Dynamics of the VIGS-Mediated Chimeric Silencing of the *Nicotiana benthamiana* *ChlH* Gene and of the Tobacco Mosaic Virus Vector

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The *ChlH* gene, encoding for the H subunit of the magnesium chelatase enzyme, was silenced in *Nicotiana benthamiana* plants by virus-induced gene silencing (VIGS), using tobacco mosaic virus (TMV) expression vector. Strong silencing of the *ChlH* target gene was initiated only in the apical tissues, in which the endogenous transcription level of the target gene and the level of TMV vector RNA were both very high. The virus vector was also targeted by VIGS, and its suppression was correlated with the silencing of the *ChlH* mRNA. In the apical tissues, the suppression of both the virus vector and the *ChlH* mRNA led to a reduction of the silencing pressure and, consequently, to partial recovery of the new growth from the silencing. As the virus vector and the target mRNA levels increased, silencing was reestablished. The feedback regulation system, caused by the transient increase and reduction in levels of the virus vector and *ChlH* mRNA, led to a fluctuation of the silenced and recovered phenotypes in the plant apex. This TMV-vector mediated silencing system differed from previously analyzed VIGS systems; although the TMV vector was initially targeted by the silencing system, it was not permanently suppressed, indicating that, in this system, TMV was able to effectively escape post-transcriptional gene silencing.

Additional keywords: chlorophyll biosynthesis pathway, co-suppression.

Sequence-specific post-transcriptional gene silencing (PTGS) occurs in many eukaryotic organisms as a defense mechanism against invading foreign genetic elements. Particularly in plants, the PTGS mechanism appears to function as a defense against RNA viruses (Dalmay et al. 2000, 2001; Fagard et al. 2000; Kooter et al. 1999; Ratcliff et al. 1997, 1999). In many organisms, the PTGS mechanism is activated by the formation of dsRNA structures (Chicas and Macino 2001). In plants, it can be activated by expression of dsRNA transcripts from transgenes inserted into the plant genome (Chuang and Meyerowitz 2000; Hamilton et al. 1998; Stam et al. 1997; Waterhouse et al. 1998) or by replicating viral RNAs. PTGS-mediated silencing of endogenes, or co-suppression, can also be induced by homologous transgene sequences. The activation mechanism of the mRNA-induced PTGS is not completely understood, but it probably involves production of dsRNA forms from the mRNA templates by host-encoded RNA-dependent RNA polymerase (Carrington 2000; Dalmay

et al. 2000, 2001; Kooter et al. 1999; Mourrain et al. 2000; Vaucheret et al. 1998; Waterhouse et al. 1999). PTGS is thought to be activated by degradation of dsRNA and by production of small interfering RNA species of both polarities, which are about 21 to 25 nucleotides in length (Chicas and Macino 2001; Hamilton and Baulcombe 1999). These, in turn, lead to the degradation of any single-stranded RNAs that are homologous to the activating dsRNA sequence (Kooter et al. 1999; Zamore et al. 2000).

In infections caused by several different groups of viruses (e.g., nepo-, tobra-, and caulimovirus infections) a PTGS-mediated defense mechanism is able to suppress the invading virus to a large extent and, after initial acute infection, leads to plant recovery (Ratcliff et al. 1999). However, in many virus-host combinations viruses are able to suppress PTGS by specific gene products. The functional mechanisms of these suppressors appear to vary, but in several cases, they are determinants of virus pathogenicity and also function as cell-to-cell or long distance movement proteins or as maintenance factors for viral replication (Brigneti et al. 1998; Kasschau and Carrington 2001; Voinnet et al. 1999, 2000). The balance between the rate of virus replication and the rate of spread in a given host, the host defense reactions, and the ability of the virus to suppress one or more host defenses determines the outcome and severity of the infection in each virus-host combination.

