nucleic acids in 1 μl template were mixed with 1 μl Taq polymerase buffer (10 ×), 0.4 μl of dNTPs (5 mM each), 0.5 μl primer 1 (2 pmol/μl), 0.5 μl primer 2 (2 pmol/μl), 0.5 μl Taq polymerase (0.5 U/μl in 1 × buffer) and 6.1 μl ddH_2O and left to react as described in Suppl. Table 1. PCR products were separated by agarose gel electrophoresis and stained afterwards with an 0.5 μg/ml ethidium bromide solution.

Rolling circle amplification and restriction fragment length polymorphism

RCA and RFLP were performed as described (Haible et al., 2006) using Illustra TempliPhi Amplification kit (GE Healthcare, Munich, Germany) and restriction enzymes (5–20 U/μl; New England Biolabs, Frankfurt/Main, Germany).

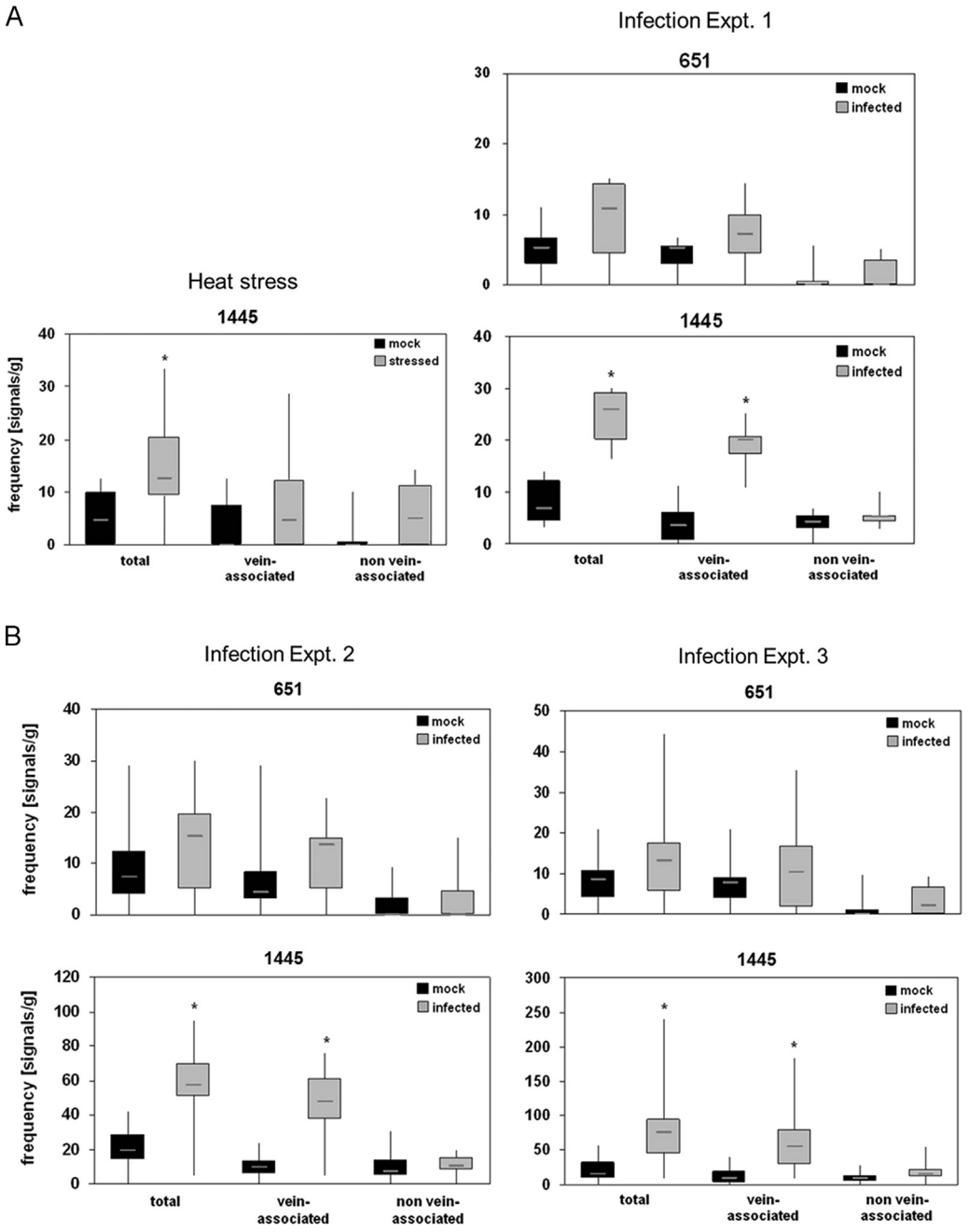

Fig. 6. Statistical analysis of recombination events in SHR reporter plants. Box plot graphs of total, vein- and non-vein-associated GUS signal frequencies per gram plant material for heat-stressed, mock-inoculated, or EuMV-MGS1 infected plants of lines 651 and 1445 at 21 dpi are compared. (A and B) Independent replicate experiments (Expts. 1–3) are shown for the infection study. The following numbers of plants were tested: 10 (Expt. 1), 35 (Expt. 2) and 28 (Expt. 3) for line 651; 11 (Expt. 1), 37 (Expt. 2) and 29 (Expt. 3) for line 1445. *T*-tests or Mann–Whitney rank-sum tests, if data were not distributed normally, were used to confirm significant differences ($p < 0.05$ for heat stress, $p < 0.001$ for infection; indicated by asterisks). Boxes comprise 50% of data, bars imply minimum and maximum, "-" median.

Fixation, paraffin embedding and sectioning of A. thaliana leaf segments

Leaf segments of EuMV-MGS1 and mock infected *A. thaliana* plants (lines 1445 and 651) at 33 dpi were embedded in paraffin, cut with a microtome 1208 (Leica Microsystems, Wetzlar, Germany) into 7–8 μm sections and processed for *in situ* hybridization as described (Zhang et al., 2001).

Blotting and hybridization of nucleic acids

Southern blotting with alkaline (Chomczynski and Qasba, 1984) or neutral transfer (Sambrook and Russell, 2001) was followed by hybridization with DIG-labeled probes as described (Kleinow et al., 2009). For tissue blotting, plant tissue sections were dappled onto N+ membranes (GE Healthcare, Munich, Germany) and DNA was UV-crosslinked to the membrane using UV light of 70,000 mJ/cm^2 for 2 min. Viral probe DNA was derived from circular DNA of infected *A. thaliana* (Col) plants which was amplified by RCA, linearized and gel-purified