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DEVELOPMENT OF A VIRUS INDUCED GENE SILENCING VECTOR FROM A LEGUMES INFECTING TOBAMOVIRUS

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Medicago truncatula, the model plant of legumes, is well characterized, but there is only a little knowledge about it as a viral host. Viral vectors can be used for expressing foreign genes or for virus-induced gene silencing (VIGS), what is a fast and powerful tool to determine gene functions in plants. Viral vectors effective on *Nicotiana benthamiana* have been constructed from a number of viruses, however, only few of them were effective in other plants. A Tobamovirus, *Sunnhemp mosaic virus* (SHMV) systemically infects *Medicago truncatula* without causing severe symptoms. To set up a viral vector for *Medicago truncatula,* we prepared an infectious cDNA clone of SHMV. We constructed two VIGS vectors differing in the promoter element to drive foreign gene expression. The vectors were effective both in the expression and in the silencing of a transgene Green Fluorescent Protein (GFP) and in silencing of an endogenous gene Phytoene desaturase (PDS) on *N. benthamiana.* Still only one of the vectors was able to successfully silence the endogenous Chlorata 42 gene in *M. truncatula.*

Keywords: VIGS – SHMV – *Medicago truncatula* – gene silencing – functional genomics

INTRODUCTION

Medicago truncatula is a model plant of legume research [4] because it has a small, diploid genome, autogamous genetics and a fast life cycle. Its genome has been sequenced; *M. truncatula* genome 3.0 assembly release is available at www.medicago.org. Although it is widely used in genome research there is no detailed description about it as a viral host.

RNA interference (RNAi) is an ultimate tool to determine the function of genes. RNAi is triggered by double-stranded RNA molecules that either enters or is generated in the cell and eventually triggers the cleavage of cognate mRNAs [1]. Virus infection in plants also generates 21–25 nt long small interfering (si) RNAs [11]. These siRNAs are recruited by the RISC complex which guides the sequence specific breakdown of any RNA species showing sequence similarity to the inducing

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virus. In virus induced gene silencing (VIGS) a virus vector engineered to contain a piece of a trans- or endogenous gene is used to infect a host plant, and the artificially introduced sequence element will induce the degradation of target mRNAs resulting in a complete or partial loss of function phenotype [2].

VIGS has a number of advantages over other gene function analysis tools. It is rapid, because it does not need stable transformation of the plant. Genes causing a lethal phenotype can also be tested because already developed plants are infected. The main challenge in setting up a VIGS system is usually to find the appropriate virus which infects the targeted host plant efficiently but does not induce severe disease symptoms which could interfere with the detection of the silenced phenotype. VIGS has been developed for many plants, including *Pea early brown virus* (PEBV) for *Pisum sativum* [3]; and more recently *Bean pod mottle virus* (BPMV) for *Glycine maximus* [18] for legumes. Although a functional VIGS vector could be highly beneficial to study gene functions in the model legume *M. truncatula,* no such system has been reported so far for this plant. Gronlund and co-workers [8] tried to use PEBV vector for *M. truncatula*. They have analysed 21 ecotypes for infectivity and for RNAi, but they have found only 4 to be a systemic host of the virus. Jemalong A17, the one with the sequenced genome, was not included. The aim of the present work was to find a virus which systemically infects *M. truncatula* and is a good candidate for developing a VIGS vector.

Sunnhemp mosaic virus (SHMV) is a positive strand tobamovirus. Its genome is 6483 nts long and contains four open reading frames (Fig. 1a). The replicase (from nt 75 to 3465 and from 75 to 4964) is translated from the genomic RNA, whereas the movement protein (MP – from nt 4957 to 5808) and the coat protein (CP – from nt 5780 to 6274) are translated from the co-terminal 3′ subgenomic RNAs (Fig. 1a). Although it was known that SHMV is a tobamovirus what infects legumes, detailed study about its host range and symptoms is still missing.

In this work we show that SHMV systemically infect *M. truncatula* (Jemalong A17) and describe the cloning of the virus and its utilization as a VIGS vector.

