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Additional Information

# Optimal viral strategies for bypassing RNA silencing

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The RNA silencing pathway constitutes a defense mechanism highly conserved in eukaryotes, especially in plants, where the underlying working principle relies on the repressive action triggered by the intracellular presence of double-stranded RNAs. This immune system performs a post-transcriptional suppression of aberrant mRNAs or viral RNAs by small interfering RNAs that are directed towards their target in a sequence-specific manner. However, viruses have evolved strategies to escape from silencing surveillance while promoting their own replication. Several viruses encode suppressor proteins that interact with different elements of the RNA silencing pathway and block it. The different suppressors are not phylogenetically nor structurally related and also differ in their mechanism of action. Here, we adopt a model-driven forward-engineering approach to understand the evolution of suppressor proteins and, in particular, why viral suppressors preferentially target some components of the silencing pathway. We analyzed three strategies characterized by different design principles: replication in the absence of a suppressor, suppressors targeting the first protein component of the pathway and suppressors targeting the small interfering RNAs. Our results seed light into the question of whether a virus must opt for devoting more time into transcription or into translation and on which would be the optimal step of the silencing pathway to be targeted by suppressors. In addition, we discussed the evolutionary implications of such designing principles.

# 1. INTRODUCTION

RNA viruses are difficult to control and eliminate because of their rapid evolution. This high evolvability is a consequence of their high mutation rates, large population size and short generation times (Domingo & Holland 1997; Elena & Sanjuán 2007) that confer them an astonishing ability to explore genotypic space. Indeed, RNA viruses typically have mutation rates orders of magnitude higher than their DNA hosts (Drake & Holland 1999). Eukaryotic organisms have developed a sequence-specific mechanism to modulate gene expression based on RNA interference (RNAi) which was first found in the nematode *Caenorhabditis elegans* (Fire *et al.* 1998) and later on in many other eukaryotes including plants (Vaucheret *et al.* 2001) and mammals (Elbashir *el al.* 2001). Likewise, this molecular mechanism is able to silence viral or aberrant genes.

The underlying working principle of RNA silencing relies on the repressive action triggered by the intracellular presence of double-stranded RNAs (dsRNA) (Fire *et al.* 1998). In the case of single-stranded RNA viruses (ssRNA), dsRNAs are byproducts of genome replication mediated by virus-encoded RNA-dependent RNA polymerases (RdRp). During viral genome replication, the dsRNA intermediates become the target of the first component of the silencing pathway, DICER, a type-III RNase that degrades these dsRNA into units of 21 to 24 nucleotides called small interfering RNAs (siRNA) (Hamilton & Baulcombe 1999). Subsequently, the cellular RNA-induced silencing complex (RISC), that contains the argonaute (AGO) endonuclease (Bohmert *et al* 1998), loads the antisense siRNAs resulting in an active form. Using the antisense siRNA as a guide, AGO cleaves the target viral ssRNA (Rand *et al*. 2005). Furthermore, in a secondary cycle of amplification, the host's RNA-dependent RNA polymerase VI (RDR6) uses siRNAs as

primers, together with partially degraded ssRNAs, to produce long dsRNAs that serve as new substrates for DICER, a process known as transitivity (Voinnet *et al*. 1998). siRNAs systemically move from cell-to-cell immunizing new cells against infection (Voinnet *et al.* 1998; Himber *et al.* 2003). Given the properties of the RNA silencing pathway (specificity and amplification), it represents a sort of innate immune system for plants (Lecellier & Voinnet 2004; Ding & Voinnet 2007).

Not surprisingly, viruses have evolved strategies to actively evade the RNA silencing surveillance while promoting their own replication (Li & Ding 2006). Many viruses encode a suppressor protein (viral suppressor of RNA silencing or VSR) that interacts with elements of the silencing pathway blocking it (Brigneti *et al*. 1998; Kasschau & Carrington 1998; Baulcombe 2002). The targets of these VSRs within the RNA silencing pathway are diverse: DICER, the dsRNA, the siRNA, RISC, or the systemic signal (Moissiard & Voinnet 2004; Li & Ding 2006; Díaz-Pendón & Ding 2008). For example, the helper component-protease (HC-Pro) encoded by the *Potyvirus* works as suppressor by sequestering siRNAs (Mallory *et al.* 2002; Chapman *et al.* 2004; Dunoyer *et al.* 2004; Lakatos *et al.* 2004). This binding prevents the incorporation of siRNAs into RISC. Furthermore, by also binding plant endogenous micro-RNAs (miRNA) and controlling the expression of other genes, HC-Pro may interfere the expression of DICER proteins (Deleris *et al.* 2006), reducing the degradation of dsRNAs and, thus, favoring potyvirus replication. Similarly, the *Nodavirus* B2 suppressor also sequesters siRNAs (Li & Ding 2002). The *Tombusviridae* P19 and *Cucumovirus* 2b suppressors interfere with the systemic spread of the 24 nucleotides siRNAs produced by DCL3 (Qi *et al*. 2004). Some suppressors act on RISC, either avoiding the upload of siRNAs into AGO, like the *Closterovirus* P21

(Peremyslov *et al.* 1998), by binding to AGO1 and avoiding its interaction with other proteins required to assemble the RICS, as the coat protein (CP) of *Tombusvirus* (Azevedo *et al.* 2010), by inhibiting the RISC activity after its maturation, like the *Begomovirus* AC4 (Vanitharami *et al.* 2004), or by targeting AGO for degradation, as it is the case for *Polerovirus* P0 protein (Baumberger *et al.* 2007; Csorba *et al.* 2010). It has also been recently shown that V2 suppressor of *Geminivirus* competes with SGS3, a key component of the secondary cycle of siRNAs amplification, in binding dsRNAs and thus interferes with transitivity (Fukunaga & Doudna 2009). Finally, the CP of some carmoviruses (Meng *et al.* 2006) and the P14 of *Aureusvirus* (Mérai *et al.* 2005) can also bind long dsRNAs, resulting in the protection of the intermediaries of replication from DICER activity. Accordingly, VSRs have been divided into three families (Díaz-Pendón & Ding 2008): (*i*) those enhancing within cell virus accumulation, (*ii*) those essential for cell-to-cell movement but dispensable on virus accumulation in single cells, and (*iii*) those that facilitate virus long-distance movement and/or intensify disease symptoms but are not essential for viral replication and cell-to-cell movement.

The first mathematical models of the RNA silencing pathway focused on aberrant cellular mRNA as triggers of the silencing response (Bergstrom *et al.* 2003; Groenenboom *et al.*, 2005). More recent models consider viral RNAs as triggers of the response and focused in virus' spread in the plant (Groenenboom & Hogeweg 2008a; Groenenboom & Hogeweg 2008b). However, on the one hand, these studies did not analyze in detail the possible effect that different viral suppressor strategies may have in the outcome of the interaction. On the other hand, although many several kinetic models of intracellular growth have been proposed for different viruses, none of them specifically incorporates the

silencing response (e.g., Reddy & Yin 1999; Sidorenko & Reichl 2004; Lim *et al*. 2006; Dahari *et al*. 2007; Sardanyés *et al*. 2009). In this work, we present the first model that incorporates the interaction of different suppressor proteins with components of the silencing pathway. We perform a dynamical analysis and show the time course of viral RNA accumulation under a wide set of parameter states. We also show phase diagrams for different combinations of parameters and focus our discussion in the behavior of the system for different viral replication and translation rates in the presence/absence of different suppressor strategies. These analyses allow us to rationalize why different viruses may opt for different strategies in their investment into producing new genomes (i.e., transcription via antigenomic strains) or into producing large amounts of protein from a few initial sense genomes (i.e., translation). Such models are important to unveil defense strategies and design principles of viral systems.


