A quantitative analysis of complementation of deleterious mutants in plant virus populations

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Abstract

Complementation can be defined as the process by which the function affected by a mutation is provided in *trans* by fully competent genotypes. Complementation can, thus, counter the effects of selection of deleterious mutants. Complementation of mutants defective for replication, movement and transmission has been often described in experiments with viruses and, occasionally, has been reported to occur in their natural populations. However, the role of complementation in virus evolution has been overlooked. Here is provided a quantitative estimate of the efficiency of complementation, defined as the probability that a non-functional mutant accomplishes a function relative to a functional one. For this, the frequency of mutants of *Tobacco mosaic virus* (TMV) defective for cell to cell movement was estimated in wild type tobacco plants and in transgenic plants expressing the TMV movement protein (MP) from a transgene. Mutants lethal for cell-to-cell movement were complemented by wild-type TMV in the first case, and by the transgene-expressed MP in the second case. Assuming that complementation is fully efficient in the transgenic plants, a value for the efficiency of complementation of 0.34 was obtained. Thus, complementation can efficiently counter selection on lethal mutants, and may have an important role on virus evolution. Complementation may be relevant for management of viral diseases if the complemented deleterious mutation is linked to other functions affecting the pathogenicity or epidemiology of the virus.

Additional key words: mutant complementation, selection, *Tobacco mosaic virus*, virus evolution.

Resumen

Análisis cuantitativo de la complementación de mutantes deletéreos en poblaciones de virus vegetales

La complementación puede definirse como el proceso por el cual una función afectada por una mutación es provista en *trans* por genotipos competentes para dicha función. La complementación de mutantes defectivos para replicación, movimiento o transmisión se ha descrito con frecuencia en poblaciones experimentales de virus y, ocasionalmente, en poblaciones de campo. Sin embargo, el papel de la complementación en la evolución de los virus no se ha considerado. En este trabajo presentamos una estima de la eficacia de la complementación, definida como la probabilidad de que un mutante no funcional realice la función respecto a un genotipo funcional. Para ello se estimó la frecuencia de mutantes del virus del mosaico del tabaco (TMV) defectivos para el movimiento de célula a célula en plantas silvestres de tabaco y en plantas transgénicas que expresan constitutivamente la proteína del movimiento (MP) de TMV. En el primer caso, los mutantes letales para movimiento serán complementados por genotipos silvestres de TMV, y en el segundo, por la MP transgénica. Suponiendo que la complementación en las plantas transgénicas es totalmente eficaz, se obtuvo un valor de la eficacia de complementación de 0,34. Esto muestra que la complementación puede contrarrestar eficazmente el efecto de la selección sobre mutantes deletéreos y tener un papel importante en la evolución de virus, con importantes implicaciones prácticas si la mutación deletérea complementada está ligada a otras funciones que afecten a la patogenicidad o epidemiología del virus

Palabras clave adicionales: complementación de mutantes, evolución de virus, selección, virus del mosaico del tabaco.

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Introduction[1]

Viruses are important pathogens with a deep socio-economic impact, as viral diseases may severely affect the welfare of people and of domestic animals and plants. In addition, viruses may play an important role in shaping the population dynamics and structure of their host species and, hence, in ecosystem composition and dynamics (Dobson and Hudson, 1986; Mitchell and Power, 2003; Hudson *et al.*, 2006). The genetic structure of virus populations may change with time, what may result in changes of the impact of virus infection on host populations, and on the success of management strategies of viral diseases. Thus the interest in virus evolution, which has resulted in an important body of literature in the past 25 years, and in the publication of frequent reviews and several books on the subject (e.g. Gibbs *et al.*, 1995; Domingo *et al.*, 1999; Roossinck, 2008).