MATERIALS AND METHODS

Cloning of SHMV, in vitro *transcription and plant inoculation*

Based on the available sequence data [13] we cloned the virus into pUC18 adding a T7 RNA polymerase promoter sequence to the 5′ end and an additional *Pml*I site to the 3′ end to facilitate the linearization of the plasmid for *in vitro* transcription. After reverse transcription (Revert aid First strand cDNA synthesis Kit Fermentas) using 1 μg total RNA extracted from virus infected plants, we made three different reverse transcription using SHMV2170AS, SHMV4063AS and SHMV6483ASPmlI oligonucleotides as primers according to manufacture's recommendation. From the first strand we amplified the virus genome in three overlapping pieces in PCR reactions

Table 1
Sequences of the used oligonucleotides

T7SHMV1S	taatacgactcactataggtatgattaacttcacaaca
SHMV2170AS	gcgtgtaattcagataactc
SHMV2006S	cgatttggtttgtctgtctg
SHMV4063AS	aaatctaatttggccttagg
SHMV3924S	gtgcaaaactttgatgactgg
SHMV6483ASPmlI	cacgtgggccctgtatccccaggg
SHMV5826SmaNheI	gctagcccgggtaaaatacacaagttgactaggag
ToMV5540S	gctagccaaatcctcaaaaagaggtccg
ToMV5738AS	atttaaatacacaaattgcgatggagaagtg
SHMV5824S	gtatactgaaaattacgctgattac
SHMV5395S	tgatcgacctgccggtcaaag
PDS80S	gggaaggtgattgaaggggatg
PDSAS	cccctttccagttctcagg
MtCH42S	acagaagatagggtgtgtggaac
MtCH42200S	gttgacgaagttaatcttttgg
MtCH42100S	ctgctcggtttatcttaattgg
MtCH4240S	aggttggaacacagtggag
MtCH4240AS	gcgagatagaaataccttctc

using Pfu turbo (Stratagene): with primer pairs T7SHMV1S and SHMV2170AS (1–2170 nt), with SHMV2006S and SHMV4063AS (2006–4063 nt) and SHMV3924S and SHMV6483ASPmlI (3924–6483 nt) (Table 1) (PCR conditions was denaturation at 94 °C 3 min, amplification in 40 cycle having denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 68 °C for 4 min.) Using restriction endonuclease sites *Bst*BI (cleaves at 2086 nt) and *Sac*II (cleaves at 4006 nt) the entire virus genome was cloned into pUC18 generating pSHMV1-6483. The recombinant plasmid was *Pml*I linearized and subsequently used for producing capped *in vitro* RNA transcripts using T7 RNA polymerase (Fermentas) and a CAP analogue (New England Biolabs). For *in vitro* transcription we used 0.5 µg linearized plasmid in 25 µl reaction (containing the reaction buffer of T7 polymerase, 1.25 µl 100 mM DDT, 0.25 µl 40 U/µl Ribolock (Fermentas), 2.5 µl 20 mM rATP, rCTP, rUTP, 2.5 µl 2 mM rGTP, 5 µl 5 mM CAP analogue (New England Biolabs) and 2.5 µl 20 U/µl T7 polymerase (Fermentas). After incubation of the transcription mixture at 25 min at 37 °C we added 2.5 µl 20 mM GTP and the reaction was further incubated at 37 °C for 1 hour. Transcripts were used to infect plants with sap inoculation method. We added 25 µl inoculation buffer to the transcription and inoculated two leaves of the plants with 12.5 µl materials each.

SHMV vector building

We have introduced a new *Sma*I site into the wild type virus at 5826 nt, just after the CP promoter. SHMVPro^{SHMV} was made by using *Sal*I natural cleavage site at 5537 nt position, upstream of the start of the CP promoter, we cloned SHMV fragment (5537–6483) after the *Sma*I site, generating a duplicated promoter. Based on similarities, ToMV CP subgenomic RNA promoter was amplified from a ToMV infected tobacco plant total RNA extract in an RT-PCR reaction using primers ToMV5540S and ToMV5738AS (Table 1). This fragment was cloned between the *Sma*I cloning site and the CP coding region to replace the original SHMV CP promoter.

Cloning endogenous genes

PDS (Phytoene desaturase) from *N. benthamiana* was cloned from total RNA from the leaves after reverse transcription with oligodT primer in a PCR reaction using PDS80S and PDSAS (Table 1). (PCR conditions was denaturation at 94 °C 3 min, amplification in 40 cycle having denaturation at 94 °C for 30 sec, annealing at 48 °C for 30 sec and extension at 72 °C for 30 sec.)

CH42 (Chlorata 42) from *M. truncatula* was amplified with Mtch42S, MtCH42200, MtCh42100 and MtCH4240S paired with MtCH42AS (Table 1). (PCR conditions was denaturation at 94 °C 3 min, amplification in 40 cycle having denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 30 sec.) from first strand what was made in reverse transcription from total RNA of the *M. truncatula* leaves with oligodT.