PTGS induced by replicating viral RNAs, or virus-induced gene silencing (VIGS) (Ruiz et al. 1998), can be applied to silence virtually any host gene; the sequence of interest is inserted into a viral vector and the induced PTGS degrades mRNAs that share homology with the insert (Baulcombe 1999). The VIGS system has been developed for several different viral vectors and has been used to silence various plant transgenes and endogenes. For instance, neomycin phosphotransferase, β -glucuronidase (GUS), and green fluorescent protein (GFP) marker genes have been silenced using potato virus X (PVX) or tobacco rattle virus (TRV) vectors, or both (Dalmay et al. 2001; English et al. 1996; Jones et al. 1999; Ratcliff et al. 1999; Ruiz et al. 1998; Voinnet et al. 1999). Polygalacturonase (PG), phytoene desaturase (PDS), the small subunit of rubisco (*rbcS*), and cellulose synthase endogenes have been silenced using either tobacco mosaic virus (TMV)-, PVX-, or TRV-derived viral vectors (Burton et al. 2000; English et al. 1996; Jones et al. 1999; Kumagai et al. 1995; Ratcliff et al. 2001; Ruiz et al. 1998). Furthermore, the luciferase gene, and the Su-gene coding for a subunit of the magnesium chelatase enzyme regulating the chlorophyll biosynthesis pathway have been silenced using a tomato golden mosaic (TGMV) geminivirus vector (Kjemtrup et al. 1998). The outcome of these silencing experiments has

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varied in terms of the persistence and systemic spread of silencing and the targeting of silencing to the replicating viral vector. Usually the new growth of the target plant has eventually reverted back to a nonsilenced phenotype, and supposedly, the inducing viral vector has been eliminated through silencing (Ratcliff et al. 2001).

We have recently reported the silencing of the *ChlH* gene, the H subunit of the magnesium chelatase enzyme, by expression of different lengths of the *ChlH* sequence in a TMV-derived vector (Hiriart et al. 2002). Similar to the earlier reports of the VIGS-mediated silencing of photosynthesis-related endogenes, the target plants showed a chimeric phenotype of chlorophyll deficiency. In this silencing system, the PTGS was also targeted to the replicating viral vector and led to strong reduction of the virus construct in the silenced tissues (Hiriart et al. 2002). Here, we report the strong correlation of the *ChlH* mRNA and virus vector RNA suppression, the dependency of the silencing efficiency on the high initial *ChlH* mRNA and TMV RNA levels, and the feedback dynamics of the VIGS-mediated silencing in this system.

RESULTS

Symptoms.

Previously, the *ChlH* gene of *Nicotiana benthamiana* was silenced by the VIGS system (Hiriart et al. 2002) by expressing different fragments of the gene's coding region in a TMV-derived vector called TMV-30b (Shivprasad et al. 1999). The most efficient gene silencing was induced with the longest (549 nt) fragment (Hiriart et al. 2002), and therefore, the same TMV-*ChlH* inoculum was used here to obtain maximal silencing of the target gene. Two weeks postinoculation (p.i.), the plants inoculated with the TMV-*ChlH* RNA started to show distortion of leaves, mosaic, and chlorosis, i.e., symptoms similar to those caused by TMV-30b. Three weeks p.i., the apical parts of the TMV-*ChlH*-inoculated plant started to show strong discoloration, indicating a lack of chlorophyll, and the apical part of the stem and the small apical leaves turned white (Fig. 1A and B). The new stem and leaf tissues produced in the following week were predominantly white (Fig. 1B and C). However, by week 6, the new

Fig. 1. Systemic symptoms and *ChlH*-silenced phenotypes observed in TMV-*ChlH* inoculated plants. **A**, Systemic symptoms at 2 weeks postinoculation (p.i.), resembling those caused by infection with TMV-30b. **B** and **C**, The newly silenced apex at 3 and 4 weeks p.i., respectively. **D**, Apex at 6 weeks p.i. showing recovery from the earlier silenced phenotype and the stem and leaves below showing the silenced phenotype. **E** through **G**, Apical tissues at 7 weeks or more p.i., showing the fluctuating phenotype in leaves and stem (**E** and **F**) and in stem after removal of the larger leaves (**G**).

