

Comparing p20's RNA silencing suppressing activity among five phylogenetic groups of *Citrus Tristeza virus*

Natália T. Marques · Ângela A. Costa ·
Débora Lopes · Gonçalo Silva · Gustavo Nolasco

Accepted: 8 September 2011 / Published online: 18 January 2012
© KNPV 2011

Abstract The p20 protein encoded by the *Citrus Tristeza Virus* (CTV) was previously identified as a RNA silencing suppressor. In this study, we analyzed the p20's suppressing activity from five phylogenetic groups of CTV, using the co-infiltration assay of Green fluorescence protein (GFP) gene and the suppressor gene in 16C line *Nicotiana benthamiana* plants. Green fluorescence, GFP mRNA relative levels and GFP specific siRNAs were compared showing in most cases, only slight differences. Contrary to previous studies, the p20 suppressor was not able to impede neither short range nor systemic spreading of RNA silencing. The suppressor from the phylogenetic group 4 revealed a much reduced activity when compared with the others. At present we still don't know whether this property is a characteristic of this group or an atypical feature due to a unique point mutation. The differences in the symptom type and intensity originated by isolates

belonging to the phylogenetic groups assayed could not be related to differences to the p20 suppressor's activity.

Keywords RNA silencing suppressor · VSR · p21-like · GFP · Closterovirus

Introduction

One of the RNA silencing mechanism functions is to protect plant cells against viruses. The RNA silencing mechanism involves the cleavage of incoming double-stranded RNA regions and the formation of 21–24 nucleotide (nt) short interfering RNAs (siRNAs), which guide, in a sequence specific manner, the degradation of cognate RNAs. A signal moves from the cells, undergoing the initial silencing, to the neighbouring cells as well as to distant cells through the phloem, priming the RNA silencing in naive cells (Baulcombe 2004; Brodersen and Voinnet 2006). To counteract this defence, viruses produce RNA silencing suppressors (VSR), which allows them to block the silencing mechanism at several points (Li and Ding 2006). VSR activity has, in several cases, been related to viral symptoms (Li and Ding 2006; Diaz-Pendon and Ding 2008).

Citrus tristeza virus ((CTV; family *Closteroviridae*, genus *Closterovirus*) has a single-stranded, positive-sense RNA genome of approximately 19.3 Kb (Karasev et al. 1995). This phloem-limited virus is the causal agent of tristeza, the most damaging disease of

N. T. Marques · Â. A. Costa · D. Lopes · G. Silva ·
G. Nolasco
Center for Biodiversity, Functional and Integrative
Genomics, Universidade do Algarve,
Campus de Gambelas,
8005-139 Faro, Portugal

G. Nolasco (✉)
Faculdade de Ciências e Tecnologia,
Universidade do Algarve,
Campus de Gambelas,
8005-139 Faro, Portugal
e-mail: gnolasco@ualg.pt

modern citriculture (Moreno et al. 2008). The virus replicates in phloem cells of a few species within the family *Rutaceae* and is transmitted by a few aphid species. The CTV exists as multiple strains within infected trees (Niblett et al. 2000). The outcome of the disease is related mainly to the CTV isolate and to the infected *Citrus* scion variety and rootstock combinations, resulting generally in three distinct syndromes named seedling yellows (SY), quick decline (QD) and stem pitting (SP) (Roistacher and Moreno 1991). The so called mild strains do not produce noticeable symptoms of any kind. A study on the genomic variability of the p25 gene (coat protein gene, CP) obtained from worldwide isolates revealed the existence of seven phylogenetic groups that could be correlated with the distinct syndromes caused by the virus (Nolasco et al. 2009). Lu et al. (2004) showed that the CTV genome has three genes encoding VSR activity: CP, p20 and p23 which affect the RNA silencing mechanism differently. CP suppresses intercellular silencing without interfering with intracellular silencing; p23 targets the intracellular silencing mechanism and p20 was described to inhibit both the intracellular and the intercellular RNA silencing mechanism. These proteins have also other functions in viral infection; for instance, p20 is a cytoplasmic protein that accumulates in infected cells as inclusion bodies (Gowda et al. 2000) and p23 is a pathogenicity determinant for the SY syndrome (Albiach-Marti et al. 2010). Considering that: *a*) the phylogenetic groups described by Nolasco et al. (2009) are related to differences in symptoms; and *b*) most of the previous work with CTV VSRs has been done solely on T36 isolate, which belongs to the phylogenetic group (Gp) 1, we decided to compare the suppressor activity of the p20 from five phylogenetic groups: 1, 3a, M, 4 and 5.