RNA analysis

Total RNA was extracted from virus infected plants [14]. RNA was resolved on TBE agarose gels (for virus RNA analysis) [14] and on polyacrylamid gels (for small RNA analysis) [16]. We have amplified the 3´ 1000 bp fragment of the virus (with the help of SHMV5395S and SHMV6483ASPmlI, see Table 1. PCR conditions was denaturation at 94 °C 3 min, amplification in 40 cycle having denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 60 sec.) Radiolabelled random DNA probe from this PCR fragment was made with Hexalabel kit (Fermentas) according to manufacturer's recommendation using radioactive dCTP. For small RNA analysis of CH42 (negative strand probe) was made from the cloned CH42 with the aid of T7 RNA polymerase in the presence of radiolabelled rUTP. (In 10 µl reaction we used 0.2 µg linearized plasmid, T7 RNA polymerase buffer, 1 µl 10 mM rATP, rCTP,rGTP, 2 µl radioactive rUTP, 0.2 µl 40 U/µl Ribolock and 0.3 µl 20 U/µl T7 polymerase.)

Semiquantitative PCR

Semiquantitative PCR was done using MtCH42200 and MtCH42AS for CH42 gene and PDS80 and PDSAS for PDS as an endogen control. The amplification was on reverse transcribed cDNA from virus infected or control plants with both oligopairs in the same tube (PCR conditions was denaturation at 94 °C 3 min, amplification in 40 cycle having denaturation at 94 °C for 30 sec, annealing at 48 °C for 30 sec and extension at 72 °C for 30 sec.). The resulting product was resolved on agarose gel and quantified using IMAGE Quant software.

In situ *analysis*

In situ hybridization of 10 μm paraffin embedded root and nodule of *M. truncatula* was carried out using digoxigenin-11-UTP-labelled virus-specific RNA probe as described [9].

RESULTS

SHMV infects M. truncatula *systemically without symptoms*

M. truncatula (Jemalong A17) was infected with an isolate of SHMV. Six days after inoculation (6 dpi) we could detect the virus in the systemically infected leaves. In spite of high level of virus accumulation in leaf, root and stem, no visible symptoms were observed on *M. truncatula* up to 40 days (Fig. 1b, c). We used mock inoculated plant (inoculated only with water/inoculation buffer mixture) for comparison. The infected plants grew further, flowered and formed nodules in the presence of the bacteria (*Shinorhizobium meliloti*). Moreover, the presence of the virus was detected even in the symbiotic nodules by *in situ* hybridization (Fig. 1d).

Preparation of infectious SHMV cDNA clone

Based on the available sequence data [13] we cloned the virus into pUC18 adding a T7 RNA polymerase promoter sequence to the 5′ end and an additional *Pml*I site to the 3′ end to facilitate the linearization of the plasmid for *in vitro* transcription. The recombinant plasmid was *Pml*I linearized and subsequently used for producing capped *in vitro* RNA transcripts. Upon infection with *in vitro* RNA transcripts viral replication could be detected in systemically infected leaves of *N. benthamiana* and *M. truncatula* at seven days post inoculation (data not shown).