## 2. THE MODEL

We have constructed a mathematical model based on nonlinear differential equations to describe the interplay between the silencing pathway and a positive-sense RNA virus that encodes for a single polyprotein that is processed into mature peptides, as it is the case for picorna-like viruses (e.g., poliovirus, hepatitis C virus, foot-and-mouth disease virus and the potyviruses, which are the largest and more important family of plant viruses). The model involves the following molecular species: genomic and antigenomic ssRNA ($S^+$ and $S^-$, respectively), dsRNA ($D$), antisense siRNA ($I$), viral proteins ($P$), virions ($V$), primed ssRNA ($S^*$), and secondary dsRNA ($D^*$). Three different viral proteins are considered, the nonstructural replicase and VSR and the structural CP. Their corresponding relative

abundances are $p$, $q$ and $1 - p - q$ respectively. This constraint is biologically relevant for picornaviruses as all proteins are self-processed from a single polyprotein and, thus, their relative abundances remain constant during infection. In addition, the model accounts for several cellular components: the ribosomes ($Z$), the RDR6 polymerase involved in transitivity ($Y$), DICER-like proteins ($C$), and inactivated and activated RISC ($R$ and $R^*$, respectively). We assume that at the beginning of infection, a single viral ssRNA genome is present, which in our particular model must be genomic. Notice that genomic strands are those that encode for proteins whereas antigenomic strands are the complementary and, for simplification, we will assume are not coding. To accommodate negative sense RNA viruses, the model can be straightforwardly modified (in this case the negative strand is encapisdated and cleaved by RISC) and changing the initial conditions. For retroviruses or DNA viruses, the model must be conveniently modified.

The model is constructed following a generalized enzyme kinetics scheme where both substrates and enzymes are limited in the medium (DeAngelis *et al.* 1975), and there are competitions between different enzymes for the same substrate and different substrates for the same enzyme (MacRae *et al.* 2007). This gives a highly coupled formulation. In Fig. 1 we show the scheme of RNA silencing pathway, and the kinetic parameters are shown in Table 1, with parameter values taken from different sources.

Viral replication is a process involving multiple reactions aiming to bypass the defense systems of the cell. The RNA replication rates ($J$), for both polarities, are given by the following set of equations:

$$J^+_{replication} = \cfrac{\dfrac{\alpha p P S^+}{K_P}}{1 + \dfrac{S^+}{K_P} + \dfrac{S^-}{\omega K_P} + \dfrac{pP}{K_P} + \dfrac{S^+ R^*}{\left(S^+ + S^*\right)K_R} + \dfrac{S^+}{S^+ + S^-}\left\{\dfrac{Z}{K_Z} + \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}\right\}},$$

$$J^-_{replication} = \cfrac{\dfrac{\alpha p P S^-}{\omega K_P}}{1 + \dfrac{S^+}{K_P} + \dfrac{S^- + pP}{\omega K_P}}, \qquad (2.1)$$

$$J^*_{replication} = \cfrac{\dfrac{\alpha Y S^*}{K_P}}{1 + \dfrac{S^* + Y}{K_P} + \dfrac{S^* R^*}{\left(S^+ + S^*\right)K_R}},$$

where $\alpha$ is the maximum replication rate per molecule of ssRNA, $K_P$, $K_R$, $K_Z$, and $K_C$ are the binding constants for the replicase, the activated RISC, the ribosomes and the CP, respectively. The affinity of the replicase for the antigenomic strands is incorporated into the model by the parameter $\omega$. If $\omega = 1$, then the RdRp has the same affinity for both strains, whereas $\omega > 1$ would imply a lager affinity for the antigenomic strain. By doing so, we can model replication modes ranging from the geometric ($\omega = 1$) to the stamping machine one ($\omega \gg 1$) (Sardanyés *et al.* 2009).

A molecule of dsRNA can be separated into two ssRNA molecules of complementary polarity at a first-order rate with constant parameter $\beta$

$$
\begin{aligned}
J_{dissociation} &= \beta D \quad J_{dissociation} = \beta D \quad J_{dissociation} = \beta D, \\
J^*_{dissociation} &= \beta D^* \quad J^*_{dissociation} = \beta D^* \quad J^*_{dissociation} = \beta D^*.
\end{aligned} \qquad (2.2)
$$

In addition, genomic ssRNAs are translated into viral proteins with rate

$$J_{translation} = \frac{\dfrac{\mu Z S^*}{K_Z}}{1 + \dfrac{S^+}{K_Z} + \dfrac{\omega p P S^+}{(\omega S^+ + S^-)K_P} + \dfrac{S^+ R^*}{(S^+ + S^*)K_R} + \dfrac{Z}{K_Z} + \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}},$$ (2.3)

where $\mu$ is the maximum translation rate per molecule of genomic ssRNA.

The process of RNA silencing is initiated when DICER cleaves dsRNA into siRNAs. The rates describing this process are given by the following set of equations:

$$J_{dicer} = \frac{\dfrac{\delta CD}{K_D}}{1 + \dfrac{D + D^* + C}{K_D}}, J_{DICER} = \frac{\dfrac{\delta CD}{K_D}}{1 + \dfrac{D + D^* + C}{K_D}},$$

$$J_{dicer}^* = \frac{\dfrac{\delta CD^*}{K_D}}{1 + \dfrac{D + D^* + C}{K_D}}, J_{DICER}^* = \frac{\dfrac{\delta CD^*}{K_D}}{1 + \dfrac{D + D^* + C}{K_D}},$$ (2.4)

where $\delta$ and $K_D$ are the catalytic and binding constants of DICER, respectively. Afterwards, RISC is activated by uploading the antisense siRNAs produced by DICER into AGO according to the equation

$$J_{risc} = \frac{\dfrac{\rho RI}{K_I}}{1 + \dfrac{I + R}{K_I}} J_{RISC} = \frac{\dfrac{\rho RI}{K_I}}{1 + \dfrac{I + R}{K_I}},$$ (2.5)

where $\rho$ and $K_I$ are the catalytic and binding constants of RISC, respectively. Following the activation of RISC, it is now able of directing the cleavage of the viral ssRNA with rate

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$$J_{cleavage}^{+} = \frac{\dfrac{\upsilon R^* S^+}{K_R}}{1 + \dfrac{S^+ + R^*}{K_R} + \dfrac{\omega p P S^+}{\left(\omega S^+ + S^-\right)K_P} + \dfrac{Z}{K_Z} + \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}},$$

$$J_{cleavage}^{*} = \frac{\dfrac{\upsilon R^* S^*}{K_R}}{1 + \dfrac{S^*}{K_R} + \dfrac{Y}{K_P} + \dfrac{R^* S^*}{\left(S^+ + S^*\right)K_R}}, \qquad (2.$$

$$6)$$

$$J_{cleavage}^{+} = \frac{\dfrac{\upsilon R^* S^+}{K_R}}{1 + \dfrac{S^+ + R^*}{K_R} + \dfrac{\omega p P S^+}{\left(\omega S^+ + S^-\right)K_P} + \dfrac{Z}{K_Z} + \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}},$$

$$J_{cleavage}^{*} = \frac{\dfrac{\upsilon R^* S^*}{K_R}}{1 + \dfrac{S^*}{K_R} + \dfrac{Y}{K_P} + \dfrac{R^* S^*}{\left(S^+ + S^*\right)K_R}},$$

where $\upsilon$ is the catalytic constant of RNA cleavage. CPs are pre-assembled with ssRNA to produce immature virions at a rate given by

$$J_{capsides} = \frac{\lambda S^+ \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}}{1 + \dfrac{S^+}{K_C} + \dfrac{\omega p P S^+}{\left(\omega S^+ + S^-\right)K_P} + \left[\dfrac{(1-p-q)Z}{2K_R K_Z}\right]^{k_0} + \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}}, \qquad (2.7)$$

$$J_{encapsidation} = \frac{\lambda S^+ \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}}{1 + \dfrac{S^+}{K_C} + \dfrac{\omega p P S^+}{\left(\omega S^+ + S^-\right)K_P} + \dfrac{R^*}{2K_R} + \dfrac{Z}{K_Z} + \left[\dfrac{(1-p-q)P}{K_C}\right]^{k_0}},$$

where $\lambda$ is the maximum assembly rate and $k_0 < k$ is the number of CP monomers associated to the immature virions, $V_{immature}$. Then, virions are produced at rate

