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**Host-virus interactions: Effects on the evolution of tobacco
mosaic virus in *Nicotiana sylvestris***

Khan, Iqrar Ahmad, Ph.D.

University of California, Riverside, 1988

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UNIVERSITY OF CALIFORNIA
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Host-Virus Interactions: Effects on the Evolution
of Tobacco Mosaic Virus in Nicotiana sylvestris

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Botany

by

Iqrar Ahmad Khan

June, 1988

Dissertation Committee:

Professor Gary E. Jones, Chairperson

Professor Laverne F. Lippert

Professor William O. Dawson

The Dissertation of Iqrar Ahmad Khan is approved:

Wm O. Dawson
Laverne F. Lippert
Gary E. Jones
Committee Chairperson

University of California, Riverside
June, 1988

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Last, but not least are my wife Gulnaz and son Hilal, who suffered the most from the long ordeal of waiting while I had to work in the Laboratory.

DEDICATION

To my family and friends.

ABSTRACT OF THE DISSERTATION

Host-Virus Interactions: Effects on the Evolution
of Tobacco Mosaic Virus in Nicotiana sylvestris

by

Iqrar Ahmad Khan

Doctor of Philosophy, Graduate Program in Botany
University of California, Riverside, June, 1988.

Professor Gary E. Jones, Chairperson

For many years, it has been known that changes in plant RNA virus populations can result from replication in different host plants or in different environments, but the genetics underlying such changes could not be explored. Recent developments in the molecular biology of tobacco mosaic virus (TMV) make such studies possible.

A typical population of "wild type" TMV (U1) contains a small subpopulation of genetic variants (necrotic lesion-NL) that evoke the hypersensitive reaction on Nicotiana sylvestris, a systemic host for wild type. This variant is strongly selected for during replication in plants that are undergoing development of shoot axes during bolting. This

selection changes the composition of the virus population dramatically. No accumulation of NL-type occurred when N. sylvestris plants were inoculated with TMV204, a clonal population derived from RNA transcripts of a plasmid carrying a cDNA copy of the TMV genome or when U1 infected other systemic hosts.

Tissue cultures initiated from virus infected explants are stressed. The extent of stress varies with the age of infected explants. NL-type virus is also strongly selected for during the regeneration of plantlets from leaf disks of plants infected with TMV U1. When whole plantlets were transferred into a greenhouse, those containing NL variants were severely stunted, showed mosaic symptoms, and most of them died slowly. Some that had originally contained lower titers of NL-type virus survived to flower but produced only a few seeds. Another set of these plants contained only wild type and grew well. Repeated assays of virus in these plants before or after bolting revealed no detectable NL-type virus. Apparently, during callus development and organogenesis in culture, partial segregation of the mixed U1 population occurred, and NL variants preferentially infected the developing tissues. This represents another situation in which dramatic change in the genetic structure of an RNA virus population occurs during development of the host plant.

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CHAPTER I

General Introduction and Literature Review

Overview

Agriculture is mankind's oldest profession. In the modern world it is an industry, a science, and an art. Its social and economic impact has and will remain crucial, and it has moved from a subsistence role to a major contributor of GNP around the globe.

The exponential growth of human population; continuing reduction of aerable lands due to construction, soil erosion, water logging, and salinity; crop losses caused by insects, pests, and disease; and the increasingly damaging effects of air pollution and acid rains are some of the serious problems facing agriculture. The present agricultural surpluses in some regions of the world are not likely to remain available indefinitely unless a consistent effort is made through research to stimulate agricultural development.

Crop losses due to diseases are becoming more and more significant. Plants and their pathogens coevolve, and man practices selection for better yield and quality while disregarding the preservation of defense mechanisms plants have evolved. The success of crops has been ensured, when possible, through artificial means of plant protection. All these practices together have created serious selection pressures on pathogens to evolve further. The cost of pest management through protection is soaring. The extinction of genetic resources, the narrow genetic base of modern crops,

and the practice of monoculture have resulted in several recent disease epidemics (Singh 1986) and uncertainties in the face of evolving pathogens (Klinkowski 1970).

Conventional plant breeding and genetics have played a leading role in the progressive development of agriculture in this century (Borlaug 1983). According to one estimate, during the last 50 years, yield increases of our major crops are attributed up to 50% to genetic improvements alone. Breeding for disease resistance has a special significance as a cheaper way of disease control and as a distinctly controllable genetic situation. Research in agricultural sciences has been primarily of an applied nature (Reagan 1967), but the advent of new technologies is bringing it close to and more directly involved with basic research (Hansen et al. 1986).

There is general agreement that many diseases, including many caused by viruses, cannot be controlled efficiently without the use of resistant varieties. A precondition for the breeder to succeed in breeding resistant varieties (or for the development of other control methods) is to understand the basic interaction mechanisms and variability of genetic entities. In the past, plant virus diseases remained largely underexplored by agricultural researchers due to their relatively less importance to major crops. Nevertheless, landmark discoveries have been made in the areas of biochemistry and

molecular biology of viruses (Fraenkel-Conrat 1986). The impact of virus diseases has been especially strongly felt in the horticultural industries (Weintraub 1985). This review focuses on some approaches toward understanding the dynamics of plant-virus interactions and thus of the control of viruses through management and/or breeding varieties resistant to these infectious agents.

Plant Virus Diseases

Historically, virus have been recognized by their symptoms. The first virus ever identified was made a trade secret and used in horticultural practice by Dutch florists to induce colors in tulips; this agent later turned out to be mechanically transmitted tulip mosaic virus. Although viruses were properly recognized less than a century ago, there are about 300 virus diseases of plants now known. Defining what a virus is has been a controversial issue (Matthews 1982; Lwoff and Tournier 1966). It is, however, accepted that a virus is an obligate parasitic disease-causing agent which is a nucleic acid (DNA or RNA) encapsidated in a protein or lipoprotein protective coat. The viruses are highly evolved in typical host and vector situations. Once infected, the susceptible plant has no escape. Although there has been considerable interest in the development of chemotherapy of infected plants, no such control or cure is available. Resistance and prevention of

infection are the only alternatives for control of viruses. Before adapting either of the two measures, it is important to properly diagnose the disease, and to learn the host range, transmission characteristics and vector relations, replication strategies, movement mechanisms in the host and vector, and subcellular and molecular interactions in which the virus might participate. Some aspects of diagnosis and epidemiology of virus diseases are noted here.

Diagnosis. Traditionally, disease symptoms on differential host species have been considered the primary criteria for detection and identification of viruses (Bos 1978). There are serious limitations to this approach. The proper indicator host may not be available, or the infection may remain latent or symptomless (Converse 1985). There may be a mixed viral infection causing ambiguity. Mineral deficiency symptoms can also confuse the diagnosis (Chapman 1973). With the advent of electron microscopy and the use of viral nucleoprotein as antigens, accurate diagnosis has become simpler (Van Regenmortel 1982). The combination of symptoms on differential hosts with EM and/or immunological studies can provide positive identification. The efficiency of immunology has been greatly improved with ELISA and other solid support serological techniques (Clark 1981), and monoclonal antibodies are now being used to detect individual strains. Double stranded RNA can be analyzed to separate the viral infection agent from other pathogens

which might be confusing the interpretation of symptom development (Ralph 1969). Recent advances in biotechnology have offered an even more versatile diagnostic tool, the use of cDNA probes (Martin 1985). In this technique, random or highly conserved regions of viral genome are cloned into bacterial plasmids (Maniatis 1982). The clone is nick translated to incorporate radioactive or biotin (vitamin H) conjugated nucleotides, and the labeled nucleic acid is hybridized to viral nucleic acid immobilized on nitrocellulose paper. Only homologous DNA/RNA will hybridize, and excess label is removed by washing. The immobilized label can be detected by autoradiography or by staining for biotin using an appropriate enzyme/substrate combination (Langer et al. 1981; Leary et al. 1983).

Epidemiology. A virus disease results from a complex interaction between many physical and biological factors. The dominant ecological considerations in controlling viral diseases are mechanisms of spread. Physical stability, rate of movement, severity of disease, mutability and selection, host range, vectors (air borne and soil borne), cultural practices, season and weather, survival through seasonal cycles, and alternate hosts are important areas of information regarding the dynamics of spread. The role of cropping practices and other activities of man in the spread of virus diseases have been reviewed (Thresh 1982). Based on such information, viruses can be described as

mechanically transmitted and vector transmitted (insects, mites, nematodes, fungi, etc.). There are about 100 viruses now known to be seed or pollen transmitted (Bos 1983). Most of the viruses can be further classified as having more than one mode of transmission, one being primary and others secondary.