Selection is most often invoked as the evolutionary force shaping viral populations. Selection is a directional process by which genotypes that are fittest in a certain environment will increase their frequency in the population (positive selection) whereas less fit genotypes will decrease their frequency (negative or purifying selection). It is widely recognised that the effects of selection on viral populations can be tempered by random genetic drift associated to small population sizes, e.g. during host colonisation or between host transmission events. Less attention has received the role of complementation of deleterious mutants in countering the effects of selection and in shaping the genetic structure of virus populations.

We define here complementation as the process by which the function affected by a mutation is provided in *trans* by fully competent genotypes in multiple-infected cells. This process should be particularly important for RNA viruses, which will generate a large number of mutants sharing the cell environment with the competent parental variant. Complementation could result, for instance, in the maintenance of more virulent mutants even if they are less fit that the wild-type, what could have important consequences in pathology at large. Complementation of replication, within-host colonisation and transmission has been often described in experiments, but its role in virus evolution has been overlooked. An obvious case of complementation is the maintenance of satellite viruses and satellite nucleic acids, or of defective RNAs, by helper viruses (Roux *et al.*, 1991; Roux, 1999; Simon *et al.*, 2004).

To quantify the efficiency of complementation in an RNA virus *Tobacco mosaic virus* was used as a model system. The analysis is based on the comparison of complementation of cell-to-cell movement of mutants lethal for this function by either wild type genotypes or by the wild-type movement protein provided in *trans* by expression from a transgene. Data indicate that the efficiency of complementation may be up to 0.34, what stresses the importance of this process for virus evolution.

Material and Methods

Virus and plants

TMV was derived from a biologically active cDNA clone (Dawson *et al.,* 1986), the gift of W. O. Dawson (Department of Plant Pathology, University of Florida, FL). RNA was transcribed by T7 RNA polymerase (Dawson *et al.,* 1986) and used to inoculate tobacco (*Nicotiana tabacum* L.) plants of four different genotypes: i) plants of cv. Xanthi-nn (*nn* plants), which are systemically infected by TMV; ii) plants of the nearly isogenic line Xanthi-NN (*NN* plants), which carry the hypersensitive-resistance gene *N;* TMV infection in *NN* plants is limited to necrotic local lesions (nll) around primary infection sites on the inoculated leaf; iii) plants of line 277 (Deom *et al.,* 1987) of Xanthi-nn plants (*nn-MP* plants); and iv) plants of line 2005 (Deom *et al.,* 1991) of Xanthi-NN plants (*NN-MP* plants). Both *nn-MP* plants and *NN-MP* plants constitutively produce the virus movement protein (MP) from a transgene. These plants were the gift of R. N. Beachy (International Laboratory for Tropical Agricultural Biotechnology, St. Louis, MO). Note that nll permit ready cloning and quantification of infectious virus particles in a manner similar to plaque formation by lytic animal viruses and bacteriophages.

Experimental procedure

The experimental procedure is described in detail in Malpica *et al.* (2002) and is summarised in Figure 1.

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[1] Abbreviations used: DIP (defective-interfering particles), HIV (Human immunodeficiency virus), MP (movement protein), nll (necrotic local lesions), SMV (*Soybean mosaic virus*), TAV (*Tomato aspermy virus*), TMV (*Tobacco mosaic virus*).
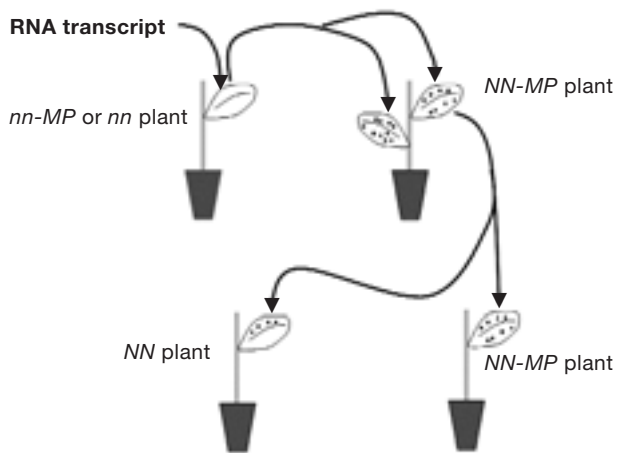
RNA transcript