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$$J_{virions} = \frac{\gamma V_{immature} \left[ \dfrac{(1-p-q)P}{K_C} \right]^{k}}{\left\{ 1 + \dfrac{V_{immature}}{K_C} + \left[ \dfrac{(1-p-q)P}{K_C} \right]^{k_0} \right\}^{k/k_0}}, \qquad (2.8)$$

where $\gamma$ is the maximum rate to produce virions, and $k$ is the number of CPs necessary to complete a mature virion. All species are thermodynamically degraded at rates $\kappa_S$ (ssRNAs), $\kappa_D$ (dsRNAs), $\kappa_I$ (siRNAs), and $\kappa_P$ (the rest of proteins or protein complexes).

The effect exerted by different VSRs on DICER, RISC and RDR6 can be conveniently modeled by the following three equations, respectively:

$$C = \frac{C_0 \left( 1 + \dfrac{fqP}{\Gamma_C} \right)}{1 + \dfrac{qP}{\Gamma_C}},$$

$$R = \frac{R_0 \left( 1 + \dfrac{fqP}{\Gamma_R} \right)}{1 + \dfrac{qP}{\Gamma_R}}, \qquad (2.9)$$

$$Y = \frac{Y_0 \left( 1 + \dfrac{fqP}{\Gamma_Y} \right)}{1 + \dfrac{qP}{\Gamma_Y}},$$

where $C_0$, and $R_0$ and $Y_0$ are the corresponding amounts of each protein in the cell, which are assumed to be in large excess, and $\Gamma_C$, $\Gamma_R$ and $\Gamma_Y$ are the binding coefficients of the corresponding VSR to their substrate protein, DICER, RISC, and RDR6, respectively. The parameter $f$ determines the efficiency at which the suppressor precludes the activity of its target. For example, in the equation of DICER, an $f = 0.01$ means that even at saturating

concentration of the suppressor, 1% of DICER molecules will still be active. To account

for the suppression on siRNA, we modify $J_{RISC}$ and introduce a new equation to model the

sequestration of siRNAs.

$$J_{RISC} = \frac{\dfrac{\rho RI}{K_I}}{1 + \dfrac{I+R}{K_I} + \dfrac{qP}{\Gamma_I}},$$

$$J_{suppression} = \frac{\dfrac{\psi qPI}{\Gamma_I}}{1 + \dfrac{I+qP}{K_I} + \dfrac{R}{K_I}},$$

(2.10)

where $\rho$, $\psi$ are, respectively, the rates at which RISC and the suppressor attach to the

siRNA and $\Gamma_I$ the binding affinity of the suppressor for the siRNAs.

After defining all the relevant rate equation, it is now possible to write a system of

coupled differential equations describing the dynamics of the system:

$$\frac{dS^+}{dt} = J_{dissociation} + J^*_{dissociation} - J^+_{replication} - J^+_{cleavage} - J_{encapsidation} - \kappa_S S^+,$$

$$\frac{dS^-}{dt} = J_{dissociation} - J^-_{replication} - \kappa_S S^-,$$

$$\frac{dP}{dt} = J_{translation} - \kappa_P P,$$

$$\frac{dD}{dt} = J^+_{replication} + J^-_{replication} - J_{DICER} - J_{dissociation} - \kappa_D D,$$

$$\frac{dI}{dt} = n J_{DICER} + n^* J^*_{DICER} - J_{RISC} - J_{suppression} - \kappa_I I,$$

$$\frac{dR^*}{dt} = J_{RISC} - J^+_{cleavage} - \kappa_P R^*,$$

(2.11)

$$\frac{dS^*}{dt} = \sigma J^+_{cleavage} - J^*_{replication} - J^*_{cleavage} - \kappa_S S^*,$$

$$\frac{dD^*}{dt} = J^*_{replication} - J^*_{DICER} - J^*_{dissociation} - \kappa_D D^*,$$

$$\frac{dV_{immature}}{dt} = J_{encapsidation} - J_{virion} - \kappa_P V_{immature},$$

$$\frac{dV}{dt} = J_{virion} - \kappa_P V,$$

where the stoichiometric parameters $n$, $n^*$, and $\sigma$ represent, respectively, the number of siRNAs produced per molecule of dsRNA, the number of siRNAs produced in the secondary cycle of amplification, and the relative contribution of the secondary siRNA amplification to the degradation of dsRNA relative to the primary siRNAs.

The full model in MATLAB format is available as electronic supplementary material.


## 3. STABILITY ANALYSIS

The system (2.11) can be rewritten in a vectorial form as $d\mathbf{y}/dt = F(\mathbf{y}) = \mathbf{\Omega} J(\mathbf{y}) - \mathbf{\Xi} \mathbf{y}$, where $\mathbf{\Omega}$ is the matrix of stoichiometric coefficients, $J(\mathbf{y})$ the vector of production rates, and $\mathbf{\Xi}$ a diagonal matrix with the vector of degradation rates. The initial condition for the molecular

species involved in the system ($\mathbf{y_0}$) depends on the nature of the virus (i.e., the infectious particle containing a genomic or an antigenomic RNA strand). Here we have considered for our analyses viruses encapsidating genomic RNAs and therefore all the elements in $\mathbf{y_0}$ are zero except for $S^+ = 1$. In case of negative sense RNA viruses, the initial condition would be $S^- = 1$. Accordingly, we construct an initial value problem to obtain the dynamics of the system. The vector of steady states is given by $F(\mathbf{y_\infty}) = \mathbf{0}$, which serves to calculate the asymptotic behavior of the system through the eigenvalues of its Jacobian matrix $\nabla F(\mathbf{y_\infty})$. The behavior can change significantly by modifying pivotal parameters of the system. Thus, the construction of bifurcation diagrams is a useful tool for evaluating the behavior regimes under different conditions, and also to build up a sensitivity analysis of the parameters of the system.

We show that the trivial solution of the system (i.e., silenced virus) is stable. The Jacobian matrix evaluated at $\mathbf{y_\infty} = \mathbf{0}$ is given by

$$\nabla F(0) = \begin{pmatrix} -\kappa_S & 0 & 0 & \beta & 0 & 0 & 0 & \beta & 0 & 0 \\ 0 & -\kappa_S & 0 & \beta & 0 & 0 & 0 & 0 & 0 & 0 \\ \mu' & 0 & -\kappa_P & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & -\delta'' & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & n\delta' & -\rho'' & 0 & 0 & n^*\delta' & 0 & 0 \\ 0 & 0 & 0 & 0 & \rho' & -\kappa_P & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & -\alpha'' & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & \alpha' & -\delta'' & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -\kappa_P & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -\kappa_P \end{pmatrix}, \tag{3.1}$$

where $\alpha' = \alpha Y/K_P$, $\delta' = \delta C_0/K_D$, $\rho' = \rho R_0/K_I$, $\mu' = \mu Z/K_Z$, $\alpha'' = \alpha' + \kappa_S$, $\delta'' = \delta' + \beta + \kappa_D$, and $\rho'' = \rho' + \kappa_I$. This Jacobian has five negative real eigenvalues ($-\alpha''$, $-\rho''$, $-\delta''$, $-\kappa_S$,