Virus Disease Control

Management via clean culture. This preventive approach has been used for controlling several viral diseases (Hansen 1985). Each virus disease has its own characteristics and needs a specific control strategy. This approach is specially effective for viruses without a living vector, or for viruses which have an insect as a secondary vector, such as seed-transmitted lettuce mosaic virus and squash mosaic virus. A totally virus-free material (seed or vegetative plant parts) is a prerequisite for the success of this procedure. Several methods are available to produce virus free propagules. To ensure and maintain the virus-free stocks, follow-up testing called "indexing" is practised. This has been extended to clonal protection and variety improvement programs for citrus in California (Reuther 1981; Calavan et al. 1978) and many other citrus growing areas of the world.

Vector control. As mentioned before, transmission of viral diseases by animals, especially insects, is a major

disease management concern. Elimination of such agents of virus spread can be the most effective control. Production of virus free seed potatoes at aphid-free higher elevations is an example. Aphids and leafhoppers are associated with certain viruses, and a high degree of specificity is found. The stylet-borne, insect transmitted viruses are also called "nonpersistent". The acquisition period of such viruses is short, and transmission is very effective in the immediate vicinity. The control of such transmission by insecticides is difficult, because the virus is transmitted before the vector is killed.

The other major category is circulative viruses, which can be persistent or nonpersistent. Considerable success can be achieved in controlling these viruses through timely insecticide application. Insects need a long acquisition time for these viruses, which provides an appropriate period for insecticide application. The time, dose, and effectiveness of a particular insecticide are critical. However, chemicals are expensive, which can make such a control method uneconomical. They also can kill beneficial organisms and pollute the environment.

Cross protection. This concept (Shalla and Peterson 1978) of controlling plant virus diseases emerged from the use of immunization (vaccination) to control human and animal diseases. Evidence has been presented that viruses can elicit resistance-related responses in infected plants

(Ross 1961; Sela and Applebaum 1962; Fraser 1981; and Fraser and Clay 1983). The use of a mild strain of virus as a way to protect against a possible subsequent infection by a severe strain was suggested long ago (Holmes 1934). Some degree of success has been achieved in using this technique for controlling citrus tristeza virus (CTV), TMV (tomato type) in greenhouses as well as in fields, and some other viruses (Grant and Costa 1951; Gilpatrick and Weintraub 1952; Rast 1972; Tomlinson and Shepherd 1979; Ahoonmanesh and Shalla 1981; and Yeh and Gonsalves 1984). However, there are several problems associated with this procedure of virus disease management (Hamilton 1985). Breakdown of such protection has also been reported (Bar-Joseph 1978). There is general agreement that cross protection in the case of TMV is controlled by the coat protein of the virus (Sherwood and Fulton 1982). Sarkar and Smitamana (1981) presented evidence against the role of coat protein in this mechanism. But Beachy et al. (1987) produced transgenic plants carrying the coat protein gene of TMV which cross protected against TMV infection. This provides a basis for the use of genetic engineering as a tool for controlling viral diseases through cross protection. Important discoveries are now being made in this area of virus disease management (Bevan and Harrison 1987).

Breeding resistant varieties. There is an extensive terminology relevant to this area (Parlevliet 1981; Russell,

1978). Briefly, resistance has been defined as the ability of crop plants to withstand the disease attack without significant economic loss. A resistance of lesser degree would be called tolerance. At the two extremes are sensitivity or complete susceptibility, and immunity or complete resistance. In the management of viral diseases, resistance is considered as the most desirable attribute, because it is likely to be more durable than other forms (Lamberti et al. 1981). The concept of resistance must further be clarified as heritable resistance versus non-heritable or induced resistance. Heritable resistance can be genetically defined and used in breeding programs. On the other hand, plants can sometimes express induced resistance in response to a stress, which is epigenetic in nature and not available for breeding purposes. The opposite of resistance of plants is virulence in pathogens, which can be classified ranging from avirulent to lethal (Fraser and Gerwitz 1987). Variants of a pathogen can be called mutants if they can be created artificially or if their origin is precisely known, or strains if they exist in nature as distinct subpopulations (Sarkar 1986). The genetic nature of resistance can be classified as quantitative (or horizontal) and qualitative (or vertical) (Van Der Plank 1968).

Operationally, breeding for resistance is no different than other breeding practices except in conceptual detail

(Mayo 1987). The backcross method is usually employed to transfer single gene resistance into an otherwise acceptable genetic background. Traditional breeding programs have relied on land races and wild relatives of our crops as sources of resistance. If such sources are available, this is still the best approach. However, these sources are subject to depletion due to their excessive use, extinction, and absence of renewability. In addition, there are incidences of newly evolved or existing viral diseases where resistance is not available. A more virulent strain of citrus tristeza virus (Calavan, 1978) and zucchini yellow mosaic virus of cucurbits are recent examples (Provvidenti et al. 1984). Using somaclonal variation as means of inducing new mutant genes and genetic engineering to transfer genes across incompatible species or even across kingdoms are being considered as future sources of resistance. Such advances in biotechnology are likely to become important aids to plant breeding but not a replacement for plant breeding. To realize the benefits of recent advances in biotechnology, it is necessary that we develop a thorough understanding of plant-virus interactions. Plant/pathogen interactions, as expressed by Flor's gene for gene hypothesis (Flor 1956), and by evidence presented by others on the coevolution of genetic systems, demand special treatment in this area of plant breeding and disease management research.

Outlook on the Problem

A careful perusal of the brief facts presented here indicates that the issue of viral disease management is unresolved. There is no single method of control which could be considered a final solution. All possible approaches are subject to breakdown. New mutants and strains of viruses arise and present continuing disease management problem. A thorough understanding of the evolutionary mechanisms underlying the emergence of variants and new strains is needed for developing an appropriate control strategy. It is said that virus population(s) carry a heavy load of mutations, presumably due to high replication errors (Holland et al. 1982) Under certain conditions, the mutant subpopulation(s) are likely to be selected for or against, leading to the prevalence or dominance of a particular variant genotype. The evolutionary significance of such selection phenomena is the development of new disease problems.

The coevolutionary mechanisms involving pathogens other than viruses are well studied. The viruses, not being independent organisms (Whittaker 1969), cannot be studied alone. Our increasing understanding of molecular mechanisms involved in plant-virus interactions can be utilized to study viral evolution if appropriate systems are developed (Day 1987; Callow 1987). An appropriate system would be the one in which a three-factor interaction involving plant,

virus, and environment can be studied under controlled conditions. The challenge of developing a model system to study plant-virus interactions is considered here.

A Model System

Nicotianas. Many important discoveries in biology have involved model organisms. The genus Nicotiana has been an important model for research in most of the botanical disciplines for more than a century. There are several advantages of this genus which attract researchers. There is an extensive amount of information available on Nicotianas. There are 64 species in the genus, representing vast genetic diversity (Smith 1979). Most of the species are cross compatible and easy to propagate. Nicotiana tabacum is an important cash crop in many parts of the world (Akehurst 1981; Tso 1972). The genus is a member of the Solanaceae family, which includes potatoes, tomatoes, and other important vegetable crops. Most of the discoveries made with Nicotianas have been directly translatable to other solanaceous crops. Tissue culture and disease management research are especially noteworthy in this context. Fulton (1979) has listed nine landmark findings in virology that were made with Nicotiana species. This author has also discussed the suitability of Nicotiana species as experimental virus hosts.

Tobacco mosaic virus (TMV). TMV has been the choice of

virologist for reasons similar to the Nicotiana species. TMV has several 'firsts' to its credit. It was the first virus to be purified in crystalline form (Stanley 1935) and the first virus reconstituted from purified nucleic acid and coat protein (Fraenkel-Conrat and Williams 1955). The complete genomic sequence is known (Ohno et al. 1984; Goelet et al. 1982) and the complete genome has been cloned (Dawson et al. 1986). TMV is not only a model virus but also a serious disease causing agent. According to a survey conducted by Holmes (1946), 199 species across 30 families were good hosts for TMV. The virus particle is rod shaped, and was the first ever visualized under an electron microscope. TMV is the type virus for the tobamoviruses as a taxonomic group (Matthews 1985; Murrant 1985). It is extremely stable and mechanically transmitted. A large variety of mutants and strains of TMV are known (Sarkar 1986), which makes it rich in diversity. The genetic diversity of TMV combined with its wide host range makes it excellent material for studying plant-virus interactions and virus population dynamics as related to evolution.

