TECHNICAL ADVANCE

Fluorescently Tagged *Potato virus Y*: A Versatile Tool for Functional Analysis of Plant-Virus Interactions

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Submitted 24 July 2014. Accepted 28 February 2015.

Potato virus Y (PVY) is an economically important plant virus that infects Solanaceous crops such as tobacco and potato. To date, studies into the localization and movement of PVY in plants have been limited to detection of viral RNA or proteins *ex vivo*. Here, a PVY N605 isolate was tagged with green fluorescent protein (GFP), characterized and used for *in vivo* tracking. In *Nicotiana tabacum* cv. Xanthi, PVY N605-GFP was biologically comparable to nontagged PVY N605, stable through three plant-to-plant passages and persisted for four months in infected plants. GFP was detected before symptoms and fluorescence intensity correlated with PVY RNA concentrations. PVY N605-GFP provided *in vivo* tracking of long-distance movement, allowing estimation of the cell-to-cell movement rate of PVY in *N. tabacum* cv. Xanthi (7.1 ± 1.5 cells per hour). PVY N605-GFP was adequately stable in *Solanum tuberosum* cvs. Désirée and NahG-Désirée and able to infect *S. tuberosum* cvs. Bintje and Bea, *Nicotiana benthamiana*, and wild potato relatives. PVY N605-GFP is therefore a powerful tool for future studies of PVY-host interactions, such as functional analysis of viral and plant genes involved in viral movement.

Potato virus Y (PVY) is the type member of the family *Potyviridae* with a single-stranded, positive sense RNA genome of approximately 10 kb (King et al. 2011). PVY was recently classified as one of the ten most important plant viruses (Scholthof et al. 2011). It infects a wide range of plant species, predominantly members of family *Solanaceae*, and is present worldwide (Blanchard et al. 2008; Gray et al. 2010). The most economically important symptoms caused by PVY are vein necrosis on tobacco and tuber necrosis on potato, known also as potato tuber necrosis ringspot disease (Beczner et al. 1984).

A range of techniques have been used to investigate the replication and movement of plant viruses. The majority used destructive sampling and subsequent detection of viral proteins or nucleic acids in different plant organs (Ghanim et al. 2009; Leisner et al. 1992; Más and Pallas 1996; Mehle et al. 2004;

Kogovšek et al. 2011; Lucas 2006; Nass et al. 1998; Rajamäki and Valkonen 2003; Rasheed et al. 2006; Rusetsky and Blotskaya 2001; Simón-Buela and García-Arenal 1999; Tilsner et al. 2009). The infectious cDNA clone strategy has been used to construct plant viruses tagged with fluorescent proteins (Baulcombe et al. 1995; Oparka et al. 1996) and nonfluorescent tags (Bedoya et al. 2012). This has allowed noninvasive monitoring of cell-to-cell movement and systemic spread of plant viruses in living plants (Cohen et al. 2000; Tilsner et al. 2013).

The tagged virus approach, however, has two issues to address before it can be used for accurate tracking of viral movement. The first concerns the stability of the inserted tag in the viral progeny. Inserted sequences can often be deleted, recombined, or modified due to the rapid and error-prone multiplication of the viral genome (Avesani et al. 2007; Seaberg et al. 2012). This will result in functional loss of the reporter gene. The second concern is the effect of the inserted gene on virus pathogenicity, multiplication, accumulation, and movement (Cruz et al. 1996). A stably tagged virus with minimal effects on the virus fitness would provide an ideal tool to describe the viral infection process through *in vivo* virus tracking within host.

PVY infectious clone development has been less straightforward than for other potyviruses (Fakhfakh et al. 1996). The presence of cryptic prokaryotic promoters within the PVY genome results in the expression of cytotoxic proteins in *Escherichia coli* (Jakab et al. 1997). Thus, only a few infectious PVY clones have been reported to date. First, a cell-free polymerase chain reaction (PCR)-based PVY^{NTN} clone (Fakhfakh et al. 1996), followed by a bipartite infectious PVY N605 clone (Jakab et al. 1997). Subsequently, full-length infectious clones were developed to avoid cytotoxic effects in *E. coli*. This was achieved through intron insertion into the toxic regions of the genome (Bukovinszki et al. 2007) or by point mutations in the cryptic promoter regions (Chikh Ali et al. 2011).

In this study, PVY N605-green fluorescent protein (GFP) was used to estimate the rate of cell-to-cell movement in inoculated leaves. The *gfp* gene was inserted into the PVY N605 clone (Jakab et al. 1997) flanked by proteolytic sites to enable excision upon translation. Pathogenicity and viral RNA accumulation throughout the plant were compared between tagged PVY N605-GFP and the nontagged PVY N605. A reverse-transcription quantitative (RT-q)PCR approach, including a newly developed assay specific to PVY N605, was used to

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures and three supplementary tables are published online.

assess the stability of the *gfp* insertion. PVY N605-GFP was infectious in a variety of hosts, including *N. tabacum*, *N. benthamiana*, potato (*Solanum tuberosum*) cultivars, and wild *Solanum* species. PVY N605-GFP is a powerful tool for studies into plant-pathogen interactions, broadening and complementing previous knowledge of PVY movement.

RESULTS

Several cloning strategies were attempted to construct GFP-tagged PVY N605. Initially, unique restriction sites were introduced in the PVY genome between nuclear inclusion b (NIb) and coat protein (CP) coding regions for a subsequent introduction of *gfp*. Two configurations were attempted, one in which GFP was excised out of the poliprotein upon translation and a second in which GFP was linked to CP by an amino acid linker. After the gene-gun bombardment of 50 *N. clelandii* plants per construct, we observed no infection. The result was not surprising. Previous studies have also reported difficulty in the manipulation of infectious PVY clones. Problems have included low plasmid concentrations in *E. coli* (Johansen 1996) and reduced or completely abolished infectivity after manipulation (Bukovinszki et al. 2007; Moury et al. 2004). The third and successful cloning procedure utilized direct fusion PCR to construct the fragment for ligation. This encompassed a partial NIb, full GFP, and full CP coding regions, which underwent ligation into the 3' subclone of the bipartite infectious PVY N605 clone (Jakab et al. 1997). *Gfp* was flanked with a native NIa proteolytic site at its 3' end and with a modified, alternate codon, NIa proteolytic site at the 5' end. The infectivity of biolistic inoculation of the bipartite clone is generally very low at 0 to 25% and stochastic (Tribodet et al. 2005). Gene-gun bombardment of PVY N605-GFP yielded three infected *Nicotiana clelandii* plants out of 60, which is within range of the nontagged clone. PVY N605-GFP multiplied and spread systemically in *N. clelandii* and infected *N. tabacum* cv. Xanthi via mechanical inoculation.