growth was less white, composed of white and green mosaic (Fig. 1D) and continued to produce predominantly green or green and white mosaic tissue for about two weeks. At about week 8, the apex started again to show the silenced phenotype. Timing of the tissue phenotype changes varied slightly from plant to plant, but in general, the new apical growths fluctuated between completely white and green or white-green mosaic in about 2-week intervals (Fig. 1E through G). Within a three-month period, the phenotype changed three or four times from white to green or mosaic and back to white. The color changes were seen on the pigmentation of the stem (Fig. 1F) and also by production of very small, fully white leaves alternating with larger green or mosaic leaves (Fig. 1).

Fluctuation of the *ChlH* mRNA and TMV-*ChlH* RNA levels at the shoot apex.

Expression levels of the three separate subunits of the magnesium chelatase enzyme are known to be distinctly higher in young leaves than in mature leaves (Papenbrock et al. 2000). Therefore, to quantify the *ChlH* mRNA silencing levels and to relate them to the endogenous *ChlH* mRNA levels, *ChlH* mRNA accumulation was first determined from different leaves of the mock-inoculated control plants. Levels of *ChlH* mRNA and of TMV RNA were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) (discussed below). In this detection system, RNA levels were quantified by the increase of the fluorescent signal during each amplification cycle and normalized by relating

Fig. 2. Diagram of reverse transcription polymerase chain reaction (RT-PCR)-quantified amounts of *ChlH* mRNA, TMV RNA, or both of the apical and mature leaves of **A**, mock-inoculated plants, **B**, plants inoculated with TMV-30b, and **C**, plants inoculated with TMV-*ChlH*. Both *ChlH* mRNA (**A**) and TMV-30b RNA (**B**) accumulated to very high levels in the youngest leaves but were rapidly reduced in the lower leaves, down to a stable level in the sixth and subsequent leaves (*ChlH* mRNA) or in the fourth and subsequent apical leaves (TMV-30b RNA). This stable level was chosen as the standard 100% level for each type of RNA, respectively. Fluctuation of the *ChlH* mRNA and TMV RNA levels and the alteration of phenotype between nonsilenced, strongly silenced, and weakly silenced or recovered stages in the apical leaves of TMV-*ChlH*-inoculated plants is shown in **C**. Corresponding times after inoculation (p.i.) are indicated. Experiments **A** and **B** have been replicated two times. In each experiment, samples were made by mixing leaves from six individual plants, and each sample was analyzed by RT-PCR at least three times. In experiment **C**, at least four individual plants were analyzed for each percentage value and for standard deviation. For each plant, the quantitative RT-PCR assay was repeated four times to analyze *ChlH* mRNA and three times to analyze TMV-30b RNA.

them to the corresponding actin mRNA levels, amplified from the same total RNA samples and from the same RT reactions in parallel PCR runs. Actin mRNA was chosen as the internal standard for the RNA amplification, because it has been successfully used as a marker of constant transcript level in numerous studies of different plant endogenes (Hiriart et al. 2002; Lermontova et al. 1997). In addition, the actin mRNA detections from different *N. benthamiana* leaves have been constant in our parallel RT-PCR amplification reactions (data not shown).