The interaction of resistance gene(s) in plants and virulence gene(s) in parasites has been thoroughly studied for fungal (Flor 1956) and bacterial systems. The recent availability of molecular cloning techniques has enabled researchers to isolate pathogen-specific genes for resistance in hosts and the corresponding virulence genes in

bacteria. Identification of such molecular events can reshape plant breeding strategies for disease resistance in future.

TMV produces characteristic systemic mosaic symptoms on its susceptible hosts. The epidemiology and control of TMV have been recently reviewed (Gooding 1986). Holmes (1929) first discovered that Nicotiana glutinosa is resistant to TMV. The resistance response was manifested as a hypersensitive reaction forming local lesions by killing host cells surrounding the site of infection. It was later found to be a single dominant gene (N) character. The N gene was transferred to N. tabacum through an artificially constructed tetraploid to allow the development of fertile progeny from a cross with the allotetraploid N. tabacum.

Nicotiana sylvestris is a systemic host for the common strain of TMV. Variants of TMV were isolated which induced the hypersensitive reaction in N. sylvestris (Valleau 1952). This response was seen in some other TMV hosts and was said to be caused by the N' gene. Using amino acid analysis, Fraenkel-Conrat's group at the University of California, Berkeley, ascertained that most such mutants have altered coat protein (Sarkar 1986). A variable response of N' gene to different mutants of TMV has been reported (Fraser 1983). Knorr and Dawson (1988) showed that a single point mutation at nucleotide 6157 (C to U) in the coat protein gene changes the coding region to specify phenylalanine instead of serine

at position 148 of the wild type coat protein. This alteration lead to the induction of local lesions on N. sylvestris. A series of similar mutants capable of inducing differential reactions with N and N' genes are now being constructed (personal communication). The interaction of TMV coat protein mutants with the N-gene system in Nicotiana species can be envisioned as an equivalent to bacterial avirulence genes. An extensive collection of TMV mutants capable of invoking N-gene or other resistance related systems provides a basis for studying the induction mechanisms. It also offers new means of genetically engineering resistant plants via cross protection or induction of inherent resistance mechanisms.

Viruses, including TMV, are being developed as gene vectors. The major objection to the use of RNA viruses is the high potential for mutability (Holland et al. 1982; King et al. 1982). Our lack of understanding of the underlying mechanisms responsible for the emergence of new viral diseases (Lisa et al. 1981; Wallace 1978) and of resistance to existing diseases due to appearance of more virulent strains is a serious handicap. The development of a system to study evolutionary mechanisms of RNA viruses is essential if future unforeseen situations are to be dealt with appropriately.

Scope of the Dissertation

The genetics underlying plant-virus interactions as a component of viral evolution is one of the least understood phenomena of modern biology. For many years, host effects (Yarwood 1979; MacNeill and Boxall, 1974; Solberg and Bald, 1963) and the environment effects (Dawson and Jones 1976; Jones and Dawson 1978) have been known to exert selection pressures on virus populations. A precise description of one type of selection process is the major theme of this dissertation. This research will lead to the use of molecular tools in the study of virus evolution. Genetically marked viral populations can be constructed, and the fates of different components of the populations determined under changing host and environmental conditions.

In the following chapters, it is shown that positive selection for a certain class of variants of TMV occurs in rapidly developing tissues of a specific host, N. sylvestris. The effects of two major developmental processes on virus populations were studied. These were bolting, which leads to the development of the flowering axis, and regeneration from cultured leaf tissues, which leads to the development of plantlets. When these processes are initiated from tissues infected with TMV, dramatic and predictable changes in the genetic substructure of the TMV population occur as virus replicates and invades the developing tissues. One variant in this class was studied

in depth and shown to be distinguishable from other variants of TMV that have been described. The selection phenomenon described here offers a system with which genetically marked virus populations can be subjected to evolutionary pressures under controlled conditions and with which the molecular bases for evolution can be studied.

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CHAPTER II

Selection for a Specific Tobacco Mosaic Virus
Variant during Bolting of Nicotiana sylvestris

Abstract

In an isolate of TMV U1, a small subpopulation of virus that invokes the hypersensitive reaction on Nicotiana sylvestris Spegaz. & Comes exists and is strongly selected for during replication in plants that are undergoing development of shoot axes during bolting. Virus of this phenotype (NL) can infect and move systemically in rapidly developing parts of N. sylvestris plants and is strongly selected for in upper tissues of the plants only during the development of the shoot axis. This selection results in a dramatic change in the genetic composition of the virus population in the upper part of the plant. No accumulation of NL-type virus occurred when N. sylvestris plants were inoculated with TMV204, a clonal population derived from RNA transcripts of a plasmid carrying a cDNA copy of the TMV genome.

Introduction

Most isolates of an RNA virus contain a spectrum of genotypes in which one genotype predominates. Changes in the environment in which the virus replicates can lead to changes in the genetic structure of the population (Bald et al. 1974; Dawson and Jones 1976; Domingo et al. 1985; Garcia-Arenal et al. 1984; Holland et al. 1982; MacNeill and Boxall 1974; Reanney 1982; Schubert et al. 1984; Steinhauer and Holland 1986; and others). One apparent consequence of

the genetic diversity of virus populations and the mutation and selection mechanisms underlying it is that RNA virus populations can evolve rapidly. New strains of known viruses and even new viruses emerge frequently and pose new threats to humans, livestock, and crops (Hughes et al. 1986; Lisa et al. 1981; Sobrino et al. 1986; Wallace, 1978; Webster et al. 1982).

Mechanisms by which RNA viruses evolve - especially plant viruses - are not well understood. In the past, few well defined experimental systems for studying this phenomenon in plants have been available. What is needed is a host-virus system in which specific changes in the genetic structure of a virus population occur, and with which selection for or against specific genetic changes can be applied under controlled laboratory conditions. In this way, definitive experiments directed toward elucidating the effects of altering specific viral gene functions can be designed and interpreted.

The results presented below demonstrate a situation in which selection radically alters the genetic makeup of populations of tobacco mosaic virus replicating during specific developmental changes in Nicotiana glauca. This system provides one component of a bidirectional selection system that will prove useful in relating changes in the genome of TMV to interactions between the host plant and virus leading to the evolution of viral populations.

Materials and Methods

Plants

Nicotiana tabacum L. var. Xanthi carries the n allele (lack of hypersensitivity) of the N,n allele pair (Holmes 1938); N. tabacum L. var. Xanthi-nc carries the N allele of this gene (hypersensitivity to TMV); and N. sylvestris Spegaz. and Comes carries the N' gene (hypersensitivity to some strains of TMV) (Valleau 1952; Valleau and Johnson 1943; Weber 1951). All plants initially were grown from seed under greenhouse conditions. Experiments in which growth chambers were used were performed at 27C with 16 hours of light. When the light source was low intensity tungsten light, the flux density was approximately 30 microeinsteins (μE) $\text{m}^{-2} \text{sec}^{-1}$. The flux density in growth chambers with high intensity fluorescent and tungsten light was about 210 μE $\text{m}^{-2} \text{sec}^{-1}$ and with only fluorescent lights about 180 μE $\text{m}^{-2} \text{sec}^{-1}$. Low intensity fluorescent lights used during local lesion assays provided a flux density of about 35 μE $\text{m}^{-2} \text{sec}^{-1}$.

When unbolted N. sylvestris plants were kept under low intensity or high intensity fluorescent lights, or under high intensity fluorescent light supplemented with low intensity tungsten light, they did not bolt. However, when such plants were exposed to light provided only by a low intensity tungsten source, bolting initiated rapidly, and the plants developed main stem axis 50 - 100 cm long within

the next few weeks.

Plants used in host range tests were started from seed in a glasshouse, mechanically inoculated, and incubated at approximately 23 C.

Virus

Initial stocks of viruses included TMV strain U1 and TMV204, a clonal population derived from transcripts of cDNA copies of the U1 genome (Dawson et al. 1986). Frequencies of NL variants in these stocks typically were less than 0.01 percent.

Virus was propagated in Xanthi tobacco for two to three weeks under greenhouse conditions, after which leaves from infected plants were harvested and frozen at -20C to be used as a source of inoculum. Unless otherwise stated, leaf extract inocula were prepared in sterile mortars and pestles by grinding 1.0 g fresh weight tissue in 10 ml glycine phosphate buffer (50 mM glycine, 30 mM phosphate, pH 9.2) containing 1 percent (w/v) celite for an abrasive. Purified preparations of virions (Gooding and Hebert 1967) were stored in 0.02 M sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.02 % sodium azide as a preservative and diluted in cold glycine -phosphate buffer just prior to use.