Materials and methods

Preparation of binary vectors for *Agrobacterium*-mediated infiltration

The p20 gene was amplified from clones of CTV genomic variants available in the laboratory (described in Table 1). Each p20 gene, including its termination and initiation codon, was inserted into pDONR vector and then transferred through Gateway recombination to the pK7WG2 binary vector (Karimi

et al. 2002) following Invitrogen protocols. In the pK7WG2 vector, the inserts are under the control of the CaMV 35S promoter. The primers used to amplify the p20 gene sequences were: CN182-GW-Fw: 5'-GTACAAAAAAGCAGGCTCCATGCGAGCTACTTTAGTG-3' and CTVp20-GW-Rv: 5'-GTA CAAGAAAGCTGGGTCTACACGCAAGATGGA GAG-3' (underlined bases are not virus specific and constitute part of the attB recombination sites). Similar procedures were used to construct the binary vector pK7WG2-GFP harbouring the GFP5-ER gene (Haseloff et al. 1997) identical to the one used to obtain the 16C line *N. benthamiana* plants (Ruiz et al. 1998). A binary vector harbouring the gene 2b of *Tomato aspermy virus* (TAV), kindly provided by Dr. Garcia-Arenal (Universidad Politécnica de Madrid, Spain), to be used as a positive control for suppression, was constructed in a similar way.

Plant material and *Agrobacterium* co-infiltration assay

Constructions done in the binary plasmid pK7WG2 were transformed into competent cells of *Agrobacterium tumefaciens* strain C58C1 carrying the pMP90 plasmid, which confers resistance to gentamycin. Selection was performed with gentamycin, spectinomycin and rifampicin at 50 $\mu\text{g ml}^{-1}$. *A. tumefaciens* cultures were grown on LB medium supplemented with 10 mM MES and 20 μM acetosyringone (Ruiz et al. 1998) at 28°C and at $\text{OD}_{600}=0.5$. Transgenic *N. benthamiana* line 16C plants (kindly provided by Dr. David Baulcombe, University of Cambridge, UK) constitutively expressing the GFP transgene were used for local transient expression assays as described by Voinnet et al. (1999). The pK7WG2-GFP construct was infiltrated singly or co-infiltrated with each of the pK7WG2-p20 constructs. For each modality two leaves per plant, in a total of 15 plants, were used. For the co-infiltrations, equal volumes of *A. tumefaciens* cultures containing pK7WG2-GFP and pK7WG2-p20 were grown to $\text{OD}_{600}=0.5$ and mixed. The cells were centrifuged and re-suspended in 10 mM MgCl_2 , 10 mM MES and 100 μM acetosyringone at pH 5.6, and kept at 25°C for at least 1 h. The suspension was infiltrated, at four leaf stage, on the leaves' underside using a 1-ml needleless syringe, as described by Brigneti et al. (1998).

The GFP fluorescence was observed with a 100 W, hand-held, long-wave UV lamp (Black-Ray B-100AP, Ultraviolet Products, Upland, CA).

Table 1 Relative expression of Green fluorescence protein as estimated by qRT-PCR

Agro-inoculated constructions	Origin of the CTV isolate	Phylogenetic group	Syndrome associated ^a	Relative expression ^b
Q3 + GFP	Albania	1	QD	2.2 a
11.4 + GFP	Madeira Island	3a	QD + SwO-SP (severe)	3.0 a
124.7 + GFP	Reunion Island	4	QD	0.2 b
25.11 + GFP	Spain	M	M	4.2 a
199.7 + GFP	Madeira Island	5	SwO-SP (mild)	4.6 a
Tav 2b + GFP	–	–		25 c
GFP	–	–		0.2 b

^a QD: Quick decline of trees grafted in sour orange; SwO-SP: Stem pitting in sweet orange trees. M: Mild symptoms in Mexican lime indicator; absence of symptoms in sweet orange trees

^b 16C line plants were used as the reference for the relative expression. Numbers followed by the same letter are not significantly different ($p < 0.05$)