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and $-\kappa_P$) that represent an asymptotically stable solution of the system. Three of them have multiplicity greater than one. The system also has a second non-trivial solution in which the virus beats the silencing response and replicates and accumulates in the cell. Although we have verified numerically the existence of this non-trivial solution on the full model, without lost of generality, the stability analysis for this second solution can be done analytically by simplifying the system (2.11) as

$$\frac{dS}{dt} = 2\beta D - \alpha S^2 - \upsilon R^* S - \kappa_S S,$$

$$\frac{dD}{dt} = \alpha S^2 - \delta D - \beta D, \qquad (3.2)$$

$$\frac{dR^*}{dt} = n\delta D - \upsilon R^* S - \kappa_P R^*,$$

where the non-trivial steady state is the solution of $S(\beta - \delta)/(\beta + \delta) = \kappa_S + n\delta\alpha\upsilon S^2/(\beta + \delta)(\upsilon S + \kappa_P)$. The characteristic polynomial is $-X^3 + \tau X^2 - \eta X - \Delta$, where $\tau = -2\alpha S - \upsilon R - \kappa_S - \beta - \delta - \upsilon S - \kappa_P$ is the trace of the Jacobian matrix, $\eta = (2\alpha S + \upsilon R + \kappa_S)(\beta + \delta + \upsilon S + \kappa_P) + (\upsilon S + \kappa_P)(\beta + \delta) - 4\alpha\beta S - \upsilon^2 RS$ is the trace of its adjoint matrix, and $\Delta = \left[4\alpha\beta S - (2\alpha S + \upsilon R + \kappa_S)(\beta + \delta)\right](\upsilon S + \kappa_P) - 2n\alpha\delta\upsilon S^2 + \upsilon^2 RS(\beta + \delta)$ its determinant. By applying the Routh-Hurwitz stability criterion, the system will be stable when $\tau < 0$, $\Delta < 0$ and $\eta\tau < \Delta$. Henceforth, by taking the appropriate kinetic parameters that meet these three conditions, the system is characterized by bistability and, therefore, the initial condition is pivotal to determine the outcome of the process.

## 4. RESULTS

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### 4.1. Virus replication in the absence of a VSR

We have studied the viral replication dynamics by using the mathematical model presented in the previous section. First, we considered the case of RNA viruses that do not encode suppressor proteins. In Fig. 2 we show several time course evolutions of the system species ($S^+$, $S^-$ and $P$) for two different sets of initial conditions. When the multiplicity of infection is low (one single viral $S^+$ genome per cell) and for the typical parameter values shown in Table 1, we show that the population is extinguished (Fig. 2a), after a transient where the concentration of $P$ reaches a maximum. The model predicts that in this situation, the amount of antigenomic strains $S^-$ produced is meaningless and its dynamics is dominated by the degradation term in the system of equations 2.11.

However, the virus can bypass the silencing mechanism if the multiplicity of infection just increases to $S^+ = 10$ molecules (Fig. 2b). In this case, after a latency period of about 1 day, viral proteins reach a critical concentration and promote further exponential replication. Analytically, the latency period can be estimated when $P$ reaches $\omega K_P$. In all these simulations the condition $S^+ > S^-$ holds, in excellent agreement with the observation of an excess of sense siRNAs for positive-sense viral genomes (Qi *et al*. 2010). The effect of further increasing the multiplicity of infection is to reduce the latency period (data not shown).

We performed several sensitivity analyses to study the regions in parameter space in which viral replication occurs (non-trivial solution) or for which viral silencing takes place (trivial solution). We found that the higher the affinity for the negative strand (lower $\omega$), the wider is the parameter space for viral replication (Fig. 3a). In fact, this can be rationalized because viral RdRps compete with ribosomes and with the activated RISC for

genomic strands, whereas they do not compete for antigenomic strands. In addition, high replication rates also allow the virus to escape from the silencing machinery and to minimize the effect of non-specific thermodynamic degradation (Fig. 3b).

One question that arises here is whether a tradeoff between replication and translation exists. Upon uncoating and the strictly necessary first event of translation, a viral genome can either be directed to transcription, and thus increase the concentration of RNA, or to translation, and thus increase the concentration of viral proteins (in this case only replicase and coat). In Fig. 3c we analyzed such tradeoff by considering the binding affinities to positive strands of replicase ($K_P$) and ribosomes ($K_Z$). We showed that in the absence of a silencing suppressor, silencing is the outcome favored when translation is more frequent than transcription ($K_P < K_Z$). Accordingly, the best strategy for a virus to bypass the RNA silencing response in the absence of a suppressor protein would be to increase the affinity of its RNA to the replicase rather than to optimize its binding affinity to the ribosomes. Likewise, by increasing its transcription efficiency, a virus will produce more copies of its genome up to the point in which the cleavage by DICER would no longer control the accumulation of viral genomes. Fig. 3d shows, as expected, that the higher are the catalytic constants for transcription and translation, the higher are the chances for a successful viral replication.

## 4.2. Virus replication dynamics in presence of a VSR that acts on DICER

Many, if not all, viruses encode proteins able of interacting with the cell molecular machinery. The suppression mechanism is often a protein-protein or RNA-protein interaction resulting in a sequestration or blockage of one of the many molecules involved

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in the silencing pathway that allows the virus to escape from silencing surveillance. Our general model can be used to analyze and study the effect of various suppressors encoded by different viruses. To analyze the effect of a suppressor, we considered the virus replication speed as a characteristic scoring function. This speed can be easily computed as the inverse of the time taken to produce mature virions ($TV$). In Fig. 4 we plot $1/TV$ versus $K_Z$ and $K_P$ for the case of a VSR operating over DICER. We found that such a suppressor enhances the speed of virus accumulation with respect to a virus without encoding a VSR.

To further analyze the suppressor strategy of manipulating DICER, we constructed a phase diagram between the catalytic constant of cleavage by DICER ($\delta$) and the suppressor binding constant ($\Gamma_C$) (Fig. 5a). We found that the effect of the suppressor is only significant beyond a threshold level of $\Gamma_C$ (in this case 7000 molecules). In other words, if the affinity of the suppressor is not high enough, it only represents a cost for the virus because it cannot help for its replication. Fig. 5b shows the effect that the binding affinity of the suppressor for DICER has on the time necessary for completing a virion as a function of the cellular amounts of DICER ($C_0$). For low amounts of DICER, $TV$ is insensitive to variation in $\Gamma_C$. In addition, an increase in the number of DICER molecules per cell does not have any effect on $TV$ for suppressors with weak affinity. However, if $\Gamma_C$ increases (moving rightwards in the ordinates axis in Fig. 5b), then the time to produce virions significantly grows up and becomes infinity (indicating viral silencing) for high amounts of DICER molecules present in the cell at the time of infection.

## 4.3. The effect of suppressing downstream steps of the silencing pathway

Next, we sought for the effect of VSRs operating downstream in the silencing pathway.

Surprisingly, we found that suppressors affecting at other levels of the pathway (e.g. sequestering siRNAs, interfering with RISC or with RDR6) did not enlarge the parameter space in which the virus successfully replicates within a single cell (data not shown). This result suggests that only by suppressing DICER, the first bottleneck to replication imposed by the system, viruses could widen the parameter region resulting in successful replication. Hence, the question is why do other types of VSRs, such as siRNA sequesters, have evolved? Our negative result suggests that the RNA silencing mode of action cannot be rationalized by only looking into a single cell but that a more complex situation in which cell-to-cell effects may contribute should be considered. This leads us to consider the role of the space to analyze such mechanism.

In Fig. 6a we plot the relative amount of accumulated siRNA (normalized by the amount or siRNA produced in the absence of a viral suppressor of silencing, $I/I_{r \to \infty}$ in presence of two suppressors strategies. For illustrative purposes, we have chosen the successful operation over DICER described in the previous section and one based on sequestering siRNAs. By increasing the affinity for the corresponding target molecule (moving rightwards on the ordinates axis) to the maximum value analyzed, the strategy based on blocking DICER reduces the concentration of siRNA around two orders of magnitude. However, the strategy based on sequestering siRNAs is far less efficient since at the strongest affinity it only reduces the accumulation of virus-derived siRNAs by one order of magnitude.