for EuMV-MGS1, EuMV-MGS2, ClLCrV DNA A or B (20–40 ng/μl). For Southern and tissue blot hybridization, viral DNA was DIG-labeled with the DIG High Prime DNA Labeling Kit (Roche Diagnostics, Mannheim, Germany), for *in situ* hybridization it was biotin-labeled (Biotin-Nick Translation Kit, Roche Diagnostics) and purified with the Nucleotide Removal Kit (Qiagen, Hilden, Germany). Chemiluminescence was detected with

anti-DIG AP conjugate and CSPD (Roche Diagnostics) for blot hybridization or with streptavidin-AP conjugate and NBT/BCIP (Boehringer Ingelheim, Ingelheim Germany) for *in situ* hybridization of leaf sections. Specimens were examined using an Axioskop microscope (Zeiss, Oberkochen, Germany) with differential interference contrast (DIC) equipment.

Fluorescence in situ hybridization (FISH) of virus DNA

Leaves and petioles of EuMV-MGS1- or mock infected *A. thaliana* plants (21 dpi) were cut into 75 μm sections using a hand microtome and were treated for FISH as described by Ghanim et al. (2009) but without RNase treatment. For detection of viral DNA, a 5'-Cy3-labeled primer (Cy3-5' CTCGTATTTCCCTGCTTCTTG 3'; Biomers, Ulm, Germany) hybridizing within the AV1 ORF of EuMV-MGS1 DNA A was used. Fluorescent images were merged with DIC images to show the localization of the hybridization signal using Corel Paintshop Pro X4 software (Corel, Munich, Germany).

GUS assay

Three leaves classified as older, medium and young were harvested for the first experimental set-up, whereas whole plants were harvested at 21 dpi and weighed immediately for the second experimental set-up. Samples were placed into small Petri dishes, submerged with 4–5 ml of sterile GUS staining solution (Baubec et al., 2009) containing 0.5 mg/ml X-Gluc (Duchefa, Haarlem, Netherlands) and vacuum-infiltrated 3–4 times for 2–3 min, incubated at 37 °C overnight, de-stained with 70% ethanol several times overnight at 37 °C, and stored in 70% ethanol at 4 °C. The frequencies of blue spots were evaluated under a MZ16FA stereomicroscope (Leica Microsystems, Wetzlar, Germany) with 40–50 fold magnification and it was determined whether they were close or at a distance to the veins. Assessed signals of each specimen were referred to its fresh weight and statistical analysis was performed with a t-test or a Mann–Whitney rank sum test, if data was not distributed normally, using the SigmaStat program.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.01.024.

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