Northern blot analysis of siRNAs

Total RNA was extracted, 5 days post-infiltration (dpi), from the inoculated leaf patches using 2 ml of TRI-Reagent (Sigma) per 0.7 g of plant material, according the manufacturer's recommendations. The final pellet was resuspended in 60 μ l of RNase free water. The quality and concentration of RNA preparations were determined by Nanodrop 2000 (Thermo Scientific). For the detection of GFP-specific siRNAs 7.5 μ g of total RNA were used. The detection was performed through a Northern blot assay adapted from Pall et al. (2007), using a minigel (85 mm/ 65 mm/ 0.75 mm). A synthetic 24 nt RNA containing part of the GFP sequence was used as a marker. The gel was horizontally cut. The upper part of the gel was used as a loading control after staining with ethidium bromide. The lower part was used for transfer to a neutral nylon membrane (AmershamHybond™ NX, GE Healthcare) through a semi-dry blotting (LKB Multiphor II, Pharmacia) at 4 mA.cm⁻². Crosslinking of siRNAs to the nylon membrane was done at 60°C during 45 min in the presence of EDC at 0.16 M. RNA blots were hybridized overnight at 40°C with a digoxigenin labeled GFP specific probe in 6 mL of the hybridization solution "DIG Easy Hyb granules" (Dig Northern Starter Kit, Roche). The membrane was then washed: twice with 2x SSC buffer, containing 0.1% SDS, for 10 min and with washing buffer for 5 min. Immunological detection was performed with anti-digoxigenin Fab' fragments conjugated with alkaline phosphatase (Roche Applied Science). Finally, the RNA blots were revealed by chemiluminescent detection with the CDP-Star ready-to-use (Roche Applied

Science). Luminescence was recorded with an adapted astronomical Starlight express MX7C CCD camera.

GFP mRNA quantification by real-time RT-PCR

Leaves were obtained from three plants kept in equal conditions; two leaves, of approximately 170 mg, were collected from each plant. For real-time PCR analysis, total RNA was extracted from the aforementioned leaves with 350 μ l of Tri-reagent and then eluted in 30 μ l of RNase free water. Before the reverse transcription reaction, total RNA samples (2.5 μ g) were DNase treated with the Turbo DNA-free kit (Ambion). For each biological sample (= leaves from one plant), 20 ng triplicates of total RNA, measured by Nanodrop 2000 (Thermo Scientific) were amplified by RT-PCR. The primers GFP-Taq-Fw₂ 5'-GCCAACACTTGTCCTACTTTCTC-3' and GFP-Taq-Rv₂ 5'-GTAGTTCCCGTCGTCCTT-GAAG-3' were used to amplify through RT-PCR a fragment of 147 bp. This was done in an iCycler IQ (Biorad) with the "iScript™ One-Step RT-PCR and SYBR-Green" Kit (Bio-Rad). The ubiquitin gene, amplified with primers *ubi3* and *ubi3B* (Rotenberg et al. 2006), was used as a normalizing gene. The cycling was initiated by a 10 min cycle at 50°C, followed by a 4 min cycle at 95°C and by a sequence of 40 cycles at 95°C for 15 s, and one finishing cycle of 30 s at 55°C completed the process. A six-point twofold dilution series of the RNA sample was used to obtain a standard curve for each gene, from which the amplification efficiencies were calculated. The 16C non-inoculated plants were used as a reference

for determining the relative level of GFP mRNA, according to Pfaffl (2001).

Results and discussion

Based on an *in silico* analysis, Reed et al. (2003) predicted that CTV's p20 should have VSR activity as it possesses conserved amino acid patterns similar to the ones already identified in suppressors in the genus *Closterovirus*. This was later confirmed by Lu et al. (2004), who worked with a p20 gene obtained from the monophyletic T36 isolate, which belongs to Gp 1. In this work we extend the characterization of the p20's VSR activity to other phylogenetic groups. These groups have previously been defined based only on the coat protein (CP) gene (Nolasco et al. 2009); however the genetic structure of the CTV population (in the sense of maintaining the clustering pattern) is maintained through the entire 3'-end coding region (Silva, G. unpublished), thus enabling the same cluster classification for the p20 gene as for the CP gene.

The VSR activity of p20 obtained from the five phylogenetic groups: Gp 1, Gp 3a, Gp 4, Gp 5 and Gp

M was assayed using the classical *A. tumefaciens* co-infiltration assay in 16C *N. benthamiana* plants, which constitutively express the GFP gene (Voinnet et al. 1999). In this assay, silencing is induced by a construct that expresses a transcript homologous to the transgene while the co-infiltrated VSR counteracts the silencing. TAV 2b was used as a positive control for suppression.