Pathogenicity and accumulation of PVY N605-GFP.

The effect of *gfp* was investigated to verify that PVY N605-GFP and PVY N605 were biologically comparable. Calibrated inoculations of *N. tabacum* cv. Xanthi, *S. tuberosum* cvs. Désirée and NahG-Désirée (Halim et al. 2004) with PVY N605-GFP and PVY N605 produced no obvious differences in the type or dynamics of the symptoms.

In *N. tabacum* cv. Xanthi plants, both tagged and nontagged PVY induced systemic vein clearing and vein necrosis symptoms at 4 and 9 days postinoculation (DPI), respectively. At 14 DPI, 100% of the PVY N605-GFP- and 80% of the PVY N605-inoculated *N. tabacum* cv. Xanthi plants showed vein necrosis. One PVY N605-inoculated plant was asymptomatic and two showed only vein clearing, indicating the natural variability of symptom development.

S. tuberosum cv. Désirée plants were asymptomatic when inoculated with either of the viruses. Tagged and nontagged PVY induced vein necrosis at 6 DPI on inoculated leaves and at 14 DPI on systemically infected leaves of *S. tuberosum* cv. NahG-Désirée, a cultivar with reduced concentration of salicylic acid and, thus, attenuated defense mechanisms related to salicylic acid signaling.

The concentrations of PVY N605-GFP and PVY N605 in inoculated tobacco and potato plants were measured using the universal (UNI) RT-qPCR assay (Kogovšek et al. 2008) (Fig. 1). The stably expressed cytochrome oxidase gene was detected with the COX RT-qPCR assay (Weller et al. 2000) and was used to normalize PVY genome concentrations. This was to account for variability in the amount of tissue sampled.

In tobacco, inoculated leaf (L1) and the first systemically infected leaf (L2) were analyzed between 1 and 5 DPI. No statistically significant differences in PVY concentrations were observed between the PVY N605-GFP and PVY N605 in L1 and L2 (t test, $P = 0.05$), except for L2 at 2 DPI, in which PVY N605-GFP reached significantly higher concentrations than PVY N605 ($P < 0.05$). Both isolates, however, reached comparable concentrations by 3 DPI (Fig. 1A and B). Additionally, we observed no significant differences in the viral genome concentrations between tagged and nontagged PVY in the upper systemically infected leaves L4 to L8 of *N. tabacum* cv. Xanthi at 14 DPI (Fig. 1C). All data combined indicated that *gfp* insertion did not significantly impair the virus accumulation in *N. tabacum* cv. Xanthi.

Viral genome concentrations in systemically infected leaves were comparable between tagged and nontagged PVY at 6 and 18 DPI in *S. tuberosum* cv. Désirée and at 6 DPI in *S. tuberosum* cv. NahG-Désirée (Fig. 1D). Tagged PVY, however, reached lower concentrations than nontagged at 11 DPI in *S. tuberosum* cv. Désirée and at 11 and 18 DPI in *S. tuberosum* cv. NahG-Désirée.

Stability of the tag insertion.

PVY N605-GFP has a larger genome than PVY N605, which could result in an altered multiplication or encapsidation rate of the virus. Genomes with partial or complete deletion of *gfp* could multiply faster and outcompete the PVY N605-GFP. This would result in loss of *gfp* in the PVY N605-GFP progeny.

First, fluorescence microscopy was used to estimate PVY N605-GFP stability at the protein level. GFP fluorescence was detected at 14 DPI in the systemically infected leaves of *N. tabacum* cv. Xanthi in three plant-to-plant passages and up to 120 DPI in all five *N. tabacum* cv. Xanthi plants inoculated in the second passage. Fluorescence was detected in *S. tuberosum* cv. Désirée and NahG-Desire until 30 DPI. Later observations were not conducted.

Second, the stability of the insertion was tested at the nucleic acid level, with a combination of four RT-qPCR assays to detect the presence of genomes with deleted *gfp*. The COX assay was used for cross-sample normalization. The UNI assay (Kogovšek et al. 2008) targeting the CP region was used to quantify the total PVY population. A PVY-*gfp* assay (Joshi et al. 2008) specific to the *gfp* sequence was used to quantify the PVY N605-GFP genome. Additionally, to detect the genomes with complete deletion of *gfp*, we designed a PVY-wild type (PVY-wt) assay that specifically targeted the NIb-CP junction in PVY N605. Stable insertion of *gfp* would result in equal quantities of nucleic acids detected by the UNI and PVY-*gfp* assays, while there would be no target to be detected with PVY-wt assay. Partial deletions of the *gfp* gene would not be detected with the PVY-wt assay. Studies on insert stability in PVX (Avesani et al. 2007) and PVA (Rajamäki et al. 2005), however, have reported that in the case of partial reporter deletion, only the terminal parts of the inserted gene remained in the viral genome, while the central part was deleted. In the present study, the PVY-*gfp* assay targeted the central part of *gfp*. Therefore partial deletions would result in a decreased quantity of *gfp* in comparison with the total PVY population. To confirm that assumption, classical RT-PCR fragment length analysis was conducted on selected samples (discussed below).