TMV U1 infectivity titers were quantified by local lesion assays on N. tabacum var. Xanthi-nc. Titters of variants that induce local lesions on N. sylvestris (called

NL variants here) were measured by local lesion assays on Xanthi-nc tobacco and N. sylvestris plants incubated at approximately 23C under fluorescent light. At least three half-leaves were used for each assay sample.

Molecular Hybridization

DNA-RNA hybridizations were performed at both high and low stringency essentially as described in Maniatis et al (1982) using ³²P-labeled plasmid kindly provided by A. Raffo. This plasmid contains approximately 1300 nucleotides of DNA complementary to most of the coat protein and 30 K protein genes of TMV 204 (Dawson et al. 1986).

Serological Analysis

Ouchterlony serological tests were performed as described by Ball (1974).

Results

Selection for NL-Type Virus

In N. sylvestris, wild type TMV replicates and spreads systemically. However, certain TMV variants (NL) as well as certain other tobamoviruses invoke the hypersensitive reaction on this host, with the attendant formation of local lesions. A series of preliminary experiments indicated that NL-type variants might be selected for during replication of tobacco mosaic virus in bolting N. sylvestris plants. That

this is the case was shown by the following experiment. Rosette leaves of unbolted N. sylvestris plants were inoculated with the U1 TMV stock, and the plants were placed under low intensity tungsten light for 30 days, during which they bolted. Virus in extracts from rosette and shoot axis leaves of these plants was tested on Xanthi-nc tobacco and on rosette leaves of nonbolted N. sylvestris plants. Total virus titers were high in all leaves of the test plants, but only in extracts from shoot axis leaves was a significant quantity of NL-type virus found (Table 2-1). This experiment was expanded and repeated several times, during which we found that NL-type virus accumulated in the shoot axis leaves of 17 of 24 N. sylvestris plants inoculated with TMV U1 prior to bolting. Frequently, leaves containing high proportions of NL-type virus became severely distorted and eventually necrotic.

To determine whether NL-type virus is induced by a mutagenic constituent of shoot axis leaves of N. sylvestris that is not found in rosette leaves, extracts from shoot axis and rosette leaves of uninfected flowering plants were mixed with U1 virus, stored at 4C, and assayed every two days for a month for total relative infectivity and for proportion of NL-type virus. Although infectivity remained high for the entire time, NL-type virus did not accumulate (data not presented.)

To test the possibility that the accumulation of NL

variants is related only to the number of rounds of replication of the virus in the host plant, experiments were performed in which virus was allowed to replicate but not spread systemically. A 20-25 cm long rosette leaf of each of several nonbolting *N. sylvestris* plants was inoculated with the U1 stock, and virus was allowed to replicate. After 8-10 days, a sample of tissue from the infected leaf of each plant was assayed for total relative virus content as well as proportion of NL-type virus. These samples also were used immediately to inoculate rosette leaves on new nonbolting *N. sylvestris* plants. Passage of the virus in this way was repeated many times (as many as 12 serial infections in one experiment that included three parallel replicates), always with the same result: although virus replicated prolifically, NL-type virus did not accumulate. A similar set of sequential inoculations and assays was performed in which young rosette leaves (4-5 cm long) of unbolted plants were inoculated and assayed 8-10 days later, during which the leaves expanded to a length of 15-20 cm, and the results were similar: during 12 sequential passages, NL-types did not accumulate. Finally, an experiment was performed in which 12 sequential propagations and assays of inoculated shoot axis leaves on bolted plants were performed, with the same result - no accumulation of NL-type virus occurred. These experiments showed that accumulation of NL variants does not occur simply because the virus is

replicating in N. sylvestris.

Bolting and the Accumulation of Variants

To clarify the relationship between accumulation of NL variants and bolting of the host plant, the following experiments were done. Three sets of 3 or 5 plants each were inoculated with the TMV U1 stock. One set was placed in a growth chamber at 27C under low intensity tungsten light, one was placed in a glasshouse (December 1986), and one was placed at approximately 23C under low intensity fluorescent light. After four weeks, all plants in the growth chamber had bolted and contained elevated proportions of NL-type virus in leaves on their shoot axes (Table 2-2). One plant of three in the glasshouse had undergone slight internodal extension, exhibited aberrant leaf morphology, and tested positive for NL-type virus in its upper leaves (Table 2-2). The other two plants in the glasshouse remained in the rosette form and accumulated high titers of wild type virus but not NL-type.

Plants kept under fluorescent illumination during this time showed little growth, and although they were heavily infected with virus, NL-type virus did not accumulate in them. After four weeks under fluorescent light at approximately 23C, these plants were moved to a growth chamber at 27C with high intensity fluorescent lights supplemented with tungsten lights, in which they resumed a

normal growth rate. Even after several more weeks, these plants had not bolted, and NL-type virus had not accumulated in them, although virus titers were high. Finally, the fluorescent lamps in this chamber were turned off so that the only illumination came from the low intensity tungsten bulbs: within two weeks all the plants bolted, and NL-type virus accumulated in the upper leaves of three of the five plants during development of the shoot axes (Table 2-2).

In analyzing the above experiments, it became clear that all leaves on the shoot axes of infected bolting plants did not contain the same relative frequencies of NL-type virus. The demographics of the change in virus population were determined by assaying total virus titer and NL-type virus titer in all leaves of four plants inoculated at the rosette stage of development and forced to bolt as described above (Figure 2-1). After four weeks, each leaf in the rosettes tested negative for accumulation of NL-type virus, while 31 out of 37 leaves on the shoot axes of the four plants contained elevated proportions of NL-type virus; leaves nearer the tops of the plants in general contained higher percentages of NL variants; in some individual leaves, the frequency of NL-type virus approached 100 percent.

Two further experiments showed that the observed interaction between the virus population and the host plant is not ubiquitously expressed. First, experiments similar

to those described above were repeated using the same source of TMV U1 to inoculate plants of N. tabacum var. Xanthi: NL-type variants did not accumulate during subsequent growth of these plants. Second, similar experiments with N. sylvestris were carried out using as inoculum TMV204, which was derived from infectious RNA transcripts of a TMV U1 cDNA carried in plasmid pTMV204 (Dawson et al. 1986); this population should be genetically homogeneous. NL-type variants were not recovered from these plants, either. Indeed, the clonally derived TMV population was passaged sequentially seven times through bolting N. sylvestris plants, but NL-type virus did not accumulate at any time during the experiment. The same result was observed when TMV204 was passaged systemically five sequential times through N. tabacum var. Xanthi with periodic testing for NL-type virus.

NL-Virus

Further characterization of NL-type virus required the use of purified preparations. Eighteen NL variants were passaged through seven sequential single lesion transfers on N. sylvestris. As the transfers progressed, various isolates exhibited differences in symptom induction, such as size of lesions, degree of necrosis, and temperature sensitivity. Each of them produced abundant lesions three days after inoculation of unbolted N. sylvestris plants and

did not replicate systemically in them even after extended times. Although several of the variants were distinguishable, they all reproducibly exhibited the phenotype associated with the selection phenomenon being studied. One variant representative of the group (NL-18) and the U1 wild type stock were inoculated into N. tabacum var. Xanthi, and after 3 weeks, leaves were harvested and both viruses purified. Yields and specific infectivities of both type viruses were similar.

One important question is whether the NL-type virus we observed was a contaminant, perhaps a virus that is not TMV, or perhaps another strain of TMV that has been or is in use in laboratories near ours. NL-18 and U1 were compared by electron microscopy and were indistinguishable. When they were tested against antibodies to the U1 strain by Ouchterlony analysis, they again were indistinguishable. Thus, it is unlikely that NL-18 is not TMV.

Other TMV strains are known to induce lesions on N. sylvestris and might be contaminating our U1 stock. Of particular concern were strains that are used locally, which include U2/U5 and a strain provisionally called TMV-o that replicates in orchids (M. Hilf, personal communication). The effects of NL-18 virus on a range of known TMV hosts were determined to eliminate this possibility. As summarized in Table 2-3, NL-18 produced symptoms on these hosts that distinguished it from the other strains, as well as from the

well characterized tomato strain of TMV, ToMV, that is found in many populations of TMV U1 (Brunt 1986).