Visual observations under UV light

Green fluorescence was visible in each plant's infiltration site; it reached a maximum 2 to 3 dpi. After that, the inoculation patches in the plants that were singly infiltrated with GFP, quickly turned reddish while those co-inoculated with p20 remained greenish for a longer period (Fig. 1). In this case, the persistence of the green fluorescence varied: plants expressing p20 from Gp5 maintained the greenish patch up to 9 dpi, while plants expressing Gp 4 p20 started turning red at 4 dpi. Between the 5th dpi and the 6th dpi the characteristic reddish halo that has been attributed to short range spreading of the silencing signal (Himber et al. 2003) could be seen surrounding the infiltrated areas. The halo appeared

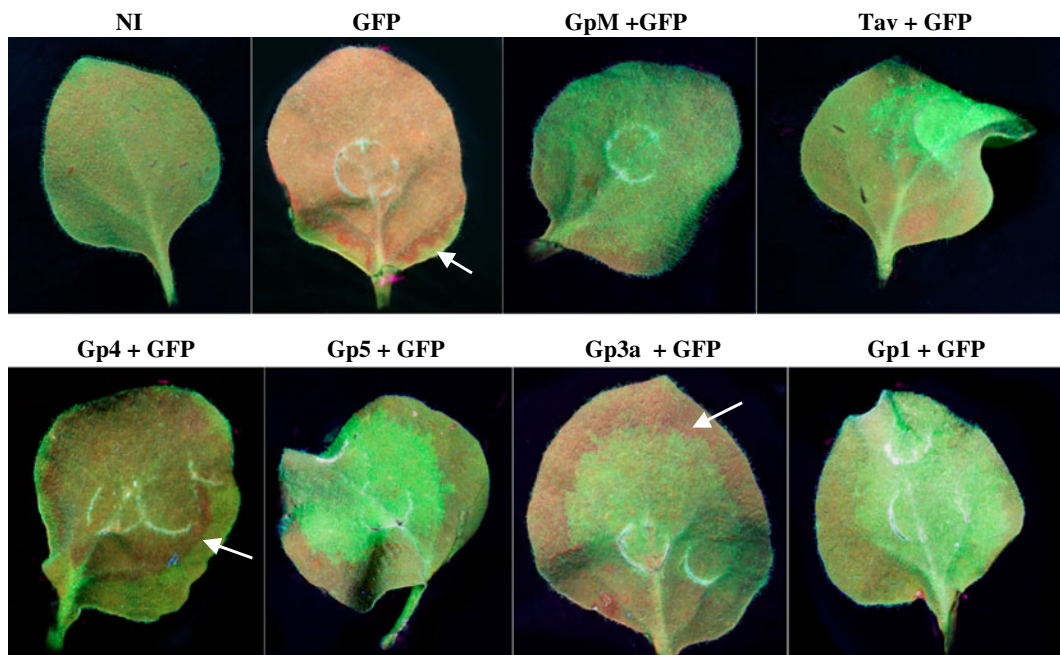
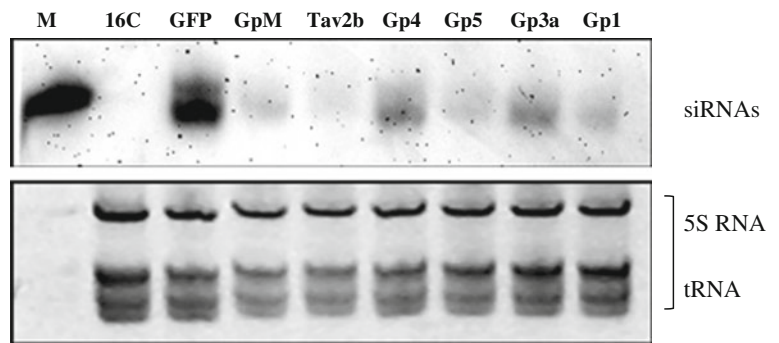


Fig. 1 Visual comparison at 5 dpi of p20's suppressor activity under UV light. NI, non-inoculated 16C plant control; GFP, 16C plant inoculated with GFP alone. The *arrow* points to the short range spread of RNA silencing, visible as a narrow red ring

Fig. 2 Northern blot analysis of GFP specific siRNAs at 5 dpi. M is a synthetic 24 nt RNA marker. The upper part of the gel was cut and stained with ethidium bromide; staining displays 5S RNA and tRNA as a loading control



first in plants inoculated with: solely GFP, GFP + Gp 4 and GFP + Gp 3a (Fig. 1). This halo could also be seen, later on, in the other co-infiltrated plants.