The concentration of *gfp* in *N. tabacum* cv. Xanthi was equivalent to that of total PVY genomes in all samples, all passages and over a wide range (10^{-10} to 10^{-9}) of PVY concentrations (Fig. 2). This is illustrated by the 0.98 slope of regression curve between the theoretical copy number measured by PVY-*gfp* and the UNI assays. The concentrations of the PVY genomes with complete deletion of *gfp*, i.e., reverted PVY N605, did not

increase either with the increased concentration of the total PVY (Fig. 2A) (slope of the linear regression for PVY-wt assay: -0.0058) or with time postinoculation. Moreover, no PVY-wt genomes were detected in plants infected with PVY N605-GFP at 120 DPI (Supplementary Fig. S1). Of 164 tested samples, 78 contained no reverted PVY N605 genomes and 74 contained PVY N605 genomes in concentrations lower than 0.05% of the total population. Only 12 samples contained PVY N605 genomes at concentrations between 0.05 and 1.2% of the total PVY population. The RT-qPCR strategy detected two additional samples, one at 5 DPI and one at 7 DPI, in which the quantity of *gfp* was $<95\%$ of the total PVY genomes and there was no PVY N605 genomes detected at the same time. This indicates that these two particular samples have a small proportion (5%) of the PVY N605-GFP genome and might have undergone partial *gfp* deletion.

Similarly, in *S. tuberosum* cvs. Désirée and NahG-Désirée, the concentration of *gfp* corresponded to the concentration of total PVY genomes (Fig. 2B). The concentration of PVY-wt genomes did not increase, either with increased total PVY concentration or with the time postinoculation. We detected three samples of *S. tuberosum* cv. Désirée with PVY-wt levels up to 40% of UNI. The concentration of PVY in those samples,

however, was near the limit of quantification of the RT-qPCR method in which accuracy can be reduced.

To further investigate stability, we amplified the fragment encompassing partial NIB, full GFP, and full CP coding regions and compared fragment lengths. The analysis was performed on a subset of samples that had either decreased levels of PVY-*gfp* or increased levels of PVY-wt. In all the 20 successfully amplified samples, we detected only a 2.2-kb-long fragment that corresponds with the presence of a full *gfp* gene.

Overall, our data indicated that *gfp* was stably maintained and multiplied in the PVY genome. Complete and partial deletions of *gfp*, however, did occur in these plants, although in most cases, the resulting genomes only reached residual concentrations.

Comparison of PVY N605-GFP tracking with symptom development and PVY RNA accumulation.

To determine the utility of PVY N605-GFP as a tracking tool, we compared the appearance of symptoms with concentrations of PVY RNA and the intensity of GFP fluorescence in *N. tabacum* cv. Xanthi.

High background fluorescence in the green and red channel was detected in the inoculated leaves during the first few days

Fig. 1. Concentration of *Potato virus Y* (PVY) N605-green fluorescent protein (GFP) and PVY N605 in inoculated and systemically infected leaves of *Nicotiana tabacum* and *Solanum tuberosum* cvs. Désirée and NahG-Désirée at different days after inoculation (DPI) as indicated. Data are means $\pm$ standard errors of three to seven data points; an asterisk (*) indicates $P < 0.05$ versus wild type.

after inoculation and gradually decreased over time (Fig. 3i). High autofluorescence was also detected in healthy and PVY N605- and mock-inoculated plants (Supplementary Fig. S2). Nonetheless, detailed fluorescence microscopy of inoculated leaves revealed increased GFP fluorescence signals specific for infected parts of the leaf as early as 2 DPI (Fig. 3ii), when first symptoms were observed at 9 DPI. Analysis of systemically infected leaves showed that PVY unloads from class I, II, III, and IV veins as early as 4 DPI.

The inoculated and systemically infected leaves were sectioned based on fluorescence microscopy analysis. The PVY genome concentration (UNI assay) and the intensity of GFP fluorescence was measured in each section (Fig. 4). The quantification of the fluorescent signal in the inoculated leaf was hampered by high background fluorescence. The PVY RNA concentration in the section containing fluorescent foci (Fig. 4, subsection 3, arrow), however, was approximately 10-fold higher than in the neighboring tissue with only background fluorescence. In systemically infected leaves, the intensity of the GFP signal showed a linear correlation with the PVY RNA concentrations in the subsections (Fig. 4, $R^2 = 0.76$).

In vivo tracking and rate of cell-to-cell movement of PVY.

To allow in vivo noninvasive tracking of PVY movement at different times postinoculation, the plants were grown in conical tubes. An example of in vivo PVY N605-GFP tracking is given in Figure 5. Under these experimental conditions, the first distinct GFP fluorescent foci were observed at 3 to 4 DPI on L1 and at 4 to 5 DPI in systemically infected leaves. The GFP signal was most intense in the inoculated leaves L1 and systemically infected leaves L3 and L4. The first systemically infected leaf above the inoculated site, i.e., leaf L2, remained fluorescence free in two-thirds of the plants used for in vivo tracking, which is in agreement with the ex vivo observations, where in five-eighths of the plants, no GFP signal was observed in L2 (data not shown).

Using in vivo PVY N605-GFP tracking technique, we estimated the rate of PVY movement in the inoculated leaf of *N. tabacum* cv. Xanthi. The measurements were carried out on 15 individual foci from two different plants at 72, 99, and 126 h postinoculation (HPI) (Fig. 6). To account for cell growth, the

mean cell areas were determined at each time point (Fig. 6A). The areas of an average cell were 7,630.5, 8,070.9, and 9,259.7 μm^2 at 72, 99, and 126 HPI, respectively. The area of the foci were measured with the circular measurement tool of the NIS Elements software (Nikon) (Fig. 6B), and the mean numbers of cells in each of the foci were estimated using the area of the average cell at each time point (Fig. 6C). Under experimental conditions, the mean rate of cell-to-cell movement across all the foci measured from 72 to 126 HPI was 7.1 ± 1.5 cells per hour. In the first interval, 72 to 99 HPI, the mean rate of cell-to-cell movement was 6.5 ± 1.5 cell per hour. This was less than in the second, 99 to 126 HPI, with a rate of 7.5 ± 2.5 cells per hour. The difference in this cell-to-cell rate, however, was not statistically significant: *t* test, $P = 0.05$. When the rate was expressed as a cellular contagion rate, i.e., newly infected cells per one initially infected cell per hour, the rate decreased from 2.1 ± 0.6 in the first interval to 0.9 ± 0.4 in the second interval.