The *ChlH* mRNA levels were found to vary greatly in the upper leaves of plants, being highest in the top leaves, declining rapidly in subsequent leaves, and then, leveling to a stable level below the fifth leaf from the top (Fig. 2A). This stable accumulation in mature leaves was chosen as the relative 100% mRNA level and used as a standard in all later quantification. Compared with this, the *ChlH* mRNA level in the uppermost leaf was about 20 times higher (2000%), in the second leaf approximately 8 times higher (794%), and in subsequent leaves gradually reduced down to the standard level (100%) (Fig. 2A). Likewise, the virus vector RNA levels were quantified from different tissues of plants inoculated with TMV-30b. The virus RNA level was constant in the systemically infected mature leaves, and this level was chosen as the standard (100%) (Fig. 2B) for the quantification of the viral vector RNA levels in further experiments. However, the content of the virus-construct RNA was higher in the apical leaves; in the topmost leaves, it was fourfold higher than in the mature leaves and, in the second leaves, about twofold higher than in the mature leaves (Fig. 2B).

Inoculation of the lower leaves with TMV-*ChlH* and establishment of local and systemic infection did not affect the level of *ChlH* mRNA in these leaves prior to the appearance of the silencing symptoms in the apical leaves (data not shown). The appearance of the silencing phenotype in the systemically infected apical leaves, however, occurred in parallel with strong reduction of both the *ChlH* mRNA and the TMV-*ChlH* RNA in these leaves. At the time of the appearance of the first small spots of silenced tissue at the apex, at 2.5 weeks p.i., the *ChlH* mRNA content was still as high as in the control plants, but a week later, it was very strongly reduced (Fig. 2C). At later stages, reduction of the *ChlH* mRNA and of the TMV vector RNA was correlated with the silenced phenotype, and the recovery of RNA levels was correlated with the recovery of mosaic or green phenotype (Fig. 2C). An increase of *ChlH* mRNA and reversion of the silenced phenotype after the suppression of virus vector indicated that the *ChlH*-silencing status did not spread systemically in the plant tissue.

The methylation stage of the *ChlH* target gene was analyzed by digestion of the genomic DNA with *AluI* restriction enzyme, known to cut the *ChlH* gene within the PTGS-targeted region, and by Southern blot analysis, using a probe corresponding to the *ChlH* fragment inserted in the TMV vector. No difference in the restriction fragment patterns was observed between the DNAs of the TMV-*ChlH*-infected silenced plant tissues and the TMV-infected or mock-inoculated control plants, suggesting that the target gene was not methylated in the silenced tissues (data not shown).

Persistence of the silenced stage in the lower leaves.

The observed patterns of the silenced phenotypes and the fluctuation in *ChlH* mRNA and TMV RNA contents suggested that silencing was induced only in the apical tissues that were systemically infected with TMV-*ChlH*. However, the established silencing status and the phenotype were maintained in these leaves as they matured, and the amounts of *ChlH* mRNA and virus vector RNA quantified by RT-PCR remained very

low (Fig. 3). In the mature leaves, the lowest *ChlH* mRNA and TMV-*ChlH* RNA contents were always detected in the fully white tissues (4% of control levels) but also in the mosaic white and, in the mosaic green tissues, the two RNA levels were reduced to 6 and 7% of control levels, respectively. In the green leaves, these RNAs were reduced to 14 and 20% of the levels detected in corresponding control leaves infected with TMV-30b (Fig. 3A and B). For comparison, the amounts of *ChlH* mRNA and virus-vector RNA were analyzed also from the mature leaves of plants infected with TMV constructs carrying shorter (214 and 67 nt) fragments of the *ChlH* gene (Hiriart et al. 2002) that induced more moderate levels of *ChlH* silencing (Fig. 3A). The white and yellow phenotype was correlated again with a strong reduction (more than 95% of nonsilenced control) of the *ChlH* mRNA in all these plants. However, the amount of *ChlH* mRNA was also reduced to 14 and 52% of the nonsilenced control level in the mosaic green and in the fully green tissues, respectively, indicating that the phenotype did not fully reflect the silencing activity. In all plants, the reduction of the TMV-*ChlH* vector RNA was correlated with the silencing of the *ChlH* gene (Fig. 3A and B).

Targeting of VIGS to the vector-specific sequences.