nn-MP or *nn* plant

NN-MP plant

NN plant

NN-MP plant

Figure 1. Outline of the experimental procedure used to estimate the frequency of TMV mutants defective in cell-to-cell movement. Circles in leaves represent necrotic local lesions. *nn* plants are systemic hosts for TMV, *NN* plants produce necrotic local lesions upon TMV infection. *MP* denotes the presence of a transgene from which TMV movement protein is constitutively expressed.

Briefly, RNA transcribed from the wild-type TMV cDNA clone was suspended in 0.1 M Na_2HPO_4 and inoculated by gently rubbing the upper epidermis of the interveinal spaces of fully expanded leaves of *nn-MP* plants. The infectivity of the inoculum was simultaneously quantified by nll assay (Matthews, 1970, pp. 12-16) on *NN* plants. Three days after inoculation, 23.5 mg of fresh tissue from the inoculated leaf were ground in 6 mL of 0.1 M Na_2HPO_4, and this extract was used to determine the virus yield ($N - N_0$, where N is the size of virus population after multiplication in the *nn-MP* leaf and N_0 is the initial infective input) by nll assay in *NN* plants. The rest was used to inoculate fully expanded leaves of *NN-MP* plants. When nll appeared in these plants, lesions were harvested individually and each lesion was ground in 30 μL of 0.1 M Na_2HPO_4 and used to inoculate leaves of both *NN* and *NN-MP* plants. If the TMV clone that caused the initial nll on the *NN-MP* plant was competent for cell-to-cell movement, new nll formed in leaves of both *NN* and *NN-MP* plants. If the TMV clone had a mutation that impaired cell-to-cell movement, nll formed in the leaves of *NN-MP* plants but not *NN* plants. Although less than 2% of transferred lesions (65 of a total of 3,400) failed to infect *NN-MP* plants, the mutant nature of clones infecting *NN-MP* plants but not *NN* plants was confirmed by transferring 5 necrotic local lesions to new leaves of both tobacco genotypes. Thus, this procedure detects mutants that are lethal because of impairment

of cell-to-cell movement. This experimental procedure was repeated several times, and in each case, a parallel control experiment was done in which the initial multiplication of the cDNA-derived clone was in leaves of *nn* plants.

Results

A total of 1,820 clones (local lesions) derived from the initial multiplication of TMV RNA in *nn-MP* plants were transferred to *NN* and *NN-M*P plants. Of these, 1,777 induced nll in both plant lines, while 43 induced nll only in *NN-MP* plants (Table 1). Thus, 43 out of 1,820 clones carried mutations lethal for cell-to-cell movement and the frequency of such mutants was $f_{+MP} = 0.0236$. Similarly, a total of 1,515 local lesions derived from virus multiplication in *nn* plants were transferred to *NN* and *NN-MP* plants. Of these, 12 were lethals for cell-to-cell movement (Table 1), resulting in a frequency $f_{-MP} = 0.0079$. The value of f_{+MP} was significantly larger than that of f_{-MP} ($P = 0.001$ in a Fisher exact test). These data show that a mutant defective in cell-to-cell movement can nevertheless move if complemented in trans by a functional MP derived from a transgene (in *nn-MP* plants) or from a non-mutant genome with which it shares the same intracellular environment (in *nn* plants), albeit with different probability.

We define the efficiency of complementation, *C*, as the probability that a non-functional mutant accomplishes a function (cell-to-cell movement in the present case) relative to a functional one. Thus, from the data in Table 1, and taking *trans*-complementation of cell-to-cell movement in *nn-MP* plants as fully efficient (i.e., $C = 1$ in *nn-MP* plants), $C = f_{-MP} / f_{+MP} = 0.335$ (95% confidence interval 0.395-0.352).