However, the transfer of siRNAs from infected to neighboring healthy cells, which allows the peripheral cells to activate RISC in the absence of viral infection, has the expected effect (Fig. 6b). In the absence of triggering siRNAs, infection progresses with

the time delay already described above. However, if the cell has been already activated, the

virus is not able of overcoming the cleavage by RISC and runs into extinction.


## 5. DISCUSSION

We have presented a deterministic model of the interplay between viral replication and the

RNA silencing pathway. For the sake of biological realism, we modeled a particular type

of viruses, the picorna-like. By doing so, the model pays the cost of reduced generality and

the conclusions may not be applicable to viruses with other genomic architectures such as

negative sense RNA, retroviruses or DNA viruses. Although our results have been

performed for positive sense RNA viruses, the model can also be used to study negative

sense viruses with minor changes in some rates and the initial conditions. Readers

interested in exploring the interplay between the silencing pathway and any of these viruses

must necessarily look this article as the starting point for developing their own models.

Nonetheless, our approximation has allowed us to study and compare different viral

suppression strategies. We have shown that the RNA silencing pathway allows a large

variety of behaviors, suggesting multiple potential evolutionary trajectories for RNA

viruses. Future models will account for different viral genomic organizations and for

inherent stochastic effects associated to small numbers of molecules (Gillespie 1977). The

model here presented differs from other models of the interaction between virus and hosts

silencing response (Groenenboom & Hogeweg 2008a; Groenenboom & Hogeweg 2008b)

in which here we have explored the role played by different suppressors of RNA silencing.

We have demonstrated and shown in Fig. 2 that the system has two stable steady states

(replication and silencing) and, thus, the initial condition of the system (i.e., the initial

amount of ssRNA in the cell) is important to determine its dynamics. Likewise, the higher is the initial amount of the viral RNA, the higher is the zone for exponential viral replication in the parameter space. This suggests that increasing the multiplicity of infection is a possible strategy for virus to escape from the control of RNA silencing.

We have shown that in the presence of an active silencing response, it is in the benefit of the virus to invest into a transcriptional strategy rather than in translation. This may be somehow counterintuitive because one may expect more replication to generate more dsRNA and, therefore, to strength the silencing response and, likewise, more translation to produce more suppressor protein. It can be argued that, after the very initial burst of translation from the infecting genomic sequence resulting in a few viral proteins, the optimal strategy involves synthesizing antigenomic strands and use them as templates for producing a large excess of genomic strands (i.e., using an stamping machine replication strategy) without diverting them into translation. If replication is fast enough, this replicative strategy works even in the absence of a suppressor protein: a positive feedback is established such as the replication overcomes the capacity of the available DICER molecules to keep virus replication under control. Once a significant amount of genomic strands has been produced, then translation may take place. If translation results in a VSR protein, then a synergistic effect between fast transcription and translation appears, resulting in a successful viral replication.

Among many possibilities, we have focused in three viral strategies. The first one, consisting of blocking DICER, turns out to be the most efficient promoting viral replication. This result is somehow logical from an optimal design perspective. By hitting the first bottleneck in the pathway the virus ensures its own replication. Hitting

downstream steps would allow DICER to still exert partial control on virus replication. The other three strategies explored, sequestering siRNA, blocking RISC and disrupting the secondary amplification via RDR6, have resulted less efficient in promoting intracellular virus accumulation, although they may gain some benefit when looking at cell-to-cell movement. This finding is in good agreement with the observation that cucumovirus 2b and tombusvirus P19, which promote systemic and cell-to-cell movements, are not required for intracellular accumulation (Li & Ding 2006).

Although mathematically convenient, it may be a biological oversimplification to assume that suppressors act at a single stage of the silencing pathway. Evidences exist showing that VSRs may well simultaneously operate at diverse stages of the pathway. For example, the potyviral HC-Pro sequesters siRNAs but also affects the expression of plant genes, including the *dcl*-like genes encoding for the different DICER proteins in *Arabidopsis thaliana* (Deleris *et al*. 2006), or by reducing the 3' methylation of siRNAs making them sensitive to oligouridilation and subsequent degradation (Ebhardt *et al*. 2005; Mérai *et al*. 2006). Another example of multiple actions is the polerovirus P0 that interferes with the silencing pathway at least at two levels: binding siRNAs and avoiding the formation of the activated AGO complex and labeling it for degradation (Baumberger *et al*. 2007; Csorba *et al.* 2010). Also, a virus may also carry more than one VSR, as it seems to be the case for some tombusviruses (P19 and CP).

We have also found that in certain regions of parameter space, a virus would be able of replicating even in the absence of a VSR. The plant subviral pathogens known as viroids do not encode for any protein at all and are still able of replication in susceptible hosts (Daròs *et al*. 2006), despite the fact that their RNA molecules are targets of DICER (Di

Serio *et al*. 2009).  It has been suggested that viroids may evade silencing because their highly complex and packed secondary structure (Wang *et al*. 2004; Gómez & Pallás 2007). Other strategies viruses may use for avoiding silencing consist in replicating within spherules in the endoplasmic reticulum membrane (Schwartz *et al.*, 2002), where they remain inaccessible to DICER.

Although we have modeled the effect of VSRs on DICER as a direct protein-protein interaction, VSRs can also interfere with DICER activity by protecting the dsRNA as it is produced.  This particular activity would be easily incorporated into our mathematical framework in two simple ways.  First, by treating the binding affinity of DICER for dsRNA ($K_D$) as a decreasing function of CP concentration.  Second, by defining a new molecular species for the complex dsRNA/CP and writing down the corresponding rates and adding a new differential equation in (2.11).

In conclusion, we have shown that from a system design perspective, the best strategy that a virus may take to ensure its replication in presence of the antiviral response mediate by RNA silencing would be to (*i*) replicate fast and by producing an excess of genomic strands, (*ii*) encode for a VSR that interacts with the DICER protein and (*iii*) exert some control on the multiplicity of infection, ensuring that multiple genomes infect each cell. Obviously evolution is not a perfect designer and viruses have acquired suppressor proteins that target at different steps of the silencing pathway.  Understanding the exact mechanisms by which these VSRs operate will allow to develop better models and to increase our ability to predict the outcome of the virus-host interaction.  Furthermore, VSRs have clear biotechnological potential as they can be used to maximize the expression of transgenes (Li & Ding 2006).  Designing optimal suppressors would benefit from the knowledge advanced

in this article.