To test whether VIGS-induced RNA degradation was targeted to the vector-specific sequences, an infectious transcript of a TMV-derived expression construct, 30b-GFP (Shivprasad et al. 1999), was mixed in a 1:1 ratio with the transcript of TMV-*ChlH* or TMV-30b and inoculated into the *N. benthamiana* plants. 30b-GFP moved in the *N. benthamiana* plants at about the same rate as the TMV-*ChlH* construct, as indicated by the appearance of a positive green fluorescent signal in the upper leaves of the plants at the same time that the virus symptoms appeared in the TMV-*ChlH*-inoculated plants, i.e., about two weeks after inoculation. In the plants inoculated with the 30b-GFP and TMV-*ChlH* mixture, the GFP signal gradually decreased and disappeared within the following week whereas, in the plants inoculated with the 30b-GFP and TMV-30b mixture, the GFP signal persisted at a high level for several weeks (Fig. 4). This indicated that the TMV-*ChlH*-induced VIGS mechanism was targeted not only to the *ChlH* sequence but also enhanced the degradation of the TMV-specific vector sequence.

DISCUSSION

Different plant endogenes and transgenes have been targeted by PTGS, induced either by homologous transgene sequences or by virus vectors carrying homologous inserts (the VIGS system). However, various silencing systems have behaved differently in several aspects; some but not all of the silenced systems have produced systemic signals that have transported the sequence-specific silencing activity throughout the plant (Jones et al. 1999; Voinnet et al. 1998) or only to such tissues that express high levels of the target mRNA (Palauqui and Balzerque 1999; Palauqui and Vaucheret 1998). In the VIGS-mediated systems, targeting of the VIGS to the inducing virus vector has also varied, depending on both the vector and the insert sequence. However, all fully analyzed VIGS systems have eventually led to recovery of the plant from silencing, apparently via PTGS-mediated suppression of the virus vector (Dalmay et al. 2001; Jones et al. 1999; Ratcliff et al. 2001; Ruiz et al. 1998).

In our experiments, the *ChlH* gene, encoding for the H subunit of the magnesium chelatase enzyme, was silenced using TMV-based expression vector (Hiriart et al. 2002). The initial mosaic phenotype obtained in this system was similar to those reported for VIGS-mediated silencing of other photosynthesis related genes (Kjemtrup et al. 1998; Kumagai et al. 1995; Ruiz

et al. 1998). Lack of chlorophyll, i.e., the white and yellow phenotype, was initially observed in the growing apex, in the young leaves, and in stems, forming mosaic tissue or fully white leaves. In the affected tissues, the amount of *ChlH* mRNA was strongly reduced. Activation of silencing in these tissues appeared to be related to the highest accumulation of both TMV-*ChlH* RNA and endogenous *ChlH* mRNA in the plant apex. Effective transgene-mediated PTGS (cosuppression) of plant genes was previously also shown to be initiated only in such tissues that had a high level of the target mRNA (Palauqui and Vaucheret 1998; Vaucheret et al. 1998).

Not only *ChlH* mRNA, but also TMV vector construct was strongly targeted for silencing in apical tissues. Eventually, this led to a strong reduction of the virus vector in the shoot apex. As a consequence, the plant apex partially recovered from the silenced phenotype, and both the *ChlH* mRNA and the TMV RNA levels increased. This led to reestablishment of the silenced phenotype and re-reduction of the two RNA species. Recovery of the new apex from silencing, or the suppression of silencing, may have been related both to the reduced target mRNA and the reduced virus vector level. Thus, fluctuation of the silenced and recovered phenotypes appeared to be regulated in a feedback loop by a reduction and increase of the target mRNA and viral RNA levels.