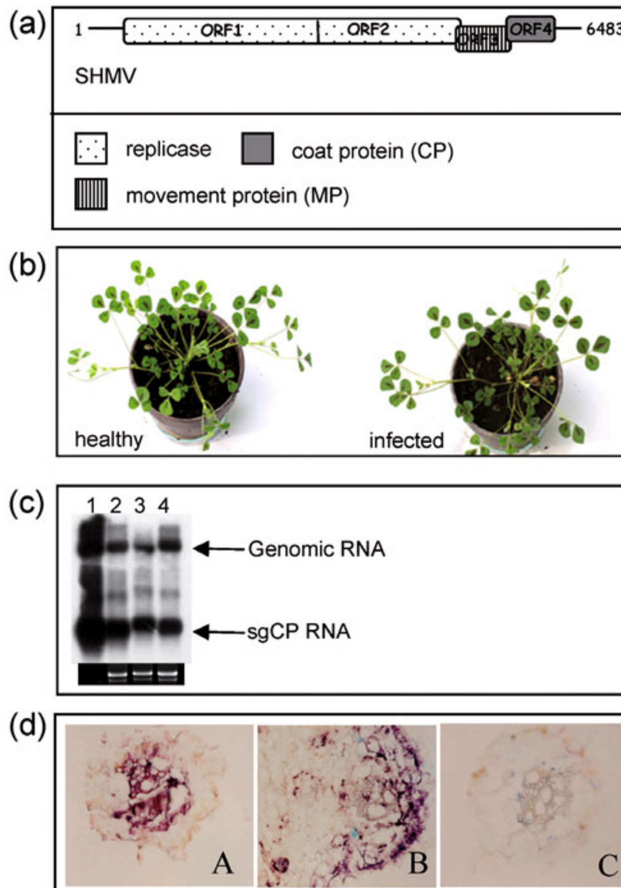

Fig 1. M. truncatula as a host of SHMV. (a) Schematic representation of the genomic organization of SHMV. (b) Picture of the healthy and SHMV infected *M. truncatula* (c) Northern blot hybridization of total RNA extracted from different organs (2-leaf, 3-stem, 4-root, 1 purified virion) of the SHMV infected *M. truncatula* and probed with random DNA probe referring to the 3´ end of the virus with ethidium bromide stained 5S rRNA as loading control. (d) Cross-sections of SHMV infected root (A, C) and nodule (B) after *in situ* hybridization with minus strand RNA probe referring to the 3´ end of SHMV (A, B) or with the positive strand RNA probe of the same SHMV segment (C) as a negative control

Building of VIGS vectors from SHMV

Considering the similarities between SHMV and TMV we constructed two vectors. Vector SHMVPro^{SHMV} contains a duplicated CP subgenomic RNA promoter with a *Sma*I cloning site (at 5826 nt) immediately downstream of the first promoter element (Fig. 2a). In the SHMVPro^{TOMV} vector the second SHMV CP subgenomic promoter was replaced with a heterologous ToMV CP subgenomic promoter (Fig. 2a). This

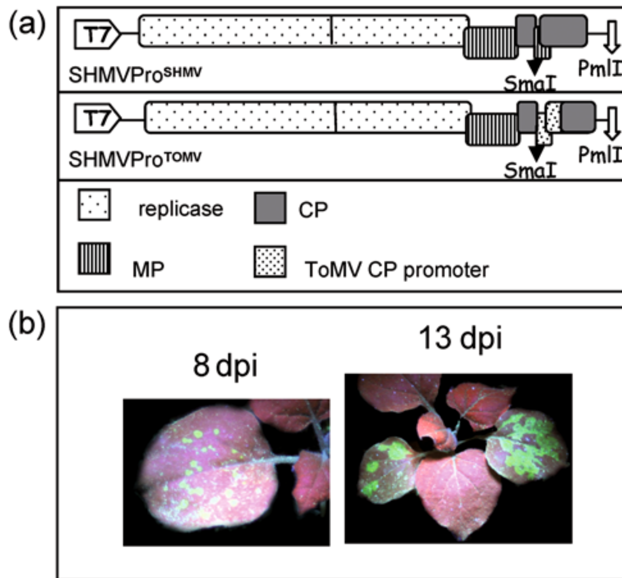

Fig. 2. Vectors from SHMV. (a) Schematic representation of the VIGS vectors. (b) Expression of GFP with SHMVPro^{TOMV}GFPs vector. Green leaves of *N. benthamiana* are red under UV light, the expression of GFP can be seen as green spots on these red leaves

modification is thought to reduce the frequency of recombination events resulting in the loss of the introduced fragment [12]. *In vitro* RNA transcripts of the constructed empty vectors were used for infection of *N. benthamiana* and *M. truncatula* to test their biological activity. SHMVPro^{SHMV} behaved similarly as the wild type virus while viral RNA transcribed from SHMVPro^{TOMV} showed a delayed infection on test plants (results not shown).

SHMV VIGS vectors are able to express and silence foreign gene in N. benthamiana

We have cloned the GFP gene into the SmaI cloning site of the vectors. Vectors SHMVPro^{SHMV}GFPs, SHMVPro^{TOMV}GFPs containing the GFP gene in sense orientations were tested on wild type *N. benthamiana.* In the case of SHMVPro^{TOMV}GFPs we could detect GFP expression (Fig. 2b) while no GFP expression was observed with SHMVPro^{SHMV}GFPs (data not shown). Inoculation with both vectors resulted in efficient silencing on GFP transgenic *N. benthamiana* 14 days after infection (Fig. 3a). In these cases the silencing effect was observed in the systemically infected leaves of the plant and was maintained for up to one month. The developing new leaves always showed this silencing phenotype.