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**Figure 1.** Schematic representation of the RNA silencing pathway and its interaction with viral replication. RNA viruses encode for replicase, suppressors of silencing (VSR) and coat proteins. Three types of suppressors are considered in the scheme: suppressors of DICER (I), sequesters of siRNA (II), and suppressors of RISC (III).
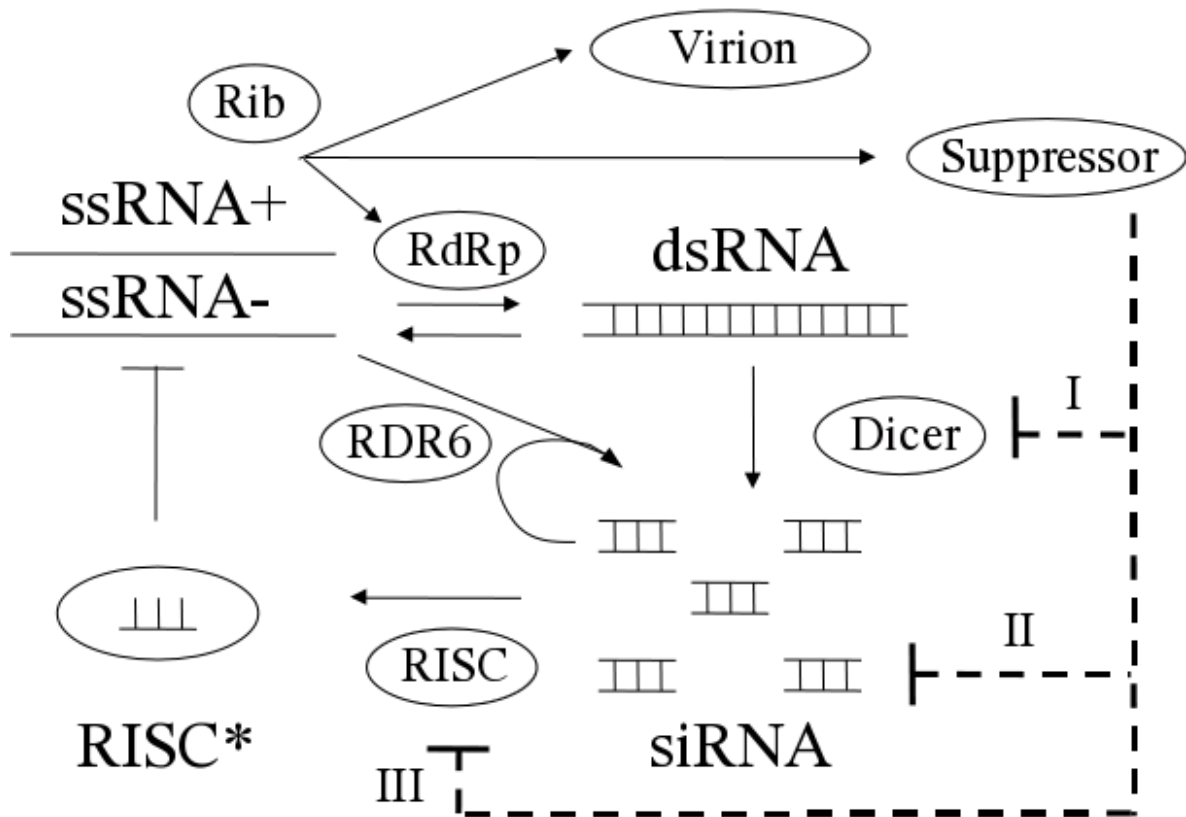
**Figure 2.** Dynamics of viral infection for different initial conditions. In (a) the starting condition of the simulation is a single viral genome; this results in the virus being silenced. In (b) the starting condition is that 10 viral genomes infect the cell; this high multiplicity of infection results in exponential viral replication after a period of latency of 1 day required to reach a threshold level of RdRp. This successful infection happens even in the absence of a VSR. The parameters values are those shown in Table 1.
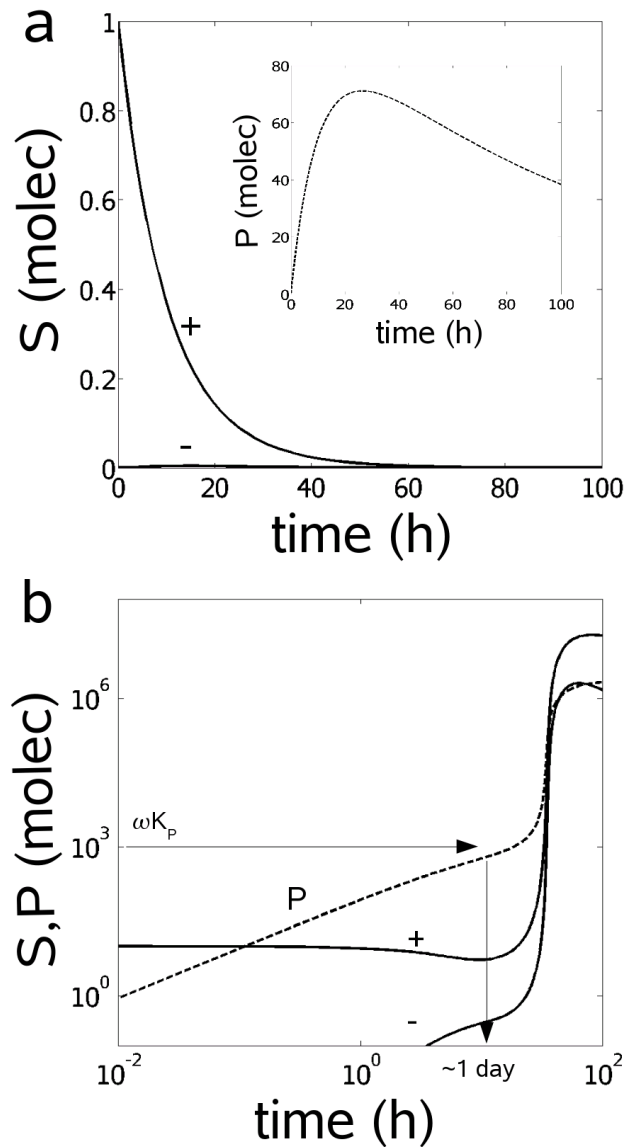
**Figure 3.** Phase diagrams identify different viral strategies. Diagram (a) illustrates the effect of the catalytic constant of DICER cleavage ($\delta$) in the replication rate ($\alpha$) and the differential affinity of RdRp for positive and negative strands ($\omega$). Diagram (b) illustrates the relationship between $\alpha$ and the ssRNA degradation rate ($\kappa_S$), for different values of the translation rate ($\mu$). Diagram (c) shows the sensitivity of the binding constants of ribosomes ($K_Z$) and replicases ($K_P$) to $\alpha$. Diagram (d) illustrates the effect of $K_P$ on $\mu$ and
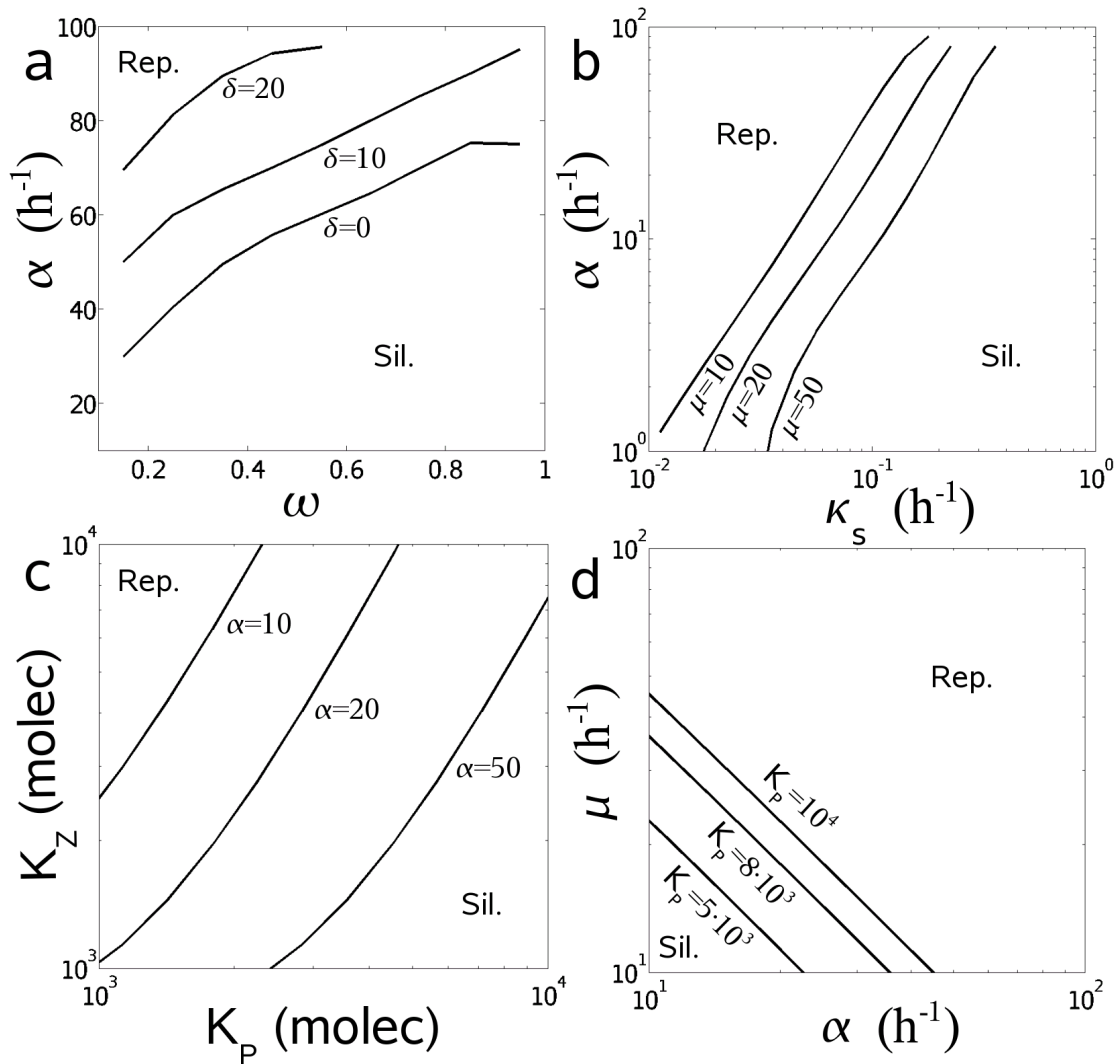
$\alpha$. *Rep* means viral replication bypassing silencing, and *Sil* viral extinction by silencing.