Although the silenced phenotypes (white, mosaic, or green) were initiated only in the apical tissues, the established phenotypes and silencing status persisted in these leaves as they matured. Separate analysis of each different phenotype present in mature leaves indicated that the fully yellow or white phenotype was produced only by the maximal (more than 95%) reduction of *ChlH* mRNA. Reduction of the *ChlH* mRNA level by 84 to 94% led to the mosaic phenotype, and reduction by 48 to 86% caused no change of phenotype (fully green leaves). In all infected tissues, the vector construct was also suppressed, as compared with the virus content of the plants inoculated with the TMV-30b vector, and the local suppression was directly correlated to the reduction of the *ChlH* mRNA. This indicates that the targeting of the VIGS-mediated silencing to the homologous endogenous mRNA also strongly enhances the degradation of the replicating viral RNA. Similar silencing enhancement is involved in RNA-mediated transgenic virus resistance, in which transcription of a fragment of viral sequence from the host genome strongly enhances the sequence specific PTGS, as compared with that caused by the native virus alone (Baulcombe 1996; Lindbo et al. 1993). However, coinoculation of two different TMV-expression constructs, TMV-*ChlH* and 30b-GFP, into the same plant demonstrated that the enhanced silencing was targeted not only to the *ChlH* insert but also to vector (TMV)-specific sequences. Voinnet and associates (1998) reported that PTGS-mediated systemic silencing of a truncated GFP transgene was also targeted to the PVX vector carrying the GFP sequences, and the authors suggested that the suppression of adjacent vector sequences was mediated by (methylation) interaction between the epigenetic and integrated target sequences. In our case, the vector-specific sequences were targeted by VIGS when the silencing was initiated by endogenous and TMV-integrated transcripts of *ChlH*. It is possible that the strong silencing, induced in the apical tissues by high levels of the target mRNA and the replicating virus construct, led to amplification of the different components employed in the sequence-specific RNA degradation and that this also enhanced the TMV sequence-specific silencing.

TMV-*ChlH*-mediated silencing led to strong suppression of the virus vector but did not lead to stable recovery of the plant from virus infection or from the VIGS-mediated silencing of the target gene. This differed from the silencing systems mediated by PVX or TRV vectors (Ratcliff et al. 2001). The efficiency of vi-

rus vector targeting has, however, varied also in the PVX- and TRV-mediated silencing systems; PVX-mediated silencing of the GFP-marker gene has led to rapid, strong, and permanent suppression of the vector (Ruiz et al. 1998), whereas silencing of GUS, NTP, or PG transgenes has led to suppression of the vector only in such plants in which the targeted transgenes were previously posttranscriptionally silenced (English et al. 1996). PVX-mediated silencing of the PDS and *rbcs* endogenes was initially not targeted to the inducing virus vector (Jones et al. 1999; Ruiz et al. 1998), but eventually, the silenced plants reverted back to wild type, suggesting that the virus vector also became suppressed by PTGS (Ratcliff et al. 2001). TRV vector-mediated silencing of the PDS and *rbcs* endogenes has followed the same pattern as with the PVX vector but has persisted on a higher level and for a longer time than in the plants silenced with PVX-vector (Dalmay et al. 2001; Ratcliff et al. 2001). The longer duration of VIGS may be because the TRV vector was able to evade, on a low level, the PTGS-mediated suppression (Ratcliff et al. 2001). In the TMV-mediated VIGS system, the propagation of TMV-*ChlH* was reinitiated as soon as the VIGS-mediated silencing was reduced. This suggests that TMV may escape from PTGS-mediated suppression more effectively than PVX- and TRV-based vectors, maybe by means of its more efficient replication and spread or its more stable particle structure, which protects the genome from the sequence-specific degradation. On the other hand, it is possible that this escape phenomenon is strongly related to the silenced target gene. Expression of the *ChlH* gene is