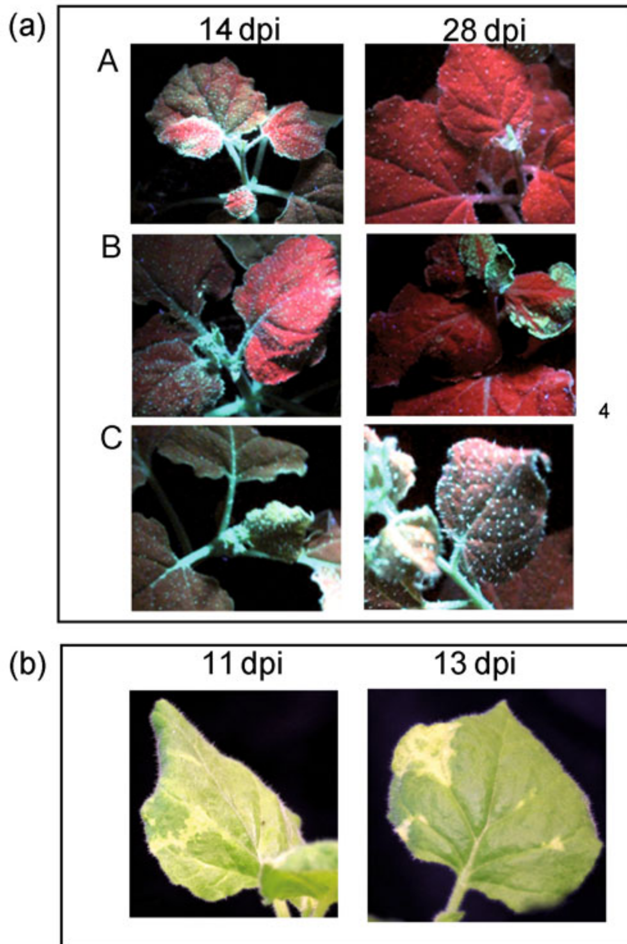

Fig. 3. VIGS studies on *N. benthamiana.* (a) Silencing of GFP using SHMV VIGS vectors on GFP transgenic *N. benthamiana.* (A) SHMVPro^{SHMV}GFPs, (B) SHMVPro^{TOMV}GFPs, (C) mock (only water inoculated). GFP transgenic plants under UV light are faint green as mock plants. Silencing of GFP emerges as red colour first in the veins, but later spreads in the whole leaf. (b) Silencing of PDS using SHMVPro^{SHMV}PDSas on *N. benthamiana.* Silencing of PDS manifests in white colour on the leaf

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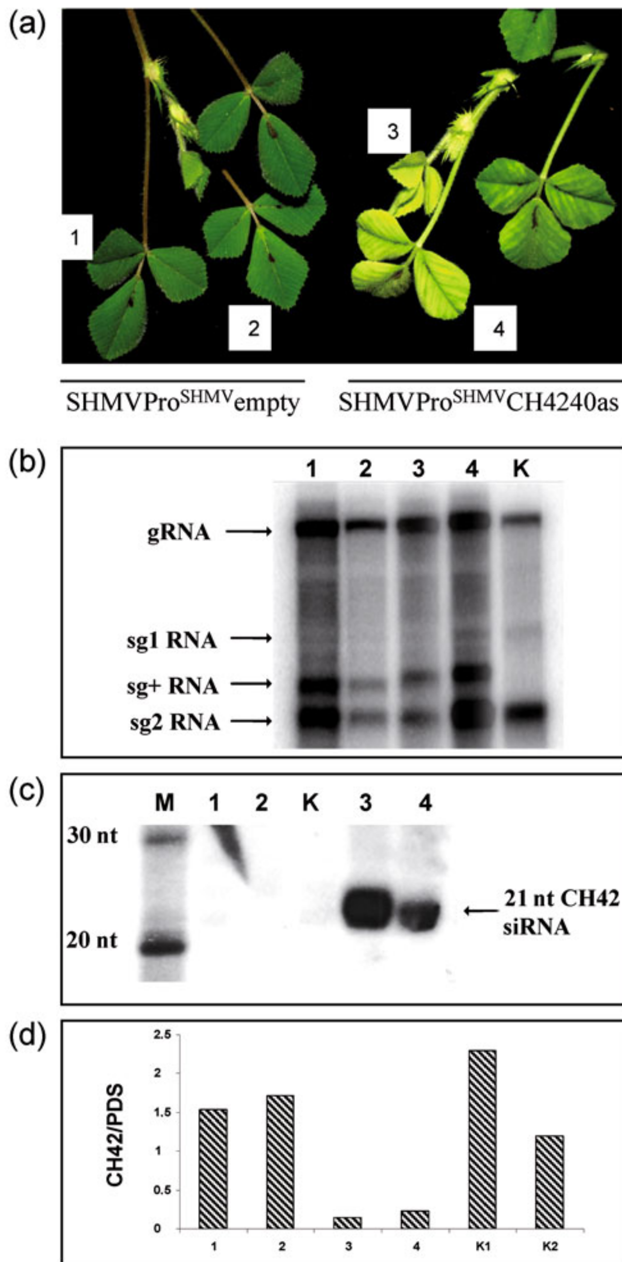
Fig. 4. VIGS study on *M. truncatula.* Silencing of CH42 in *M. truncatula.* (a) *M. truncatula* leaves from plants infected with empty SHMVPro^{SHMV} (left side) and SHMVPro^{SHMV}CH4240as (right side) at 30 dpi. (b) Northern blot of total RNA samples from the numbered leaves and wild type SHMV infected plant (K) probed with SHMV 3′ random DNA probe. (c) Small RNA blot using extracted total RNA samples from the numbered leaves and wild type SHMV infected plant (K) probed with CH42 antisense RNA probe, (M) molecular weight marker. (d) Schematic representation of CH42/PDS ratio in total RNA samples from the numbered leaves and wild type SHMV infected plant (K) using semiquantitative RT-PCR