Finally, to confirm the above results, a ^{32}P -labeled cDNA probe containing approximately 1300 bases complementary to a region of the TMV U1 genome containing most of the coat protein and 30K protein genes was hybridized to RNA from strains U1, NL-18, TMV U5, and TMV-o, which are used extensively on our campus. Under conditions of both high and low stringency, dot blot analysis showed that the probe hybridized well to RNA from NL-18 (although not as strongly as with U1 RNA), did not hybridize with U5 RNA, and hybridized only weakly at low stringency with TMV-o RNA (Figure 2-2). This evidence, combined with the above results, shows that NL-18 is a strain of TMV that, while somewhat different from U1, is not likely to be a contaminant of extraneous origin.

Systemic replication of NL variants

The results presented so far imply that NL-type variants replicate systemically in N. sylvestris, even though when inoculated onto rosette leaves of nonbolting plants, they induce the hypersensitive response and are contained within local lesions. To further delineate conditions under which NL-type virus can replicate systemically in this host, a series of experiments was performed in which rosette or shoot axis leaves

of N. sylvestris plants forced to bolt prior to inoculation were inoculated with the U1 stock or with purified NL-18. A set of 5 or 6 plants was used for each inoculation. After 4 weeks, all plants were assayed for total virus and for NL-type virus in leaves in the rosettes and on the shoot axes (Table 2-4).

When rosette leaves were inoculated with NL-18, local lesions formed, and the virus was completely restricted from further movement; no systemic spread occurred into rosette leaves or leaves on the flowering axes. In contrast, when leaves on flowering axes approximately 15 cm long were inoculated with NL-18 and the bolting allowed to continue, NL-type virus spread throughout the plant, although titers in younger leaves above the inoculated ones were substantially higher than in rosette leaves of these plants.

When shoot axis leaves were inoculated with NL-18, severe chlorosis and incomplete lesion development occurred on the inoculated leaves. These plants did not continue to grow normally, bolting was slowed, and the plants were stunted, showed severe distortion of newly developing leaves, and developed necrosis on their stems.

Bolting plants similarly were inoculated with the U1 laboratory stock. These plants continued to grow nearly normally, and whether shoot axis or rosette leaves were inoculated, the virus rapidly spread systemically into all parts of the plants, and the usual mosaic symptoms

developed. Three of the six plants inoculated in a shoot axis leaf accumulated substantial quantities of NL-type virus in leaves higher on the flowering axis, and in one plant, a small quantity of NL-type virus was detected in rosette leaves (Table 2-4).

In all experiments so far presented in which rosette leaves of *N. sylvestris* plants were inoculated, relatively large (6-10 cm) leaves were exposed to the virus. To test whether the age of rosette leaves affects the spread of NL variants, young nonbolting plants with expanding leaves about 2 cm long were inoculated with purified NL-18 virus. In these plants, the virus replicated and spread systemically into newly developing leaves in the rosette. Lesions did not form, and typical mosaic symptoms developed. Subsequently, these leaves developed severe necrosis. This experiment confirms that the ability of NL-18 to spread systemically depends upon the developmental stage of tissues exposed to it.

Degree of Selection

In most cases discussed thus far, when rosette leaves were inoculated with the U1 stock and the plants forced to bolt, not all plants accumulated NL-type virus in the upper leaves. (Overall, NL-type virus accumulated in 65 of 79 plants.) To determine whether the frequency of plants accumulating NL-type virus could be increased, six

nonbolting N. sylvestris plants were inoculated with a 50:50 mixture of U1 and NL-18 virus and then forced to bolt. In all six plants, NL-type virus accumulated to high titers in the upper leaves of the bolted plants, and a small amount of NL-type virus apparently moved into other leaves on the rosettes (Table 2-5). Also, in three of six control plants inoculated with NL-18 alone and forced to bolt, NL-type virus accumulated in upper leaves, in contrast to what occurred when rosette leaves of plants already forced to bolt were inoculated with NL-18. In the latter situation, NL-18 did not move systemically through the plants (see above). Finally, in this particular experiment, in none of six plants inoculated with TMV U1 and forced to bolt was accumulation of NL variants observed. This result presumably reflects the previously observed statistical nature of the occurrence of accumulation when plants are inoculated with a virus population containing a low proportion of NL variants.

Discussion

The evidence presented above strongly suggests that the population of U1 virus used in these experiments includes a small subpopulation of virus of the NL-type that can be selected for during replication in N. sylvestris plants undergoing development of shoot axes. The data also suggest that one or a few mutations occurring after inoculation of

the plants is not responsible for the accumulation of NL variants: accumulation of variants in the TMV204 populations should have occurred if that were the case.

Taken together, these experiments confirm that NL-type virus can infect and move systemically in rapidly developing parts of N. sylvestris plants, that NL-type virus is strongly selected for only during replication and systemic movement in the shoot axis or into newly developing leaves, and that the dynamics of accumulation of NL-type virus depend upon the genetic structure of the initial inoculum. This structure can be modulated by using artificially constructed populations of virus containing wild type and variant virus in known proportions. Our results also imply that some of the NL-type virus present at low frequency in the U1 inoculum can infect, replicate, and systemically move in older rosette leaves of N. sylvestris plants when the plants are forced to bolt.

While much work on host effects on virus populations has been done (Yarwood 1979), including elegant experiments using genetically marked strains of TMV (MacNeill and Boxall 1974), the effects we have described appear to be unique first in the nature of the selection phenomenon itself and second in the implied strength of the selective pressure, whatever its biological basis. Many strains and mutants of TMV are known to invoke the N' gene carried by N. sylvestris and certain tobacco varieties (Fraser 1983; Sarkar 1986;

Siegel and Wildman 1954; and others). Most such variants have altered coat proteins, and recently, it has been shown that a single base change in the TMV coat protein gene can generate a mutant that induces lesions on this host (Knorr and Dawson 1988). We have yet to determine the molecular change responsible for local lesion induction in the NL variants we have described here and to test whether the selection we have observed is a pleiotropic effect of mutations that lead to the induction of local lesions on N. sylvestris. It is possible that selection acts on a different function of the virus that is not directly related to lesion induction. Such experiments might contribute significantly to our understanding of how virus - host interactions affect the evolution of RNA plant viruses and perhaps to our knowledge about viral pathogenesis.

Another important feature of this work is a more general one. In the past, it has been difficult to investigate the evolution of RNA virus populations due to lack of genetically homogeneous populations with which to initiate controlled experiments (Garcia-Arenal et al. 1984) and to lack of a well defined system for studying factors that might perturb the genetic structure of populations. Recent advances in determining the complete nucleotide sequence of TMV (Goelet et al. 1982; Ohno et al. 1984) as well as the molecular cloning of the complete genome of TMV (Dawson et al. 1986; Meshi et al. 1986) help alleviate the

former problem, and the system we have described in this report helps solve the latter.

Many questions about the relationship between a particular gene and its environment can be answered by using a system in which, under some conditions, the function of that gene is selected against, and under others, selected for. Such bidirectional selection systems have proven to be immensely valuable: the "HAT" system developed for use in animal somatic cell genetics research is a classical example (Weiss and Green 1967); myriad plasmid and viral constructs that easily can be selected for or against are others. Not only have these systems been used as tools in experiments to study other genetic phenomena, they also have been the source of much information about the structures and functions of the genes involved. In this report, we have described a way in which one class of variants can be selected for during replication. Elsewhere, we will describe a way in which the same variants can be selected against (Aldaoud, Dawson, and Jones, unpublished). Thus, we now have a bidirectional genetic selection system with which to capitalize on advances in the molecular biology of plant viruses in studies of host -virus interactions and of plant virus evolution.

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Table 2-1. Local lesion assay of virus in leaf extracts from N. sylvestris

Plant No.	Rosette leaf ^a extracts		Axis leaf extracts	
	<u>N. syl-</u>	<u>N. tabacum</u>	<u>N. syl-</u>	<u>N. tabacum</u>
	<u>vestris</u>	Xanthi-nc	<u>vestris</u>	Xanthi-nc
1	0	117	42	43
2	0	142	82	144
3	0	108	36	80
4	0	68	24	43
Mean $\pm$ SE	0	109 $\pm$ 15	46 $\pm$ 12	78 $\pm$ 24

^a 1.0 g leaf tissue was ground in 10 ml buffer and applied to leaves as described in Materials and methods. Numbers are average numbers of lesions on three half-leaves of assay plants. A zero represents <1 lesion per assay plant.