Fig. 3. Relative levels of **A**, *ChlH* mRNA and **B**, TMV-*ChlH* RNA in mature leaves of plants inoculated with the TMV constructs carrying different lengths of the *ChlH* insert (TMV-*ChlH* with a 549-nt insert and the other constructs with 214- and 67-nt inserts, as indicated). The RNA levels are expressed in relation to the RNA levels detected in corresponding mature leaves of the nonsilenced control plants inoculated with TMV-30b (100%). Amounts of RNA have been determined separately by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) from fully white (FW), mosaic white (MW), mosaic green (MG), and fully green (FG) leaves. The experiment was repeated three times. For each repetition, samples have been obtained by pooling leaves from 10 plants, and the cDNAs have been analyzed at least two times by quantitative PCR for each gene.

known to be strongly regulated by the diurnal cycle (Gibson et al. 1996). This daily fluctuation of the target gene transcript level could cause parallel alteration in the silencing efficiency, and this may facilitate the escape of the replicating virus.

In TMV-mediated VIGS, the fluctuation of the plant apex between green and yellow phenotypes indicated that the silencing was not systemically spread or induced in the plants. Previously, it has been shown that both VIGS and transgene-induced silencing of a GFP transgene produce a systemic signal that spreads throughout the plant and initiates silencing even in the absence of the original inducing agent (Voinnet and Baulcombe 1997, Voinnet et al. 1998). In these systems, the systemic silencing is enhanced in a relay race fashion, by repeated production and amplification of the systemic signal in the newly silenced tissues (Jones et al. 1999; Kooter et al. 1999; Voinnet et al. 1998). PTG silencing (cosuppression) of plant-derived transgenes can also spread systemically, but initiation of silencing in remote tissues requires a high concentration of transcripts of the target gene at this site (Palauqui and Balzergue 1999; Palauqui and Vaucheret 1998; Vaucheret et al. 1998). Neither the systemic

silencing signal or the signal that mediates the maintenance stage of VIGS have been identified as yet, but they appear to be related to methylation of the coding region of the targeted transgene (Jones et al. 1999, 2001; Mallory et al. 2001; Thomas et al. 2001). Coding region methylation has been associated also with the PTGS-mediated cosuppression of different plant endogenes and transgenes (Hobbs et al. 1990; English et al. 1996; Stam et al. 1998; Voinnet et al. 1998; Fagard et al. 2000). However, the susceptibility of different target genes to DNA methylation appears to vary greatly and usually has not been observed in VIGS applied to endogenes. Also, in our experimental system, the *ChlH* DNA sequence was not methylated in the silenced tissues, suggesting that the established silencing can persist in the same tissues even without target gene methylation.

MATERIALS AND METHODS

TMV constructs.

The TMV-based virus expression vector TMV-30b and its derivative, 30b-GFP, were obtained from W. O. Dawson

Fig. 4. Visualization of the positive green fluorescent protein (GFP) signal in the apical leaves of two infected plants. **A** and **C**, One plant was inoculated with a 1:1 mixture of TMV-30b + 30b-GFP, and **B** and **D**, the other one with a 1:1 mixture of TMV-*ChlH* + 30b-GFP. Plants are pictured at 2 (**A** and **B**) and 3 (**C** and **D**) weeks postinoculation (p.i.). **E**, Visual symptoms of the same plants infected with TMV-30b + 30b-GFP or **F**, with TMV-*ChlH* + 30b-GFP at 3 weeks p.i. Each double infection has been successfully repeated in six plants.

(Shivprasad et al. 1999). Previously (Hiriart et al. 2002), three different constructs were generated by cloning into pTMV-30b different lengths of the *ChlH* cDNA. These constructs, named pTb67s, pTb214a, and pTb549s, contain respectively 67-, 214-, or 549-nt-long *ChlH* inserts. For clarity, virus derived from the pTb549s construct was renamed TMV-*ChlH* in this manuscript.

Plant inoculations.

In vitro transcriptions of pTMV-30b, p30b-GFP, pTb67s, pTb214a, and pTMV-*ChlH* from linearized template plasmids and TMV transcript inoculations onto *N. benthamiana* plants were performed as described previously (Hiriart et al. 2002). Plants were grown at 22°C and with a 16 h light and 8 h dark cycle.