Host range of PVY N605-GFP.

To investigate the use of GFP-tagged PVY in other plant species, a range of *Solanum* and *Nicotiana* species and cultivars were inoculated (Table 1). *Nicotiana benthamiana*, systemically infected with PVY N605-GFP at 6 to 8 DPI, was tested, as it is a model for plant-virus interaction studies. To study the movement of PVY in *S. tuberosum*, two additional cultivars, i.e., 'Bintje' and 'Bea', were chosen due to their different sensitivities to PVY. PVY N605-GFP was detected as small fluorescent foci in the inoculated leaves of 'Bintje' at 6 to 8 DPI. In inoculated leaves of 'Bea', the background fluorescence was too high for a reliable detection. Discreet fluorescent patches were detected in systemically infected leaves of both 'Bintje' and 'Bea' at 18 to 20 DPI. In addition, wild potato relatives were inoculated with PVY N605-GFP. The tested species were chosen based on their potential use as models to study potato-PVY interactions. GFP signals were observed in the inoculated leaves of all 15 tested wild *Solanum* species at 14 DPI. GFP fluorescence was detected systemically in *Solanum mochiense*, *Solanum papita*, and in three of six tested *Solanum venturii* accessions at 14 DPI.

Fig. 2. Analysis of the viral progenies in plants inoculated with *Potato virus Y* (PVY) N605-green fluorescent protein (GFP). Stability of the *gfp* insertion is shown as correlations between total PVY concentration (UNI assay) and concentrations of PVY N605-GFP (PVY-*gfp* assay) or reverted PVY N605 (PVY-wt assay). **A**, Linear regression curves with equations and R^2 are for PVY-*gfp* and PVY-wt assays on *Nicotiana tabacum* cv. Xanthi and **B**, on *Solanum tuberosum* cvs. Désirée and NahG-Désirée. The PVY-*gfp* and PVY-wt regression curves in **B** represent samples from both 'Désirée' and 'NahG-Désirée'.

DISCUSSION

In the present study, *gfp* was inserted in the PVY genome between the potyviral *NIb* and *CP* and was flanked by the Nla-specific protease recognition sites, allowing excision from the polyprotein and release into the cytoplasm after translation, as has been reported for the majority of other potyviruses (Supplementary Table S2). This insertion site has been described to yield very high transgene protein levels (Chen et al. 2007). The construction of the PVY N605-GFP, however, was successful only at the third attempt. This indicated that the production of fluorescently tagged PVY is still not a straightforward process. The stability of the reporter gene and its biological effects on

viral pathogenicity and accumulation were characterized prior to the monitoring of viral movement of the virus in its host plant. To ensure insert stability, the protease recognition site at the 5' of *gfp* was modified, using alternative codons to decrease the possibility of recombination due to sequence repetitions.

When used for localization and movement studies, it is essential that tagging does not compromise viral pathogenicity or accumulation and that the tag is stably maintained in the progeny. The potyviral genome is highly prone to recombination (Chare and Holmes 2006), and *gfp* could, therefore, be partially or fully deleted during the infection cycle. Here, the inclusion of *gfp* in the PVY N605 genome did not affect symptom development either in *N. tabacum* cv. Xanthi or in *S. tuberosum* cvs. Désirée and

Fig. 3. Detection of *Potato virus Y* (PVY) N605-green fluorescent protein (GFP) (left) and observation of the symptoms (right) for the same leaf of *Nicotiana tabacum* cv. Xanthi. Fluorescence observations in green and red are shown together with the overlay of green and red channels; pixels with signals in both the red and green channels are yellow in the overlay. Detailed sections: (i) Examples of unspecific background fluorescence, (ii) examples of specific GFP signal. Images are identically scaled to accurately present the size of the GFP fluorescence foci. Scale bars: 5 mm (main image) and 250 μ m (i and ii).

NahG-Désirée. Additionally, all *N. tabacum* cv. Xanthi plants inoculated with the PVY N605-GFP were systemically infected, demonstrating good overall infectivity. In *N. tabacum* cv. Xanthi, the concentrations of PVY-*gfp* were comparable to the total PVY concentrations and did not decrease with the time postinoculation indicating steady multiplication of the GFP tag. RT-qPCR analysis showed that very low background levels of the reverted genome (genome with deleted *gfp*) were present in *N. tabacum* cv. Xanthi. These low concentrations did not increase, either with increased total PVY concentrations or with the time postinoculation. Moreover, tagged PVY reached concentrations comparable to nontagged PVY in *N. tabacum* cv. Xanthi at the beginning of the infection in L1 (1 to 5 DPI) and in the upper systemically infected leaves at 14 DPI, which reflects the comparable dynamics of the systemic infection and viral accumulation of nucleic acids for PVY N605-GFP and PVY N605. Taken together, we can conclude that PVY N605-GFP is an appropriate tool for studying virus movement in *N. tabacum* cv. Xanthi.

Potato is regarded as a less-permissive host for PVY than tobacco. The concentration of PVY N605-GFP in systemically infected leaves of *S. tuberosum* cv. Désirée was comparable to the concentration of nontagged PVY N605 at 6 and 18 DPI. Lower concentrations of tagged PVY were detected at 11 DPI. In 'NahG-Désirée', PVY N605-GFP reached generally lower concentrations than did PVY N605, especially at later stages of the virus cycle between 11 and 18 DPI. We can speculate that the hindered defense mechanisms of salicylic acid-deficient 'NahG-Désirée' were still sufficient to reduce accumulation of the presumably slower tagged virus but not the wild-type one. In 'Désirée', the plant defense is high enough to prevent the

accumulation of both viruses, reflected in lower overall concentration of tagged and nontagged PVY than in 'NahG-Désirée'. When applying PVY N605-GFP on potato cultivars, the accumulation should, therefore, be tested for each cultivar, as each can affect viral multiplication differently. The stability, reflected by the ratio between PVY-*gfp* and total PVY concentrations, followed the same trend as in tobacco.