Footnotes to Table 2-2.

- a Experimental conditions are described more fully in the text.
- b 1.0 g leaf tissue was ground in 10 ml buffer and applied to leaves of assay plants. Numbers are means $\pm$ SE of means of counts of lesions on half-leaves. N/A = not applicable (bolting did not occur). A zero represents <1 lesion per assay plant.
- c NL-type virus was detected in only one plant.

Table 2-2. Relationship between NL variant accumulation and bolting of N. sylvestris plants.

Incubation ^a conditions	Extract from	Titer on ^b		
		<u>N. sylvestris</u>	Xanthi-nc	
Tungsten light; growth chamber	Stem axis leaves	15 ± 5	38 ± 15	
	Rosette leaves	0	47 ± 8	
Glasshouse	Stem axis leaves	6 ^C	23	
	Rosette leaves	0	45 ± 3	
Fluorescent light, 23C for four weeks; then growth chamber with fluorescent and tungsten light for three weeks.	Stem axis leaves	N/A	N/A	
	Rosette leaves	0	48 ± 12	
Same plants as above two weeks after turning off fluorescent light.	Stem axis leaves	11 ± 3	52 ± 7	

Table 2-3. Host ranges of TMV U1 and NL-18.

Host	U1 ^a	NL-18	Remarks ^b
<u>N. sylvestris</u>	SYS	LL	U2/U5, ToMV, and TMV-o induce LL
<u>N. tabacum</u> Xanthi	SYS	SYS	U2/U5, ToMV, and TMV-o spread SYS
<u>N. tabacum</u> Xanthi-nc	LL	LL	U2/U5, ToMV, and TMV-o induce LL
<u>N. glutinosa</u>	LL	LL	U2/U5, ToMV, and TMV-o induce LL
<u>Lycopersicon</u> <u>esculentum</u>	SYS	SYS	U2/U5 does not infect
<u>N. glauca</u>	Infects poorly	Infects poorly	U2/U5 rapidly spreads systemically
<u>N. benthamiana</u>	Lethal necrosis	Lethal necrosis	TMV-o infection yields typical green mosaic
<u>Petunia</u> <u>hybrida</u>	SYS	SYS	ToMV induces LL

^a Abbreviations: SYS = systemic spread. LL = local lesions

^b Brunt (1986); M. Hilf, personal communication; Wetter (1986)

Table 2-4. Replication and accumulation of NL-type virus in N. sylvestris plants.^a

Leaf Type Inoc / Assay	Frequency of Plants with NL	<u>N. tabacum</u> ^b Xanthi-nc	<u>N. sylv-</u> <u>estris</u>
NL-18			
R / R	0/5	0	0
R / A	0/5	0	0
A / R	5/5	3 ± 1	3 ± 1
A / A	5/5	33 ± 4	52 ± 5
U1			
R / R	0/6	10 ± 3	0
R / A	0/6	45 ± 5	0
A / R	1/6	15 ± 5	< 1
A / A	3/6	35 ± 7	11 ± 2

^a NL-18 or U1 TMV was inoculated onto rosette (R) or stem axis (A) leaves of bolted N. sylvestris plants, and rosette or stem axis leaves above the inoculated leaves were assayed 4 weeks later on N. tabacum var. Xanthi-nc or on N. sylvestris. 1.0 g leaf tissue was ground in 10 ml buffer and applied to leaves of assay plants.

^b Numbers represent average ± SE of local lesion counts on half-leaves of N. tabacum var. Xanthi-nc or N. sylvestris. A zero represents <1 lesion per plant.

Table 2-5. Accumulation of NL variants in plants inoculated with a mixture of NL-18 and U1 and forced to bolt.^a

Inoculum	Rosette leaf extract		Stem axis leaf extract	
	assayed on:		assayed on:	
	<u>N. sylvestris</u> Xanthi-nc	<u>N. sylvestris</u> Xanthi-nc	<u>N. sylvestris</u> Xanthi-nc	<u>N. sylvestris</u> Xanthi-nc
NL-18	0.6 ± 0.2	0.3 ± 0.2	23 ± 4 ^b	30 ± 5
U1	0	16 ± 2	0	30 ± 6
Mixture	0.8 ± 0.3	8 ± 2	14 ± 2 ^c	28 ± 4

^a Rosette leaves of unbolted N. sylvestris plants were inoculated with NL-18, U1, or a 50:50 mixture of the two strains. The plants were forced to bolt, and extracts of rosette or stem axis leaves of the bolted plants (1.0 g ground in 10 ml buffer) were assayed on unbolted N. sylvestris plants or on Xanthi-nc tobacco. Numbers represent means ± SE of local lesions formed per half leaf on assay plants.

^b Mean ± SE of lesion counts from extracts of the three plants that showed accumulation of NL variants.

^c NL-type virus was detected in all six plants in this treatment. The range of titer means was 4 - 27 lesions per half-leaf.

Figure 2-1. Line diagram of a bolted N. sylvestris plant showing gradient of NL accumulation. Single rosette leaves on each of four nonbolting N. sylvestris plants were inoculated with TMV U1, and the plants were forced to bolt by placing them in a growth chamber at 27C illuminated with low intensity tungsten light. After four weeks, samples of each leaf on the four plants were assayed on Xanthi-nc tobacco for total virus titer and on rosette leaves of nonbolted N. sylvestris plants for titer of NL-type virus. Samples (1.0 g) of leaf tissue were ground in 10 ml glycine-phosphate buffer, and the extract was applied to half-leaves with cotton swabs. Titers from triplicate assays of leaves at equivalent positions on the four plants were averaged and are presented in Figure 2-1. The first number represents titer on N. sylvestris, the second number the titer on Xanthi-nc tobacco, and the number in parentheses is the percent NL-type virus calculated from the first two numbers.

In rosette leaves of the N. sylvestris plants, significant amounts of NL-type virus were not detected; the average titer of total virus in these leaves, determined on Xanthi-nc tobacco, was 77 ± 7 (mean $\pm$ SE) lesions per half-leaf.