(a)
1
2
3
4
SHMVPro^{SHMV}empty
SHMVPro^{SHMV}CH4240as
(b)
1 2 3 4 K
gRNA
sg1 RNA
sg+ RNA
sg2 RNA
(c)
M 1 2 K 3 4
30 nt
20 nt
21 nt CH42 siRNA
(d)
CH42/PDS
2.5
2
1.5
1
0.5
0
1 2 3 4 K1 K2

SHMV VIGS vectors are able to silence endogenous genes both in N. benthamiana *and in* M. truncatula

We have cloned an 80 bp fragment of *N. benthamiana* endogenous PDS gene and created $\mathrm{SHMVPro}^{\mathrm{SHMV}}$PDSas, $\mathrm{SHMVPro}^{\mathrm{TOMV}}$PDSas vectors. Bleaching phenotype what is characteristic for PDS silencing was produced on newly emerging leave after 11–13 dpi with both vectors, although the white zones were confined to segments of the leaves and we never got fully silenced white leaves (phenotype with $\mathrm{SHMVPro}^{\mathrm{SHMV}}$PDSas vector is shown in Fig. 3b, phenotype with $\mathrm{SHMVPro}^{\mathrm{TOMV}}$PDSas is not shown).

For VIGS studies in *M. truncatula* we have amplified 200 bp and 100 bp fragments of CH42 from the plant and cloned them into the *Sma*I site of $\mathrm{SHMVPro}^{\mathrm{SHMV}}$, $\mathrm{SHMVPro}^{\mathrm{TOMV}}$ [15]. The viability of the viruses transcribed from vectors carrying the target sequences were tested first on *N. benthamiana* by Northern blotting using an SHMV specific probe (data not shown). Both vectors $\mathrm{SHMVPro}^{\mathrm{SHMV}}$ and $\mathrm{SHMVPro}^{\mathrm{TOMV}}$ replicated well in plants irrespective of the size of the introduced target sequences, although the $\mathrm{SHMVPro}^{\mathrm{TOMV}}$ derivatives showed a significantly delayed infection (detection of virus vector in systemic leaves showed 10 days delay in the case of $\mathrm{SHMVPro}^{\mathrm{TOMV}}$, data not shown). These results demonstrated that all of the constructed recombinant vectors are biologically active.

Subsequently, the efficiency of these vectors was tested on *M. truncatula*. Unfortunately none of the investigated recombinant vectors was able to induce an effective generalized infection on *M. truncatula* although they infected *N. benthamiana* systemically (data not shown).