Red spots characteristic of systemic silencing started to appear in non-inoculated areas: in plants inoculated singly with GFP these appeared 7 dpi, and in plants co-infiltrated with p20 9 dpi. The latter was surprising as in accordance with Lu et al. (2004), p20 can suppress intercellular silencing.

Quantification of GFP expression by qRT-PCR

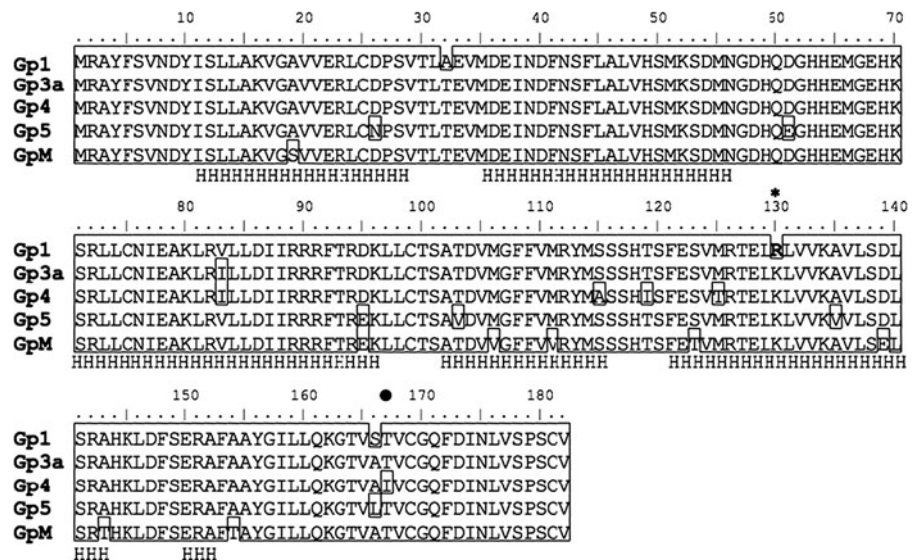
The relative expression of GFP was quantified through qRT-PCR at 5 dpi (Table 1); non-inoculated 16C plants were used as a reference. Silencing induced by agroinfiltration with GFP alone resulted in a notorious decrease of the transcript level. With exception of Gp 4, all the other suppressors led to an overexpression of GFP when compared with the non-

inoculated 16C plants. No significant differences could be proved among Gp 1, Gp3a, Gp M and GP5 VSRs. The effect between the “less active” suppressor (Gp 1) and the “most active” (Gp 5) in the level of GFP mRNA varies about twofold. Apparently, the activity of Gp 4 was already overcome by silencing at 5dpi, which is in agreement with the observations. When compared with TAV 2b, the p20 VSRs are much less active, confirming Lu et al. (2004) previous results for the T36 isolate.

Analysis of GFP specific siRNAs

The siRNAs corresponding to the GFP induced silencing (Fig. 2) appeared as at least two bands. One, less intense, corresponding to 24 nt and another one, more intense, corresponding to smaller size siRNAs, which can be 21 or 22 nt. In all cases the application of the VSRs resulted in a reduction of the

Fig. 3 Alignment of the deduced amino acid sequences of p20 from the five phylogenetic groups analysed. Non-conserved residues are surrounded by box. The asterisk at position 130 marks a non conserved residue which constitutes the p21-like suppressor family motif. The black dot at position 167 marks a unique mutation in the Gp 4 suppressor. Blocks of letters H below the alignment correspond to the regions predicted to form α -helices for Gp M. Secondary structure for the other groups is very similar