Since the RT-qPCR technique applied here did not enable reliable detection of PVY genomes with partial *gfp* deletions, we also analyzed the length of RT-PCR fragments corresponding to the 3' end of NIb, full GFP, and full CP regions. Tobacco and potato samples with increased concentrations of reverted genomes, i.e., PVY N605, or a reduced percentage of *gfp* in the total PVY population were investigated. In all cases, a single fragment corresponding to intact *gfp* was visualized on agarose gel. Due to the lower sensitivity of RT-PCR in comparison with RT-qPCR, the presence of PVY genomes with partial deletions of *gfp* cannot be excluded.

In *N. tabacum* cv. Xanthi, fluorescently tagged PVY enabled visualization of the primary infectious foci in the inoculated leaf and observation of the viral spread to the systemically infected leaves (Figs. 3 and 5, respectively). In inoculated leaves, PVY infection is difficult to detect with molecular methods, as viral RNA levels in the infected tissue are obscured by the nucleic acids present on the leaf surface from the inoculum. PVY N605-GFP proved to be a good PVY infection indicator, as the primary infective foci were definitively detected on L1 already at 2 DPI (Fig. 3), before any symptoms were observed. Moreover, the intensity of the GFP fluorescence was in linear correlation to the PVY RNA concentrations in *N. tabacum* cv. Xanthi.

Fig. 4. Correlation between green fluorescent protein (GFP) signal intensity and *Potato virus Y* (PVY) RNA concentrations in the inoculated L1 and systemically infected L3, L4, L5, and L6 leaves at different times postinoculation. **A**, Leaves were sectioned according to the fluorescence observed. The concentrations of PVY RNA and the integrated density of the GFP fluorescence for each subsection of the systemically infected leaves were measured. The images of the leaves are identically scaled and are oriented left (tip) to right (base). No significant background signals were detected in the red channel of the systemic leaves. **B**, Regression curve between PVY RNA concentration and intensity of GFP fluorescence on systemically inoculated leaves (subsection numbers 5 to 18), with the R^2 value given below. TCN = theoretical copy number.

More importantly, the *in vivo* tracking strategy enabled estimation of the rate of cell-to-cell movement of PVY in *N. tabacum* cv. Xanthi (Fig. 6). The mean rate of cell-to-cell movement was expressed as the number of newly infected cells per hour. In the first measured interval, 72 to 99 HPI, this was lower than in the second interval, 99 to 126 HPI. The estimations associated with these two intervals were not significantly different. Thus, the data here could suggest that, in the initial stages of PVY infection, more new cells are being infected each subsequent hour. A possible explanation is that, with an increase in infected area, an increasing number of noninfected neighboring cells are available for infection. In contrast, the cellular contagion rate, i.e., newly infected cells per one initially infected cell per hour, decreased in the second interval in comparison with the first. This observation is in agreement with the recent study on *Tobacco etch virus* movement, where flow cytometry was used to measure the infection rate (Tomas et al. 2014). The observed rate defines the increase in the number of cells producing viral polyprotein at detectable levels, i.e., the spread of metabolically active PVY (Forterre 2010). The rate of spread of the viral RNA may be different. Nonetheless, to our knowledge, the present study is the first in which an estimation of cell-to-cell spread was carried out nondestructively.

The use of PVY N605-GFP in other *Nicotiana* and *Solanum* species and cultivars was also investigated. The well-established protocols for transient expression of fluorescent fusion proteins by agroinfiltration in *N. benthamiana* (Du et al. 2014) combined with the use of PVY N605-GFP will enable high spatial resolution studies of plant protein expression or localization in relation to PVY. The *S. tuberosum* cultivars tested have different susceptibilities to PVY and, hence, the use of PVY N605-GFP in these cultivars will allow studies on the effects of plant susceptibility on the cell-to-cell and long distance movement of PVY. Wild-potato relatives are used in our laboratory as tools for the functional analysis of genes involved in potato-PVY interactions. A GFP signal was detected systemically in *N. benthamiana*, *S. tuberosum* cvs. 'Désirée', 'NahG-Désirée', 'Bintje', and 'Bea', and in many wild-potato relatives (Table 1), indicating wide applicability of the PVY N605-GFP. Nonetheless, to ensure reliable results obtained by using PVY N605-GFP, the correlation of GFP intensity with virus concentration and stability of the tag should be further investigated for each host and experimental condition separately.

The PVY N605-GFP developed here will contribute to advances in the functional analysis of viral and host genes involved in viral movement and aid systems biology approaches

Fig. 5. In-vivo tracking of the long-distance movement of *Potato virus Y* (PVY) N605-green fluorescent protein (GFP). Top: Symptoms on the plant at 9 days postinoculation (DPI). Bottom: GFP fluorescence of inoculated L1 and systemically infected L2, L3, and L4 leaves of the same plant at different DPI.

for studies of PVY-host interactions. To date, such studies have involved laborious RNA isolations, followed by RT-qPCR to evaluate the extent of virus spread. PVY N605-GFP will enable much faster and noninvasive observations of the localization and spread of PVY.

MATERIALS AND METHODS

Construction of GFP-tagged PVY N605.

The GFP-tagged PVY was derived from a bipartite infectious PVY N605 clone developed by Jakab et al. (1997). It consists of two overlapping PVY subclones (the 5' and 3' halves) that are ligated into a full-length PVY N605 clone before biolistic inoculation of *N. tabacum* cv. Clevelandii at the three- to four-leaf stage.