Table 1. Frequency of TMV mutants defective in cell-to-cell movement in tobacco plants

Host plant[a]	No. of clones analysed	No. of mutant clones	Frequency of lethals[b]
nn-MP	1,820	43	0.0236 (0.035-0.019)
nn	1,515	12	0.0079 (0.015-0.0045)

[a] Type of plant in which the initial virus multiplication occurred: *nn-MP* = Xanthi tobacco plants expressing the TMV movement protein from a *MP* transgene. *nn* = wild-type Xanthi tobacco plants. [b] Values between parenthesis indicate 95% confidence intervals.

In this estimate it is assumed that no more than one virus particle initiates a focus of infection. This is a realistic assumption for an inoculum dose resulting in about 30 foci per inoculated leaf (see data in Malpica *et al.,* 2002) because, from the infectivity-dilution curve of TMV it is highly improbable that more than one virus particle initiates a focus (Furumoto and Mickey, 1967). Possible differences in fitness between mutant and wild-type clones were not considered because, except for cell-to-cell movement, such differences will be equivalent in *nn* and *nn-MP* plants. Differences in the dynamics of TMV infection in *nn* and *nn-MP* plants were neither considered because both multiplication of the initial inoculum (from 30 to an average of 1.8×10^7 infectious units per inoculated leaf area), and the number of infection cycles (5.7 infection cycles) were similar in both host genotypes. Last, differences in the efficiency of complementation of different mutants were neither considered, as the mutational spectrum did not differ between cell-to-cell movement mutants obtained in *nn* and in *nn-MP* plants (see Malpica *et al.,* 2002, for details).

The above estimate of the efficiency of complementation is an upper threshold estimate as it assumes that *trans*-complementation of lethal mutants by the transgenic MP is fully efficient. This is not an unreasonable assumption, as the mutational spectrum described for these mutants includes big insertions and deletions (Malpica *et al.,* 2002) and it has been shown that TMV constructs in which the *MP* gene was deleted were complemented in *nn-MP* plants (Deom *et al.,* 1987). However, regardless that all possible mutants at the *MP* gene were complemented in *nn-MP* plants, their fitness could be smaller than that of the wild type virus. Indeed, a comparative assay of the dynamics of infection of wild-type TMV and of an engineered mutant with a frameshift in *MP* showed that the mutant accumulated in *nn-MP* plants at a rate ≈ 0.50 of that of the wild type. Thus, the efficiency of complementation of mutants defective for cell-to-cell movement in *nn-MP* plants could rather be $C = 0.50$. In this case, the efficiency of complementation in wild-type plants would be half of the previous estimate, i.e., $C = 0.168$, which is a lower threshold estimate.

Discussion

Complementation of replication, movement and transmission has been often described in experiments with viruses. Indeed, rescue of deleterious mutants by wild type genotypes has been a powerful instrument for the genetic analysis of virus functions in the past (Van Vloten-Doting and Bol, 1988; Raning, 1991). In spite of being widely documented, the role of complementation in virus evolution has been overlooked. Complementation requires that different viral genotypes share a cellular environment. It has been described that multiplicity of infection is bigger than one for bacterial and animal viruses (Horiuchi, 1975; Turner *et al*., 1999; Bull *et al.,* 2001), providing for this condition. Multiplicity of infection has not been estimated for any plant virus, the model system used in this work, but the occurrence of satellite virus and satellite nucleic acids associated to plant viruses as well as other commensalistic associations, which are much more frequent than for animal-infecting viruses (Gibbs *et al.,* 2007), suggest that multiplicity of infection should be large. Complementation could be particularly relevant for the biology of RNA viruses: even in the absence of co-infection of the same cell by different genotypes, the high mutation rates of RNA viruses (Drake and Holland, 1999; Malpica *et al.,* 2002; Furió *et al.,* 2005) would result in the sharing of the cell environment by the parental genotype and newly generated mutants. This is in fact the situation in our experiment, where inoculum dose was set for a high probability of infection foci being started by a single molecule of the wild-type genomic RNA, and mutants deficient for cell-to-cell movement would have been generated during its replication.