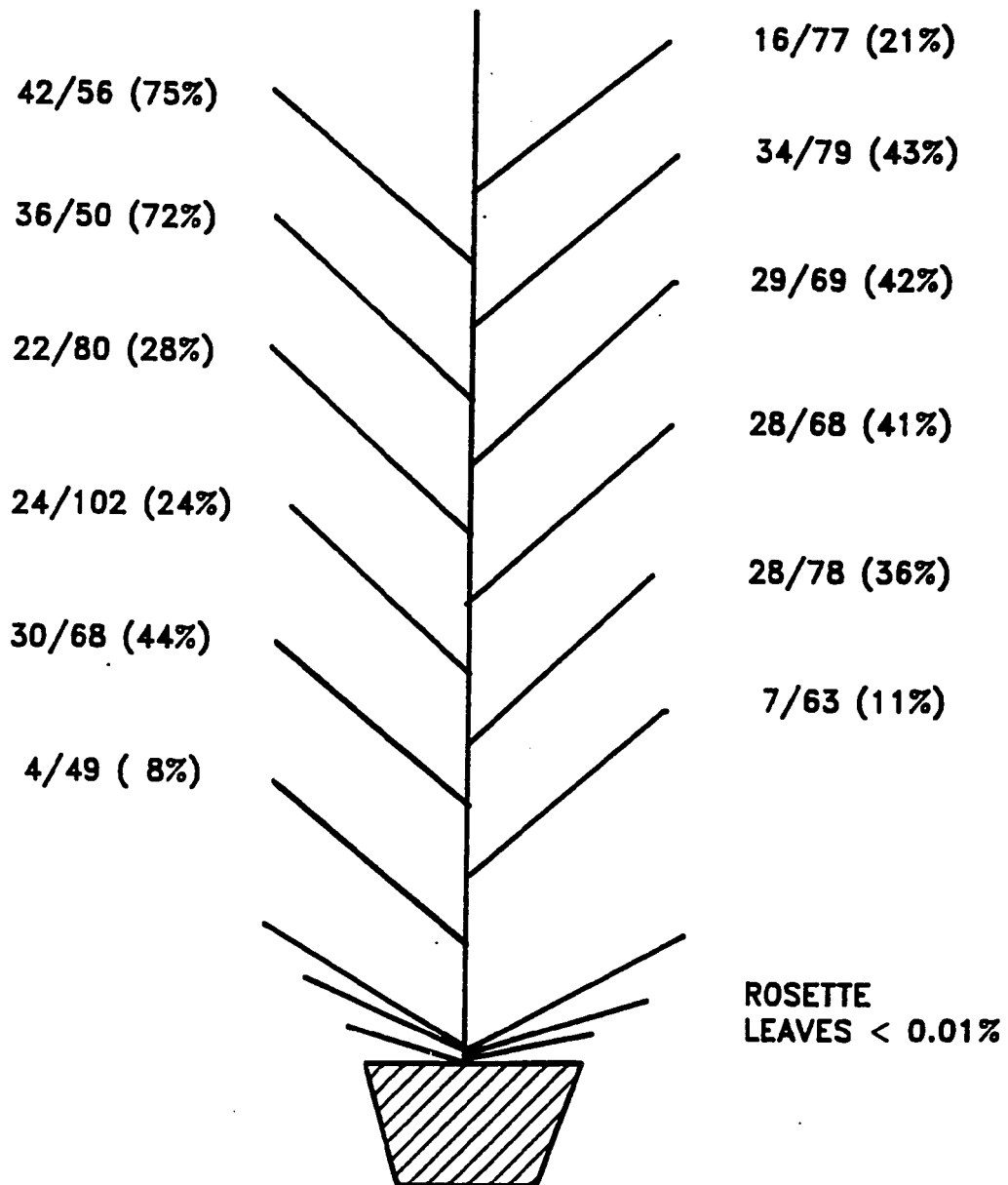


Figure 2-2. Dot-blot hybridizations of cDNA derived from TMV204 (U1) with RNA from TMV U1, TMV NL-18, TMV U5, and TMV-o. At 1X concentrations of RNA, each spot on the filter contained 75 ng of RNA.

Dilution

0.25X
0.5X
1X



U1
NL-18
U5
TMV-0

U1
NL-18
U5
TMV-0

High
Stringency

Low
Stringency

TMV Strains

CHAPTER III

Growth Dynamics of Healthy and Virus-Infected Leaf
Disks from Developmentally Different Stages of
Nicotiana sylvestris in Tissue Culture.

Abstract

Tissue culture techniques are used extensively to provide virus-free plants or plants that are resistant to viruses. However, in most situations, viral infection does not significantly reduce the growth of plant tissues in culture and does not significantly affect the ability to regenerate plantlets from somatic tissues in culture. The data presented here show that, under some circumstances, physiological stress imposed by tobacco mosaic virus replicating in Nicotiana sylvestris Spegaz. and Comes leads to reduced growth of leaf disk tissues and to reduced regeneration of plantlets from the cultures. In particular, when TMV infected leaves from the stem axes of bolting N. sylvestris plants were used as the source of disks, growth and regeneration were significantly reduced, compared to disks from similar leaves on uninfected plants. When disks from infected or healthy rosette leaves of the same plants were used, growth and regeneration were slower than for axis leaves, but viral infection did not significantly affect the rosette leaf disks. These results show that the developmental stage of plant tissues used in culture can have a significant effect on the outcome of experiments involving viruses, which might be exploited in studies of host-virus interactions.

Introduction

Tissue culture techniques have been used extensively in different areas of plant science research and as a modern technology for plant propagation (Yeoman 1986). One of its many applications involves the use of shoot-tip culture, alone or in combination with thermotherapy, to produce virus-free plants, because viruses generally are excluded from rapidly developing apical meristems. In addition, somaclonal variation, which refers to the high rate of generation of cells of variant genotype during growth of plant tissues in culture, has been used as a source of plants that express enhanced resistance to some viruses (Murakishi and Carlson 1982; Carlson and Murakishi 1978). In general, however, infection by plant viruses seems to have little or no direct effect on the growth of cultured plant tissues, and little progress has been made in developing ways to distinguish infected cells from uninfected ones in culture on the basis of their growth properties alone (Dawson, personal communication). Such procedures might be highly useful for developing screening techniques to detect virus-free materials.

Previously, we presented evidence that interactions between developing Nicotiana sylvestris plants and tobacco mosaic virus can dramatically change the genetic structure of the virus population, and can lead to systemic infection of the plant by virus strains that ordinarily would be

prevented from spreading systemically by the hypersensitive response (Khan and Jones 1988a,b). When infected plants enter the flowering stage, or when plantlets are regenerated from infected callus in culture, a particular class of TMV variants much more readily populates the developing tissues than does wild type virus. This implies that tissues at different developmental stages respond differently to TMV. Thus, it was of interest to determine whether TMV infection affected the growth and development of N. sylvestris tissue in culture. If so, TMV replicating in N. sylvestris tissues in culture might prove useful as a model system for the study of interactions between plants and their viral pathogens (Callow 1987). We present here the results of an experiment conducted to study the relative dynamics of growth of healthy or TMV infected leaf disk cultures initiated from two developmentally distinct stages of leaves of Nicotiana sylvestris.

Materials and Methods

Plants

N. sylvestris Spegaz. and Comes was used in this study. It carries the N' gene (hypersensitivity to some strains of TMV) (Valleau and Johnson 1943; Weber 1951). Plants initially were grown from seed under greenhouse conditions.

Virus

The virus strain used was wild type strain TMV U1. Virus was propagated in Nicotiana tabacum L. var. Xanthi for two to three weeks under greenhouse conditions. Leaves from infected plants were harvested, frozen at -20C, and used as a source of inoculum.

Disk cultures

Young Nicotiana sylvestris plantlets in a seed pot were transplanted into soil in pots and cultured in a greenhouse for four weeks during a summer month, then infected with the U1 strain of TMV and placed in a growth room under fluorescent lights (16 h day) at 27C. Infected plants and healthy controls were allowed to grow for an additional four weeks in the growth room. During this time, the plants bolted and developed flowering stem axes. Then, with an 11 mm cork borer, leaf disks were taken from fully expanded rosette leaves and from expanding leaves on the stem axes of the plants. The surface sterilized disks were placed onto agar medium in plastic petri dishes. The culture medium contained MS salts (Murashige and Skoog 1962) supplemented with 3% sucrose; 0.1% myo-inositol; 1 mg per liter each of thiamine.HCl, naphthalene acetic acid, and benzyladenine; 80 mg per liter adenine sulfate ($.2\text{H}_2\text{O}$); 100 mg/l L-tyrosine; and 0.17 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$. Culture plates were placed under 16H light with a constant day and night temperature of 27C.

Fresh weight and dry weight of individual disks were recorded after 0, 7, 14, 21, and 28 days of cultures. Numbers of plantlets containing at least 2 leaflets on the disks were counted at 21 and 28 days. Each data point was based on measurements using six disks, and the entire experiment was replicated four times.

Results and Discussion

Two sets of healthy or virus infected Nicotiana sylvestris plants (four plants per set) were allowed to grow for four weeks after the time of inoculation. Leaf disks were taken randomly from leaves of both sets of plants and placed into culture. In less than 10 days, some of the disks from uninfected plants had expanded and grown much more rapidly than the virus-infected disks. Virus-infected disks were expanding and growing more slowly, and disks taken from leaves of different developmental states were responding differently to viral infection (Figure 3-1).

To investigate this phenomenon further, two factorial experiments were performed that included disks from leaves of different developmental state from healthy and infected plants. The data presented in Figures 3-2 and 3-3 show that, for the first three weeks after culturing, disks taken from uninfected leaves from the stem axis grew considerably faster than disks from virus -infected axis leaves. Disks taken from rosette leaves at the base of the plant grew more

slowly still, and infection by the virus did not affect their growth. In the fourth week of culture, the growth of disks from axis leaves of infected plants reached about the same level as that of the uninfected axis leaf disks. Thus, although growth rates of infected axis leaf disks were reduced significantly by virus infection, the weight at which further growth ceased was the same for uninfected and infected tissues.

The number of plantlets produced on each type disk is a parameter of practical interest. The results reported in Table 3-1 show that the presence of the virus significantly slowed the development of plantlets on disks from axis leaves. After three weeks, the number of plantlets on virus infected disks from axis leaves was only about 50 % of the number on disks from uninfected axis leaves. Plantlets developed even more slowly on disks from rosette leaves, but virus infection appeared to have no significant effect on regeneration from disks of this type.

These experiments show that both infection by TMV and the developmental stage of the host explant are important factors affecting the growth and regeneration of plantlets from cultured leaf disks of this species. Also, interactions between these factors can vary with different stages of growth of the host plants. Under conditions used here, then, a clear distinction between infected and uninfected tissues from axis leaf explants could be discerned. How the

virus reduces disk growth and plant regeneration is not known, but these effects might be a reflection of the way in which growth of whole plants is reduced by viruses. Use of axis leaf explants might be of value in studies of the interactions between TMV and N. sylvestris.