The *gfp* gene sGFP S65T was inserted between the Nib and CP coding regions of the 3' PVY N605 subclone, using fusion PCR

and ligation. Three PCR fragments were amplified to insert *gfp* between *Nib* and *CP*. The schematic representation of the cloning steps is depicted in Supplementary Figure S3, and the primers used are listed in Supplementary Table 1. PCR fragment A, a partial *Nib* sequence, and C, a full *CP* and 3' untranslated region, were amplified with the FA_Fw/Rev and FC_Fw/Rev primer pairs, respectively. This was performed using the 3' half PVY N605 subclone as template. PCR fragment B was amplified with the FB_Fw/Rev primer pair from *gfp*-containing plasmid. A NlaI protease recognition site and fusion PCR hybridization sites were added to the primer tails. Fragments were amplified in 50- μ l reactions, under the following cycling conditions: 3 min at 94°C, followed by 5 cycles of 1 min at 94°C, 1 min at 49°C, and 1 min at 72°C; then, 35 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C; and a final elongation step at 72°C for 10 min. Fusion PCR was carried out in two steps. First, purified PCR fragments A and

Fig. 6. Measurements of the rate of cell-to-cell movement of *Potato virus Y* (PVY) N605-green fluorescent protein (GFP) in an inoculated leaf of *N. tabacum* cv. Xanthi. **A**, The area of the average cell was measured for three different positions of the leaf, numbered 1 to 3, at 72, 99, and 126 h postinoculation (HPI). The area of 10 cells (outlined with green line) was measured on each position. **B**, The diameter of each foci was measured at 72, 99, and 126 HPI. **C**, Number of cells in each foci from plant 1 (1a to 1j) and plant 2 (2a to 2e) was estimated, using the area of the average cell at that time postinoculation. In the case of foci 1a and 1j the size at 72 HPI was not measured due to high background fluorescence. **D**, Estimated rates of cell-to-cell movement in all measured foci. R1 and R2 denote first (72 to 99 HPI) and second (99 to 126 HPI) measured interval, respectively.

B were fused using the FA_F and FB_R primer pair, and the fusion product (F_A-B) was excised from the agarose gel and cleaned, using the GeneJET (Thermo Scientific) gel extraction kits. Second, PCR fragment C was fused with F_A-B using the FA_Fw and FC_Rev primer pair. The PCR conditions for the first fusion step were as described above, with the annealing and elongation times prolonged to 2 min. The second step of fusion PCR was carried out with a single annealing at 52°C for 2 min, with 15 cycles only, and the primers were added to the reaction after cycle 5. The full fusion PCR product, 2,236 bp, was sequentially restricted by the restriction endonucleases PflFI (NEB) and BglII (Invitrogen), in 50-µl reactions, according to the manufacturer's protocol. After each restriction step, the restriction reaction was cleaned using MinElute gel extraction kits (Qiagen), 150 µl of QG buffer and 50 µl of isopropanol were added to the 50-µl reactions, and the mixtures were loaded into MinElute columns. The cleaning was finalized following the manufacturer's instructions. The 3' half PVY N605 subclone was restricted with PflFI (NEB) and BglII (Invitrogen), with an additional alkaline phosphatase treatment step. One microliter of calf intestinal phosphatase (NEB) was added to the BglII and PflFI reaction mixtures at the beginning and after 30 min of incubation. After the restriction, the appropriate fragment was excised from the agarose gel and was cleaned using GeneJET gel isolation kits. The fusion PCR product was ligated into the opened, phosphatase treated 3' half PVY N605 subclone, with T4-DNA ligase (NEB), at an insert plasmid molecular ratio of 3:1. The ligation mixture was according to manufacturer's instructions, with overnight incubation at 16°C. Afterward, the ligase was inactivated by 10 min of incubation at 65°C, and the ligation mixture was dialyzed for 60 min on MF-Millipore discs of 0.22 µm pore size comprised of mixed cellulose esters against double-distilled H₂O, before electroporation to electro competent *E. coli* DH5α. The transformed bacteria were plated on Luria Bertani (LB) media supplemented with 200 µg of ampicillin per milliliter. Colonies were screened using colony PCR and the FA_Fw/FC_Rev primer pair flanking the fusion PCR insert. The colonies containing the 3' half PVY N605 subclone plasmid with

the expected insert size, approximately 2.2 kb, were transferred to 5 ml of liquid LB with 200 µg of ampicillin per milliliter and were incubated overnight. The next day, the plasmids were isolated with the NucleoSpin Plasmid kit (Macherey-Nagel), following the instruction manual, and were sequenced with all three primer pairs (FA, FB, FC), to determine whether PCR induced errors into the sequence. Only the error-free 3' half PVY N605 subclone with the inserted *gfp* was used to construct the full-length infectious PVY N605-GFP clone. The ligation of the bipartite system, biolistic inoculation of *N. clelandii*, and enzyme-linked immunosorbent assay testing of the inoculated plants were carried out according to Tribodet et al. (2005).

Plant material and inoculation.

Nicotiana tabacum cv. Xanthi, *N. clelandii* and *N. benthamiana* were grown from seed and were inoculated at the three- to four-leaf stage. All *Solanum* species and cultivars (Table 1) were grown in stem node tissue culture. Two weeks after node segmentation, they were transferred to soil in growth chambers and were grown as previously described by Baebler et al. (2009). Briefly, plants were grown at 24°C with a long day (16-h) photoperiod of light (light intensity 4,000 lm/m²) and 60 to 70% relative humidity. After four weeks in soil, the plants were inoculated at the four- to six-leaf stage.