Since the empty vectors replicated well in *M. truncatula* systemically, we hypothesized that the length of the introduced target sequence can play a pivotal role in the determination of the biological activity of the recombinant vector. Therefore using $\mathrm{SHMVPro}^{\mathrm{SHMV}}$, the most effective vector, we constructed a vector $\mathrm{SHMVPro}^{\mathrm{SHMV}}$CH4240as carrying an antisense 40 nts fragment from CH42. RNA samples derived from systemic leaves both from the control $\mathrm{SHMVPro}^{\mathrm{SHMV}}$ and from $\mathrm{SHMVPro}^{\mathrm{SHMV}}$CH4240as infected plants were analysed for the presence of the virus on Northern blot, hybridized with virus DNA probe (Fig. 4b), and also for the introduced CH42 sequence (data not shown). Both vectors replicated well in systemically infected leaves (Fig. 4b). Ten percent of the *M. truncatula* plants inoculated with $\mathrm{SHMVPro}^{\mathrm{SHMV}}$CH4240as showed yellowing on the emerging new leaves at 30 dpi (Fig. 4a). Moreover from these leaves we could detect 21 nt long siRNAs on a 12% acrylamide gel using CH42 antisense RNA probe demonstrating that RNAi was activated against this sequence (Fig. 4c). To quantify the CH42 mRNA levels in control (mock inoculated, empty vector infected) and silenced plants, we performed a semi-quantitative PCR (Fig. 4d). Our results demonstrated that CH42 mRNA level dramatically decreased in the developing yellow leaves.

DISCUSSION

Medicago truncatula with its sequenced genome is one of the best characterized plants, representing legumes. However, there is no detailed description about it as a viral host. VIGS is a powerful tool to study function of genes, but VIGS vectors cannot be used universally, because every virus–plant interaction is special and differs at the molecular level. To find an appropriate virus candidate is crucial for VIGS vector development. The best candidate infects the plant systemically; replicates at high level and cause no or mild symptoms. SHMV behaves like this on *M. truncatula.* Its infection does not cause severe symptom and it has no effect on the nodule formation as we demonstrated with *in situ* hybridization. There are sequence data about SHMV, but infectious transcript from the virus has not been reported. Using sequence data available in the literature we have cloned the virus and it was infectious on both *N. benthamiana* and *M. truncatula*.

The founder virus of Tobamoviruses, Tobacco mosaic virus (TMV) was among the first viruses which were used for construction of viral vectors [5]. When the promoter of the coat protein encoding subgenomic RNA was duplicated and used to drive the expression of a reporter gene, the virus replicated and the foreign gene was expressed. However, the inserted foreign sequence element was quickly deleted due to a recombination mechanism [5]. To avoid this problem the CP subgenomic RNA promoter was replaced with a heterologous promoter originating from an other tobamovirus [6, 12] and these constructs were used successfully to silence the phytoene desaturase (PDS) gene in *N. benthamiana* [10].

SHMV is a unique member of the Tobamovirus family as its ORFs are overlapping. One part of the coding sequence is a promoter of the next one. This feature made SHMV extremely difficult to handle as a recombinant template for VIGS vector. The TMV 2nd subgenomic RNA promoter expands from nt –157 to +54 [7] containing a region encoding the first 18 amino acids of the virus CP. Insertion of foreign sequence elements into this region therefore requires special consideration. In spite of all these difficulties our vector constructs were infectious although $SHMVPro^{TOMV}$ **s**howed a delayed pattern in the infection. It is possible, that alteration of the beginning of the CP causes a delay in the movement of the virus.

Our vectors as expression vectors for transgene behaved ambiguously, as we could detect GFP expression after infection with $SHMVPro^{TOMV}GFPs$ but not with $SHMVPro^{SHMV}GFPs$. The reason of this can be that the two vectors behaved differently according to how big fragment can be tolerated in their cloning site. $SHMVPro^{SHMV}GFPs$ can be more sensitive in this respect. Both vectors could silence the GFP transgene in GFP transgenic *N. benthamiana.* Previously, CH42 was used for VIGS experiments [15) and silencing of this gene resulted in the appearance of a yellow colour on the plants. We could not detect systemic infection with either of the vectors with 100 or 200 bp CH42 at their cloning site. The movement of the virus seems to be more sensitive for the size of the virus or for the size of the introduced sequence at the cloning site of the recombinant virus. These results indicate that the introduction of foreign sequences into these viral vectors have a more profound

impact on the biological activity on *M. truncatula* than on *N. benthamiana* perhaps because of the important differences in plant RDR6 (RNA dependent RNA polymerase 6) which may be involved in the plant antiviral defence [17].