Few attempts have been made to quantify the effects of complementation. The most studied defective mutants complemented by a wild-type virus are defective-interfering particles (DIP) or defective-interfering RNAs. DIP of animal viruses have been studied in big detail in cell culture, and for the best analysed systems such as *Vesicular stomatitis virus* or *Polio virus*, and their DIP, the parameters of DIP generation and competition have been estimated (e.g. Cole and Baltimore, 1973; Horodyski *et al.,* 1983) and used in the building of sophisticated population models (Bangham and Kirkwood, 1990; Kirkwood and Bangham, 1994). However, no attempt was made, to our knowledge, to quantify the efficiency of complementation of DIP by helper viruses. Quantitative analyses of complementation have been reported for animal viruses in cell culture (e.g., Novella *et al.,* 2004; Wilke *et al.,* 2004; Perales *et al.,* 2007), but dynamics of infection in cell culture an in the multicellular animal or plant host could vary

largely. The few reported quantitative analyses of complementation in the multicellular host come all from the plant virus field. A *Tomato aspermy virus* (TAV) mutant lethal for cell-to-cell movement that replicated more efficiently than the wild-type in tobacco protoplasts was described by Moreno *et al.* (1997). The point mutation responsible for this phenotype was maintained at high frequency (0.76) in TAV populations repeatedly passaged in tobacco. An argument of steady-state equilibrium allowed to estimate a lower threshold for the efficiency of complementation for this mutant at 0.13, which would result in the equilibrium frequency of 0.76. Mansky *et al.* (1995) have described that in soybean cultivars carrying the resistance gene *Rsv* to *Soybean mosaic virus* (SMV), previous infection by a SMV capable of overcoming *Rsv* resistance complemented infection by a second SMV genotype to which these plants were otherwise immune. From their quantification of the frequency of this phenomenon it is possible to set a lower limit of 0.20 to the efficiency of complementation occurring in a field situation, rather than in experimental populations.

The estimate of the efficiency of complementation reported here derives from an *ad hoc* experiment that also allowed the estimation of TMV's spontaneous mutation rate (Malpica *et al.,* 2002). It is to be stressed that the present estimate, bounded between lower and upper thresholds, is well in agreement with the lower threshold estimates derived for mutants of other plant viruses that differ widely from TMV in their genomic structure and gene expression strategies, as TAV and SMV, and in which different functions were complemented. Thus, data consistently show high efficiency of complementation of defective mutants by plant viruses, both under experimental and field situation, which illustrates the potential importance of this phenomenon in virus evolution. Complementation can be particularly relevant for virus evolution if the deleterious mutation has a pleiotropic effect on other virus functions, as was the case for TAV above, or if it is linked to relevant phenotypes, for instance to increased pathogenicity to other hosts, as described for canine and feline parvoviruses (Shackelton *et al.,* 2005), or decreased susceptibility to antivirals, as described for HIV (Kuritzkes, 2001). The relevance of epistatic interactions in the small genomes of RNA viruses (Sanjuán *et al.,* 2004), and the multifunctionality of the proteins encoded by RNA viruses (e.g. García-Arenal *et al.,* 2001), suggest that this may be often the case. Also, complementation may result in

host range expansion and in modified tissue tropisms. These phenomena have been documented for plant viruses for a long time (e.g., Atabekov and Taliansky, 1990) and more recently for viruses infecting mammals (Vignuzzi *et al.,* 2006).

Complementation of deleterious mutants is evidence for coinfection in viruses. Selection in coinfection groups may be viewed as selection on diploid individuals albeit with a variable and statistically distributed ploidy, and the efficiency of complementation of deleterious mutants will be linked to the level of ploidy. The high efficiency of complementation in plant viruses strongly suggest a high value for their average ploidy.

Acknowledgements

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