Four weeks after biolistic bombardment of the PVY N605 GFP infective clone, 1 g of systemically inoculated leaves of *N. clelandii* was ground with 120 mg of Carburundum (0.65 µm) (Sigma) and was used directly to inoculate the first passage of *N. tabacum* cv. Xanthi. Subsequent washing of the inoculum from inoculated leaves was not performed. The second and third *N. tabacum* cv. Xanthi passage, along with *S. tuberosum* cultivars and wild-potato relatives were inoculated with systemically infected leaves of *N. tabacum* cv. Xanthi from the first or second passage collected at 12 to 16 DPI. GFP presence on the leaves was confirmed by microscopy. Leaves were ground with a 20 mM phosphate buffer supplemented with 2% (wt/wt) polyvinylpyrrolidone, of average molecular mass 10,000 (Sigma), in a 1:5 plant material to buffer

Table 1. Detection of green fluorescent protein (GFP) fluorescence upon *Potato virus Y* (PVY) N605-GFP inoculation of different *Nicotiana* and *Solanum* species and cultivars

Species	SolRgene accession no.	GFP signal detected ^a	
		Inoculated leaves	Systemic leaves
<i>Nicotiana tabacum</i> cv. Xanthi	...	+	+
<i>Nicotiana benthamiana</i>	...	+	+
<i>Solanum tuberosum</i> cv. Désirée ^b	...	+	+
<i>Solanum tuberosum</i> cv. NahG-Désirée ^b	...	+	+
<i>Solanum tuberosum</i> cv. Bea ^b	...	n.t.	+
<i>Solanum tuberosum</i> cv. Bintje ^b	...	+	+
<i>Solanum bulbocastanum</i>	331-2	+	–
<i>Solanum hjertingii</i>	349-3	+	–
<i>Solanum jamesii</i>	355-1	+	–
<i>Solanum lesteri</i>	358-4	+	–
<i>Solanum mochiense</i>	186-1	+	+
<i>Solanum okadae</i>	970-3	+	–
<i>Solanum polytrichon</i>	378-2	+	–
<i>Solanum papita</i>	767-8	+	+
<i>Solanum species</i>	278-2	+	–
<i>Solanum venturii</i>	283-1	+	+
<i>Solanum venturii</i>	366-2	+	+
<i>Solanum venturii</i>	365-1	+	+
<i>Solanum venturii</i>	741-1	+	–
<i>Solanum venturii</i>	250-2	+	–
<i>Solanum venturii</i>	896-4	+	–

^a Results from microscopy detection of GFP fluorescence; +, fluorescence observed; –, fluorescence not observed; n.t., not tested. Detection of GFP in *Nicotiana* species and *S. tuberosum* was done at 11 and 18 days postinoculation (DPI) on inoculated and systemically infected leaves, respectively. Other *Solanum* species were examined at 14 DPI.

^b Inoculated with nondiluted sap from infected *N. tabacum* cv. Xanthi.

ratio. Classical mechanical inoculation with Carburundum was carried out with 200 μ l of inoculum per leaf and was washed with tap water after 10 min of incubation. *S. tuberosum* cultivars Désirée, NahG-Désirée, Bintje, and Bea were inoculated in the same manner as the first *N. tabacum* cv. Xanthi passage but without the use of buffer. The source of the inoculum, however, were systemically infected leaves of *N. tabacum* cv. Xanthi from passage one and two. Three lower leaves, L1 to L3, were inoculated on all of the tested species and cultivars, except for *N. tabacum* cv. Xanthi, on which only leaf L1 was inoculated.

RNA extraction.

A piece of each sampled leaf, 0.01–0.1 g of fresh weight, was cut using a fresh scalpel, transferred to chilled 2 ml microcentrifuge tubes (Eppendorf, Germany) supplemented with 1 $\times$ 5 mm stainless steel bead, and stored at -20°C . The samples were then homogenized in the storage tubes using a Tissue Lyser (Qiagen, Germany) (3 min, max speed) in 90 μ l Lysis Binding Solution concentrate and 10 μ l Plant RNA Isolation Aid RNA (MagMAXTM-96 Total RNA Isolation kits; Life Technologies, USA). After homogenization, the samples were centrifuged at 14,000 $\times$ *g* for 5 min. The RNA was extracted from 50 μ l of clear homogenate using the MagMAXTM-96 Total RNA Isolation kit and the MagMAXTM Express Magnetic Particle Processor (Life Technologies, USA), according to manufacturer instructions for plant samples. A DNase step was included in the isolation procedure, according to manufacturer instructions (MagMAXTM-96 Total RNA Isolation kit, Life Technologies, USA).

Classical and quantitative RT-PCR.

To detect potential deletions of the inserted *gfp* gene, a combination of different quantitative RT-PCR (RT-qPCR) assays were used. To quantify the content of the total PVY genomic RNA in host plants, a previously developed universal PVY real-time RT-PCR assay, UNI, was used (Kogovšek et al. 2008). Quantification of *gfp* was carried out with the assay developed by Joshi et al. (2008), here denoted as PVY-*gfp*. The forward primer sequence was modified to hybridize perfectly on the *gfp* sequence used in this study. To detect PVY genomes with complete deletion of *gfp*, we developed an assay that specifically detects only PVY N605, denoted as PVY-wt. The assay was developed according to MIQE standards (Bustin et al. 2009) with the forward primer located in *Nib*, the reverse primer in *CP*, and the TaqMan MGB probe located partly on the N1a protease recognition site and partly on the CP coding region, with amplicon length of 70 bp. The probe was designed manually, while the primers were designed using the Primer Express 3 software (Applied Biosystems). The assay specificity was tested in silico, using the BLAST algorithm, and in vitro on healthy plant RNA extracts and PVY N605-GFP-infected plant RNA extracts. The range of quantification and amplification efficiency of the PVY-wt assay were evaluated according to Kogovšek et al. (2008). An assay specific for a stably expressed plant cytochrome oxidase gene, *COX* (Weller et al. 2000), was used for normalization of the different total RNA input in the RT-qPCR reactions (Baebler et al. 2009).

All of the samples were analyzed using all four assays: UNI, PVY-*gfp*, PVY-wt, and *COX*. Theoretical copy number of the targets was determined using a dilution-series approach (Kogovšek et al. 2008), assuming that the quantification cycle value (C_q) values at the end of the linear range correspond to 10 copies in the RT-qPCR reaction. To normalize for different total RNA input in the RT-qPCR reactions, all of the determined copy numbers of UNI, PVY-*gfp*, and PVY-wt were expressed as copies per 10⁶ copies of *COX* in the same sample. Normalization was performed using 10⁶ copies of

COX due to its concentration being six orders of magnitude higher than the concentration of other targets in most samples. To assure the quality of the analysis, the appropriate controls were used, i.e., nontemplate control, negative control for isolation, positive control for amplification.