**Figure 4.** Virus replication speed (computed as the inverse of the time to form a mature virion, $TV$) versus the binding constants of ribosomes ($K_Z$) and replicases ($K_P$), with $\Gamma_C = 10^2$ molecules. (a) Virus without a VSR. (b) Virus encoding a suppressor that blocks DICER. The benefit associated with carrying such a suppressor is evaluated as the difference between both surfaces and is indicated by the dashed line and the arrow. The other parameters take the values shown in Table 1.
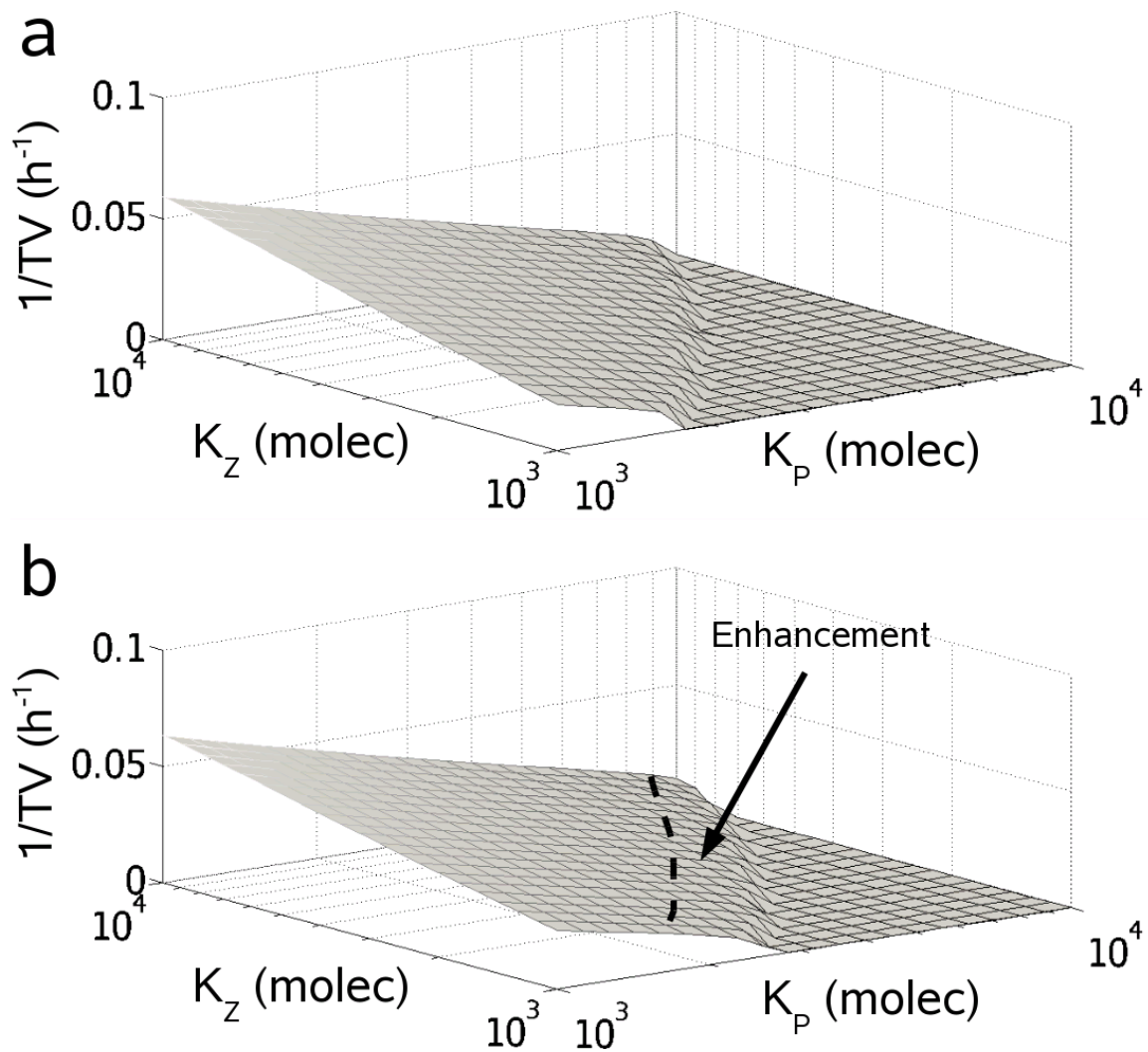
**Figure 5.** (a) Phase diagram to analyze the suppressor effect on DICER between $\delta$ and $\Gamma_C$, with $\alpha = 20$ h$^{-1}$ for different values of $\mu$. (b) Time to form one virion (*TV*) versus the suppressor constant of DICER, with $\alpha = \mu = 20$ h$^{-1}$ for different values of $C_0$ (in molecules). The other parameters take the values shown in Table 1. *Rep* means viral replication bypassing silencing, and *Sil* viral extinction by silencing.
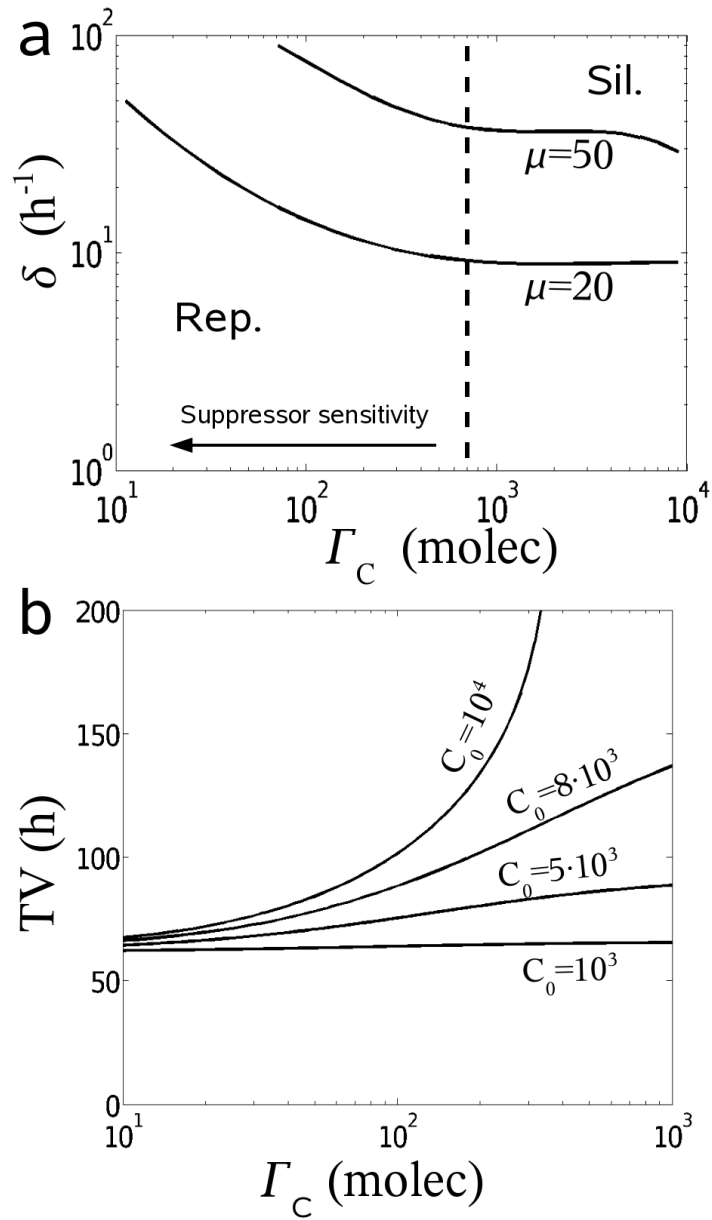
**Figure 6.** (a) Amount of siRNA (relative to the amount accumulated without a viral suppressor of RNA silencing) versus the suppressor constant ($\Gamma$) on DICER or siRNA. (b) Viral RNA dynamics in a cell which has not been immunized by receiving siRNA from neighboring cells ($R*(0) = 0$) and in a cell that has received a small input of siRNA from an infected neighbor cell ($R*(0) = 10$ molecules). The parameters take the values shown in Table 1, expect $\alpha = 50$ h$^{-1}$.
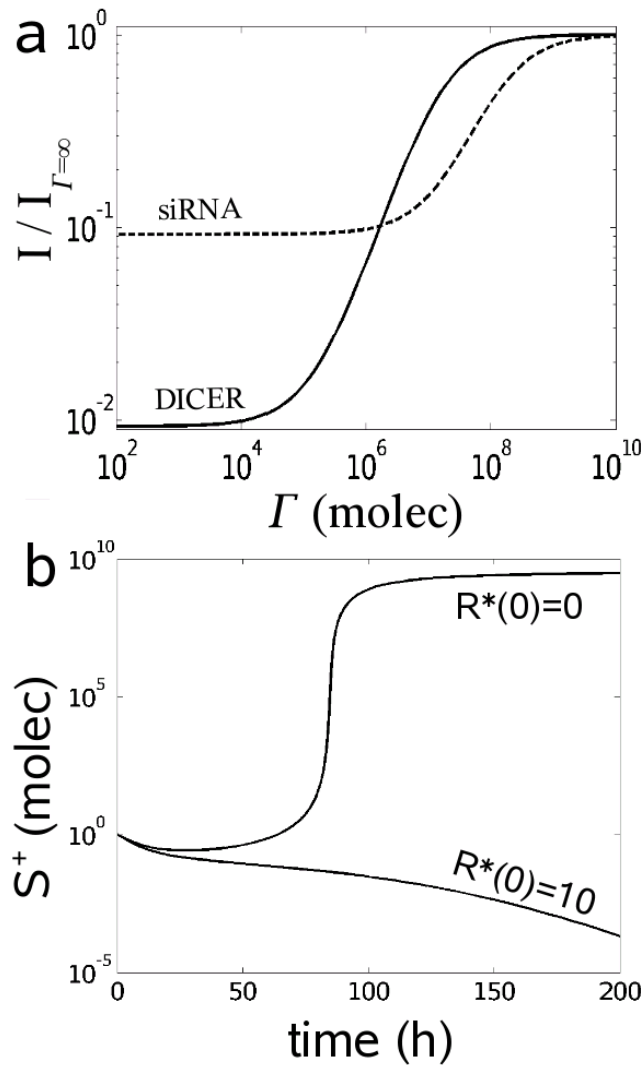
**Table 1.** Values for the kinetic parameters used in the model. Other non-kinetic model parameters are $p = q = 0.4$, $\omega = 0.1$, $n = 2n^* = 10$, $f = 0.01$, $\sigma = 0.1$, and $k = 10k_0 = 30$. The amounts of cellular resources are $Z = 10^5$, $Y = 10^5$, $C_0 = 10^4$, and $R_0 = 10^4$ molecules. In case of a virus encoding a VSR, the corresponding binding constant ($\Gamma_C$, $\Gamma_I$ or $\Gamma_R$) takes the value of $\Gamma$. The cell volume is assumed $\sim 10^{-13}$ L, then 1 nM $\sim$ 100 molecules.