Taken together we obtained an infectious cDNA clone of the virus SHMV, which replicated well in the model legume *M. truncatula* and infected it systemically. Importantly the virus was also detectable in the root nodules (Fig 1d). We constructed SHMV derivative VIGS vectors that were able to replicate in systemic leaves of *M. truncatula*. The size of the inserted foreign sequence element proved to be a crucial factor in achieving efficient viral replication and a simultaneous knock-down of gene expression. Using a 40 bps fragment of the CH42 gene in vector $SHMVPro^{SHMV}$ we were able to silence an endogenous gene in *M. truncatula* and observed the corresponding phenotype. Although the frequency of efficient endogenous gene silencing was relatively low (about 10%) in our experiments we demonstrated for the first time that a biologically active VIGS vector can be built for *M. truncatula*.

Although we have got positive results, the efficiency of our VIGS vector is below of the desired efficiency required for functional genomic approaches. Adapting the virus to *M. truncatula* with serial passages or careful selection of the size and sequence of the introduced target gene may improve the efficiency of the virus infection. As we have only tested one gene for silencing in *M. truncatula* it is difficult to ascertain that this percentage of silencing is typical for this vector, or the percentage of silencing could be higher depending on sequence of the particular target gene. Further investigation has to be made to answer this question in order to improve our vector for functional genomic studies.

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REFERENCES

1. Baulcombe, D. (2004) RNA silencing in plants. *Nature 431,* 356–363.
2. Baulcombe, D. C. (1999) Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant. Biol. 2,* 109–113.
3. Constantin, G. D., Krath, B. N., MacFarlane, S. A., Nicolaisen, M., Johansen, I. E., Lund, O. S. (2004) Virus-induced gene silencing as a tool for functional genomics in a legume species. *Plant. J. 40,* 622–631.
4. Cook, D. R. (1999) Medicago truncatula – a model in the making! *Curr. Opin. Plant. Biol. 2,* 301–304.
5. Dawson, W. O., Lewandowski, D. J., Hilf, M. E., Bubrick, P., Raffo, A. J., Shaw, J. J., Grantham, G. L., Desjardins, P. R. (1989) A tobacco mosaic virus-hybrid expresses and loses an added gene. *Virology 172,* 285–292.
6. Donson, J., Kearney, C. M., Hilf, M. E., Dawson, W. O. (1991) Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. U.S.A. 88,* 7204–7208.

7. Grdzelishvili, V. Z., Chapman, S. N., Dawson, W. O., Lewandowski, D. J. (2000) Mapping of the Tobacco mosaic virus movement protein and coat protein subgenomic RNA promoters *in vivo. Virology 275,* 177–192.
8. Gronlund, M., Constantin, G., Piednoir, E., Kovacev, J., Johansen, I. E., Lund, O. S. (2008) Virus-induced gene silencing in *Medicago truncatula* and *Lathyrus odorata. Virus Res. 135,* 345–349.
9. Havelda, Z., Hornyik, C., Valoczi, A., Burgyan, J. (2005) Defective interfering RNA hinders the activity of a tombusvirus-encoded posttranscriptional gene silencing suppressor. *J. Virol. 79,* 450–457.
10. Kumagai, M. H., Donson, J., della-Cioppa, G., Harvey, D., Hanley, K., Grill, L. K. (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. U.S.A. 92,* 1679–1683.
11. Molnar, A., Csorba, T., Lakatos, L., Varallyay, E., Lacomme, C., Burgyan, J. (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J. Virol. 79,* 7812–7818.
12. Shivprasad, S., Pogue, G. P., Lewandowski, D. J., Hidalgo, J., Donson, J., Grill, L. K., Dawson, W. O. (1999) Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology 255,* 312–323.
13. Silver, S., Quan, S., Deom, C. M. (1996) Completion of the nucleotide sequence of sunn-hemp mosaic virus: a tobamovirus pathogenic to legumes. *Virus Genes 13,* 83–85.
14. Szittya, G., Silhavy, D., Dalmay, T., Burgyan, J. (2002) Size-dependent cell-to-cell movement of defective interfering RNAs of Cymbidium ringspot virus. *J. Gen. Virol. 83,* 1505–1510.
15. Turnage, M. A., Muangsan, N., Peele, C. G., Robertson, D. (2002) Geminivirus-based vectors for gene silencing in Arabidopsis. *Plant. J. 30,* 107–114.
16. Varallyay, E., Burgyan, J., Havelda, Z. (2007) Detection of microRNAs by Northern blot analyses using LNA probes. *Methods 43,* 140–145.
17. Yang, S.-J., Carter, S. A., Cole, A. B., Cheng, N.-H., Nelson, R. S. (2004) A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana. PNAS 101,* 6297–6302.
18. Zhang, C., Ghabrial, S. A. (2006) Development of Bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. *Virology 344,* 401–411.