RT-qPCR analysis was performed in duplicates on 2 μ l of isolated RNA, in a total volume of 10 μ l, using the AgPath-IDTM one-step RT-PCR kits (Applied Biosystems) on an ABI 7900 HT Fast instrument, with AgPath-ID one-step RT-PCR reagents (Applied Biosystems). The cycling conditions were as follows: reverse transcription 48 $^{\circ}\text{C}$, 10 min; denaturation 95 $^{\circ}\text{C}$, 10 min; 45 cycles: 95 $^{\circ}\text{C}$, 15 s; 60 $^{\circ}\text{C}$ 1 min. The amplification curves for every sample were checked manually using SDS 2.3 software (ABI), with manual threshold of 0.1 for PVY-*gfp* and PVY-wt, 0.25 for *COX*, and 0.15 for UNI. Automatic baseline was used to determine the C_q values.

Two-step classical RT-PCR was done with the F1_F and F3_R primer pair to amplify the approximately 2.2-kb-long fragment, corresponding to partial N1b, complete GFP, and complete CP coding regions. The SuperScript III first-strand synthesis system (Invitrogen) was used for the first strand cDNA synthesis according to manufacturer's protocol. cDNA synthesis reactions were stored at -20°C or were used for PCR immediately. PCR was performed with Platinum Taq DNA polymerase high fidelity (Invitrogen) in 50- μ l reactions with 5 μ l of cDNA and a final primer concentration of 200 nM. The products were visualized on 1% agarose gel.

Fluorescence microscopy and image analysis.

The samples of *N. tabacum* cv. Xanthi and *S. tuberosum* were examined with a fully motorized Nikon Eclipse Ti inverted microscope, equipped with an ANDOR Clara E high sensitivity black and white camera (Andor Technologies). Two fluorescent channels were used for image acquisitions: green with excitation at 465 to 495 nm and emission at 515 to 550 nm and red with excitation at 528 to 553 nm and emission at 590 to 650 nm; exposure time of 700 ms was used for both. Imaging was carried out at 40 $\times$ magnification. Large leaf samples were imaged by taking individual images sequentially with 5% overlap between images, in both fluorescent channels, across the whole sample area. The objective was focused on one focal plane, which was defined manually for each sample. Up to 600 high-resolution images were acquired per sample and were combined together, using the NIS Elements software (Nikon) to form a detailed image of the entire sample.

For visualization purposes, the "Look Up Table" was adjusted in the NIS elements software for each channel separately; however, the same adjustment was used for all of the images (Supplementary Fig. S4).

In the wild *Solanum* relatives, the GFP was visualized using a Leica TCS SP5 laser-scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems), with a HC PL FLUOTAR 10.0 $\times$ 0.30 DRY objective. For excitation, the 488-nm line of an Argon laser was used. Fluorescence emissions with wavelengths of 505 to 520 nm and 590 to 680 nm were collected simultaneously through two channels. The images were processed and assembled using the Leica LAS AF Lite software (Leica Microsystems).

To distinguish GFP signals from autofluorescent artifacts, only the signals specific to the green channel were considered as genuine GFP signals. To exclude artifacts from the images taken on the PVY N605-GFP infected leaves, a range of control samples were examined and imaged. This included healthy plants, mock-inoculated plants (healthy *N. tabacum* cv. Xanthi leaves as a source of inoculum), and PVY N605-inoculated plants.

To correlate the RNA concentrations with fluorescence, the average intensity was measured in separate sections of the imaged

leaf. Measurements of the raw integrated density of GFP fluorescence in the selected area were carried out using FIJI software (Schindelin et al. 2012). The background fluorescence pixel intensity of the healthy leaf (800) was subtracted from the entire image. Then the integrated density of the signal (area [pix] × mean intensity of the pix) was then measured in the sections.

In vivo tracking of PVY N605-GFP.

A modified plant-growing technique enabled localization of PVY N605-GFP in the same plant at the different times post-inoculation. *N. tabacum* cv. Xanthi plants were grown in plastic 50-ml tubes (Supplementary Fig. S5). Each day, half of the inoculated L1 and systemically infected leaves L2, L3, L4 were carefully placed between two objective glasses and the whole bottom side of the leaves were scanned to produce a high-resolution composite image, as described above.

The images from in vivo tracking were used to estimate the rate of movement of PVY N605-GFP for the inoculated leaf. All of the measurements were carried out using the NIS Element software (Nikon). Fifteen fluorescent foci were chosen on two independent plants. Their diameters were measured with the circular measurement tool at 72, 99, and 126 HPI, and their areas were calculated. The area of the average epidermal cell was measured separately each day to account for the growth rate of the cells. At each time point, three groups of 10 cells were chosen on three different locations on the leaf near the fluorescent foci. Their area was measured using a freehand area measurement tool. The total area was divided by the number of cells measured to calculate the area of an average cell. To estimate the number of cells in the fluorescent foci at each time point, the area of the foci was divided by the calculated area of an average cell at that particular time point. The estimated number of infected cells in each fluorescent foci at a particular time point was used to calculate the rate of movement of PVY, expressed as the number of newly infected cells per hour for each foci separately.

ACKNOWLEDGMENTS

We thank R. G. F. Visser (Wageningen University) for providing the collection of wild-potato relatives and L. Maticic for propagating and maintaining plant tissue cultures. We would like to acknowledge A. Millar, G. Scott, and C. P. Berrie for grammatical and stylistic revision of the manuscript. The study was supported by the Slovenian Research Agency through a PhD research grant to M. Rupar (Contract number 1000-10-310196) through a research programme P165 and SLO-FR bilateral project PROTEUS (BI-FR/10-11-Proteus).

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