| Parameter | Value | Value in the literature |
|---|---|---|
| $\alpha$ | 10 h$^{-1}$ | 1.7 h$^{-1}$ for HCV (Dahari *et al*. 2007) |
| $\mu$ | 10 h$^{-1}$ | 10 h$^{-1}$ for HCV (Dahari *et al*. 2007) |
| $\beta$ | 10 h$^{-1}$ | 10 h$^{-1}$ (Groenenboom & Hogeweg 2008) |
| $\delta$ | 10 h$^{-1}$ | 228 h$^{-1}$ *in vitro* for *E. coli* (Sun *et al*. 2001) |
| $\rho$ | 1 h$^{-1}$ | ~5 h$^{-1}$ (Groenenboom & Hogeweg 2008) |
| $\upsilon$ | 25 h$^{-1}$ | 25 h$^{-1}$ *in vitro* for *D. melanogaster* (Haley & Zamore 2004) |
| $\lambda$ | 0.1 h$^{-1}$ | ~100 M$^{-1}$h$^{-1}$ for nucleation (Endres & Zlotnick 2002) |
| $\gamma$ | 10 h$^{-1}$ | ~$10^5$ M$^{-1}$h$^{-1}$ for elongation (Endres & Zlotnick 2002) |
| $\kappa_S$ | 0.1 h$^{-1}$ | 0.5 h$^{-1}$ (Groenenboom & Hogeweg 2008) |
| $\kappa_I$ | 1 h$^{-1}$ | 2 h$^{-1}$ (Groenenboom & Hogeweg 2008) |
| $\kappa_D$ | 0.01 h$^{-1}$ | 0.06 h$^{-1}$ for HCV (Dahari *et al*. 2007) |
| $\kappa_P$ | 0.01 h$^{-1}$ | 0.01 h$^{-1}$ (Rana 2007) |
| $K_P$ | $10^4$ molecules | 225 nM *in vitro* for TBSV (Rajendran & Nagy 2006) |
| $K_R$ | $10^3$ molecules | 8 nM *in vitro* for *D. melanogaster* (Haley & Zamore 2004) |
| $K_Z$ | $10^4$ molecules | 260 nM *in vitro* (Scheper *et al*. 2002) |
| $K_C$ | $10^5$ molecules | 3780 nM (Endres & Zlotnick 2002) |
| $K_D$ | $10^5$ molecules | 335 nM *in vitro* for *E. coli* (Sun *et al*. 2001) |
| $K_I$ | $10^5$ molecules | ~1000 molecules (Groenenboom & Hogeweg 2008) |
| $\Gamma$ | $10^3$ molecules | 10 – 1000 nM *in vitro* p19, p21 and HC-Pro (Lakatos *et al*. 2006) |