intensity of the siRNA bands. The greater reduction of siRNAs accumulation occurred with TAV 2b, Gp M and Gp 5 while, on the contrary, Gp 4 showed a less intense reduction. The accumulation of the 24 nt siRNAs was affected by all VSRs, but it did not disappear. Notwithstanding, there seems to be a discrepancy between the sizes of the smaller siRNAs detected. For the most active VSRs, Gp M, Gp 5 and TAV 2b the smallest size siRNAs (presumably 21 nt), are not present enabling the appearance of a band corresponding to a somehow longer siRNAs (presumably 22 nt). The less active suppressors Gp 4 and Gp 3a have the shortest siRNAs detected, which have the same size of the shortest siRNAs of the non suppressed GFP treatment (21 nt). Much controversy exists regarding the individual role of the different size siRNAs in local, short range and systemic silencing. According to a recent model proposed by Liang et al. (2011), which synthesizes previous views, the 21 nt siRNAs are the predominant mobile signals for the short range spread of silencing. Concordantly, at 5 dpi, the halo characteristic of short range silencing spread can be seen in plants treated with Gp 4 and Gp 3a p20. In the same model proposed by Liang et al. (2011) the 24 nt siRNAs are associated with amplification of silencing signal and the systemic silencing of transgenes. The fact that these do not disappear totally at 5 dpi may be related with the failure to suppress the systemic silencing. Interestingly, in the paper describing the p20 VSR activity (Lu et al. 2004) the 24 nt siRNAs are clearly visible 3 dpi as well as 6 dpi, in similar co-infiltration assay with p20 and GFP. Eventually, a dosage effect may explain this discrepancy as the assays that lead to the conclusion of p20's intercellular suppressing properties were based in transgenic plants permanently expressing the p20 protein.

All the analysed p20 VSRs are true to type members of the p21-like family

Reed et al. (2003) defined the *Beet Yellows virus* (BYV) p21-like family of VSRs and reported the existence of conserved amino acid motifs among its members. To verify whether the reported characteristics were also present in the set of p20 genes used, the deduced amino acid sequences were aligned (Fig. 3) and compared with the motif reported by those authors. Except for the residue present in

position 130, the remaining 17 residues that constitute the motif are present in the p20 sequences of the five groups considered. The arginine in position 130, which is conserved among the remaining members of the p21-like family, is substituted in groups Gp 3a, Gp 4, Gp 5 and Gp M for a similar amino acid, lysine. The predominance of alpha-helix domains characteristic of p21-like family is also observed.

The alignment also reveals a conserved set of sequences with an average 95.2% identity. Pairwise identities range from 92.9% to 97.8%. The sequence of the Gp 4 suppressor, whose activity was found to contrast with the others, appears close to Gp 3a (97.8% identity). However, a search for unique mutations among the sequences available in our lab and others retrieved from the Genbank, spanning through the seven CTV phylogenetic groups, revealed that in position 167, this particular Gp 4 sequence has an isoleucine instead of threonine. The latter is present in all the other 54 sequences analyzed which also includes other representatives from Gp 4. Without experimentally analyzing other suppressors from the same group it is not possible to know whether this particular low activity is characteristic of Gp 4 or an effect of the unique mutation found. In fact, there are single mutations described for TAV2b suppressor (Chen et al. 2008) and for *Tobacco Etch Virus* HC-pro suppressor (Torres-Barcelo et al. 2008), which originate drastic reductions in the suppressing activity.

Absence of a relationship with symptoms

The p20 genes assayed were obtained from isolates, which originate different symptoms but we could not trace any relationship between the symptoms' type kind and intensity and the VSR activity. Nevertheless, the CTV suppressing activities are encoded in three genes, and probably the severity of the interaction with the host may be governed by their coordinated action and complementarity. For instance, Satyanarayana et al. (1999) showed that p20 is not essential for CTV replication in protoplasts.

Acknowledgements This research was funded by grant PTDC/AGR-GPL/99512/2008 from Foundation for Science and Technology, Portugal. Angela Costa and Gonçalo Silva are the recipients of FCT grants SFRH/BD/62248/2009 and SFRH/BD/37310/2007, respectively.