Quantitative RT-PCR.

To determine the *ChlH*-m RNA and TMV-RNA steady-state levels in different *N. benthamiana* leaf samples, quantitative RT-PCR was performed using the Amplifluor Universal Amplification and Detection System (Intergen, Purchase, NY, U.S.A.). This system is based on a two-step reaction carried out during the same PCR amplification. First, a universal Z sequence is added to the 5' end of one of the two specific primers, in order to be incorporated into the amplicon during the first cycles. Then, a third primer, called uniprimer, with the same Z sequence tail attached to its 3' end is used for the following amplification cycles. The uniprimer also has a stem-loop structure at the 5' end. A fluorophore is attached to one strand of the stem-loop and a quencher to the other. The stem holds the two in close proximity for an efficient quenching. When the uniprimer is incorporated in the amplicon and forms dsDNA, the hairpin is unfolded, and the distance between the fluorophore and quencher is increased. A fluorescence signal is then observed, directly correlated to the amount of amplified DNA.

Total RNA extracts, purified as described by Sijen and associates (1996), were cleared of genomic DNA by a 2 h incubation at 37°C with RQ1 RNase-Free DNase (Promega, Madison, WI, U.S.A.) followed by phenol extraction. Reverse transcription was performed with the Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) following the manufacturer's procedures. Typically, 5 µg of total RNA was reverse transcribed, and one fifth of the reaction was used for real time PCR quantification of *ChlH* and actin mRNAs and one sixteenth of the reaction for TMV RNA. Quantitative PCR reactions (25 µl) contained 1× Thermo-Start Standard Buffer (ABgene, Epsom, U.K.), 1.2 mM MgCl₂, 250 µM dNTPs, 50 nM Z-tailed specific primer, 500 nM untailed specific primer, 500 nM uniprimer, and 0.05 U of Thermo-Start DNA Polymerase (ABgene) per µl. The mixture was placed in an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA, U.S.A.), and DNA amplification was done using the following thermocycling program: 1 cycle of enzyme activation at 95°C for 15 min and 50 cycles of template denaturation at 95°C for 15 s, primer annealing at 55°C for 20 s, and extension at 72°C for 40 s. The instrument was programmed to measure fluorescence only during the annealing step. At this temperature, nonincorporated uniprimer is in the silent hairpin conformation and will not contribute to fluorescence measurements.

All primers used for RT-PCR quantification were designed to function with the reaction conditions defined above. For *ChlH* amplification, primers, *QChlHFOR* (5'-CCTTTGGCTCCTTGATGTATGA-3') and *ZQChlHREV* (5'-ACTGAACCTGACCGTACAGCAAACAGTCCAACAA-CAG-3') were designed to amplify a 136-bp DNA fragment. For TMV-RNA quantification a 110-bp DNA fragment, located

in the 126-kDa helicase gene was amplified, using primers *Q30bFOR* (5'-TTACGGAGACACACAGCAGATT-3') and *ZQ30bREV* (5'-ACTGAACCTGACCGTACACCACCTCGT-CAACTTCCAAT-3'). To normalize the amount of RNA (*ChlH* and TMV) in each sample, we amplified and quantified an 89-bp DNA fragment corresponding to actin mRNA, using primers *ZQACFOR* (5'-ACTGAACCTGACCGTACATTCCGATGC-CCTGAAGTCCTT-3') and *QACREV* (5'-GTCGTTTCGTGA-ATGCCTGC-3'). Primers were defined according to sequence data previously obtained (Hiriart et al. 2002), and the exact 5' position of the Z sequence is underlined.

The relative quantifications were determined from appropriate standard curves prepared for each gene, using PCR blunt plasmids containing *ChlH* or actin sequences (Hiriart et al. 2002) or using the pTMV-30b plasmid. *ChlH* or TMV RNA amounts were calculated after normalization to the actin endogen control.

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