References

- Albiach-Marti, M. R., Robertson, C., Gowda, S., Tatineni, S., Belliure, B., Gamsey, S. M., et al. (2010). The pathogenicity determinant of *Citrus tristeza virus* causing the seedling yellows syndrome maps at the 3'-terminal region of the viral genome. *Molecular Plant Pathology*, *11*(1), 55–67.
- Baulcombe, D. (2004). RNA silencing in plants. *Nature*, *431*, 356–363.
- Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W., & Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO Journal*, *17*(22), 6739–6746.
- Brodersen, P., & Voinnet, O. (2006). The diversity of RNA silencing pathways in plants. *Trends in Genetics*, *22*(5), 268–280.
- Chen, H. Y., Yang, J., Lin, C., & Yuan, Y. A. (2008). Structural basis for RNA-silencing suppression by *Tomato aspermy virus* protein 2b. *EMBO Reports*, *9*(8), 754–760.
- Diaz-Pendon, J. A., & Ding, S. W. (2008). Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annual Review of Phytopathology*, *46*, 303–326.
- Gowda, S., Satyanarayana, T., Davis, C. L., Navas-Castillo, J., Albiach-Marti, M. R., Mawassi, M., et al. (2000). The p20 gene product of *Citrus tristeza virus* accumulates in the amorphous inclusion bodies. *Virology*, *274*(2), 246–254.
- Haseloff, J., Siemering, K. R., Prasher, D. C., & Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. *Proceedings of the National Academy of Sciences of U S A*, *94*(6), 2122–2127.
- Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., & Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO Journal*, *22*(17), 4523–4533.
- Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilf, M. E., Koonin, E. V., et al. (1995). Complete sequence of the *Citrus tristeza virus*-RNA genome. *Virology*, *208*(2), 511–520.
- Karimi, M., Inze, D., & Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science*, *7*(5), 193–195.
- Li, F., & Ding, S. W. (2006). Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annual Review of Microbiology*, *60*, 503–531.
- Liang, D., Finnegan, E. J., Dennis, E. S., Waterhouse, P. M., & Wang, M.-B. (2011). Mobile silencing in plants: what is the signal and what defines the target. *Frontiers in Biology*, *6*(2), 140–146.
- Lu, R., Folimonov, A., Shintaku, M., Li, W. X., Falk, B. W., Dawson, W. O., et al. (2004). Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proceedings of the National Academy of Sciences of United States of America*, *101*(44), 15742–15747.
- Moreno, P., Ambros, S., Albiach-Marti, M. R., Guerri, J., & Pena, L. (2008). *Citrus tristeza virus*: a pathogen that changed the course of the citrus industry. *Molecular Plant Pathology*, *9*, 251–268.
- Niblett, C. L., Genc, H., Cevik, B., Halbert, S., Brown, L., Nolasco, G., et al. (2000). Progress on strain differentiation of *Citrus tristeza virus* and its application to the epidemiology of citrus tristeza disease. *Virus Research*, *71* (1–2), 97–106.
- Nolasco, G., Santos, C., Silva, G., & Fonseca, F. (2009). Development of an asymmetric PCR-ELISA typing method for *Citrus tristeza virus* based on the coat protein gene. *Journal of Virology Methods*, *155*(2), 97–108.
- Pall, G. S., Codony-Servat, C., Byrne, J., Ritchie, L., & Hamilton, A. (2007). Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic Acids Research*, *35*(8), e60.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), e45.
- Reed, J. C., Kasschau, K. D., Prokhnovsky, A. I., Gopinath, K., Pogue, G. P., Carrington, J. C., et al. (2003). Suppressor of RNA silencing encoded by *Beet yellows virus*. *Virology*, *306*(2), 203–209.
- Roistacher, C. N., & Moreno, P. (1991). The worldwide threat from destructive isolates of *Citrus tristeza virus*. A review. *Proceedings of the 11th Conference of the International Organization of Citrus Virologists*. Riverside, 7–19.
- Rotenberg, D., Thompson, T. S., German, T. L., & Willis, D. K. (2006). Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *Journal of Virology Methods*, *138*(1–2), 49–59.
- Ruiz, M. T., Voinnet, O., & Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *The Plant Cell*, *10*(6), 937–946.
- Satyanarayana, T., Gowda, S., Boyko, V. P., Albiach-Marti, M. R., Mawassi, M., Navas-Castillo, J., et al. (1999). An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. *Proceedings of the National Academy of Sciences of United States of America*, *96*(13), 7433–7438.
- Torres-Barcelo, C., Martin, S., Daros, J. A., & Elena, S. F. (2008). From hypo- to hypersuppression: effect of amino acid substitutions on the RNA-silencing suppressor activity of the *Tobacco etch potyvirus* HC-Pro. *Genetics*, *180* (2), 1039–1049.
- Voinnet, O., Pinto, Y. M., & Baulcombe, D. C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proceedings of the National Academy of Sciences of United States of America*, *96*(24), 14147–14152.