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Table 3-1. Effects of leaf age and viral infection on regeneration of plantlets from cultured leaf disks of Nicotiana sylvestris.^a

Days Cultured	Uninfected		Infected	
	Rosette	Stem Axis	Rosette	Stem Axis
21	7 ± 1	54 ± 4	12 ± 2	25 ± 3
28	34 ± 3	77 ± 5	46 ± 4	65 ± 3

^a Numbers are means and standard errors of average number of plantlets on six disks of different types at 21 and 28 days after initiation of cultures.

Figure 3-1. Ten day old leaf disk cultures from TMV infected Nicotiana sylvestris plants. H-Y: disks from uninfected stem axis leaves; V-Y: disks from infected stem axis leaves; H-O: disks from uninfected rosette leaves; V-O: disks from infected rosette leaves.

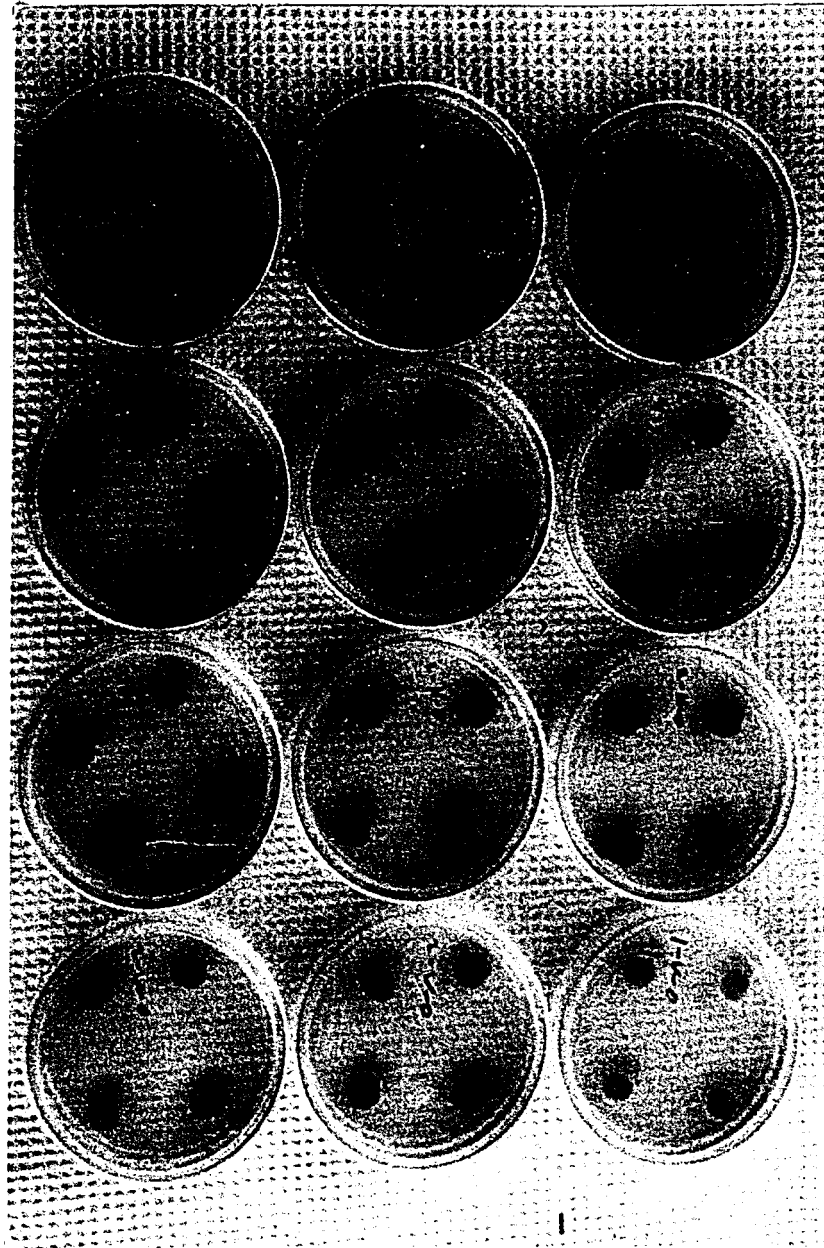


Figure 3-2. Wet weight (fresh) of leaf disks at various times after initiation of cultures. Closed squares: disks from uninfected stem axis leaves; closed circles: disks from infected stem axis leaves; open squares: disks from uninfected rosette leaves; open circles: disks from infected rosette leaves. Data are presented as means $\pm$ standard errors of means.

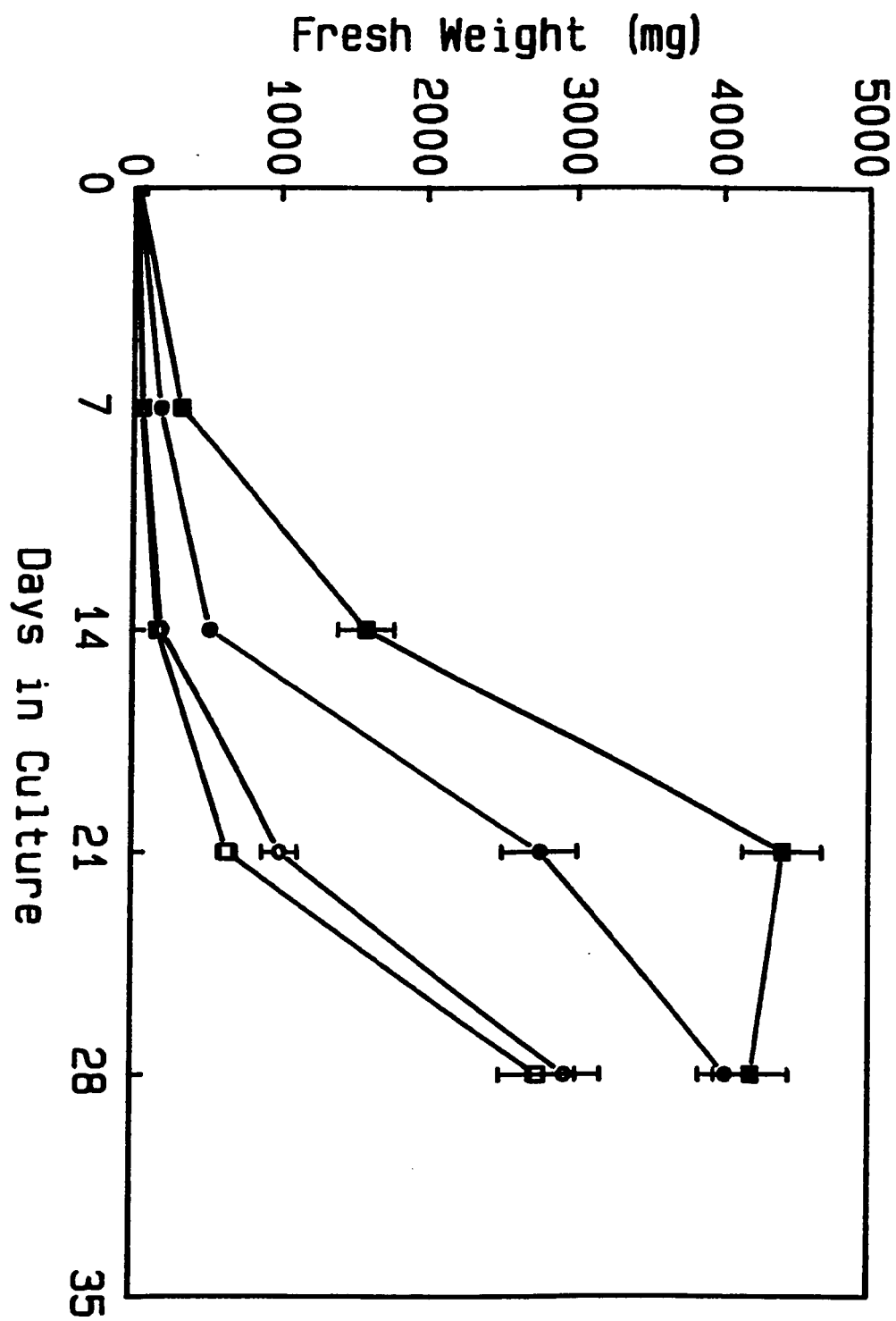
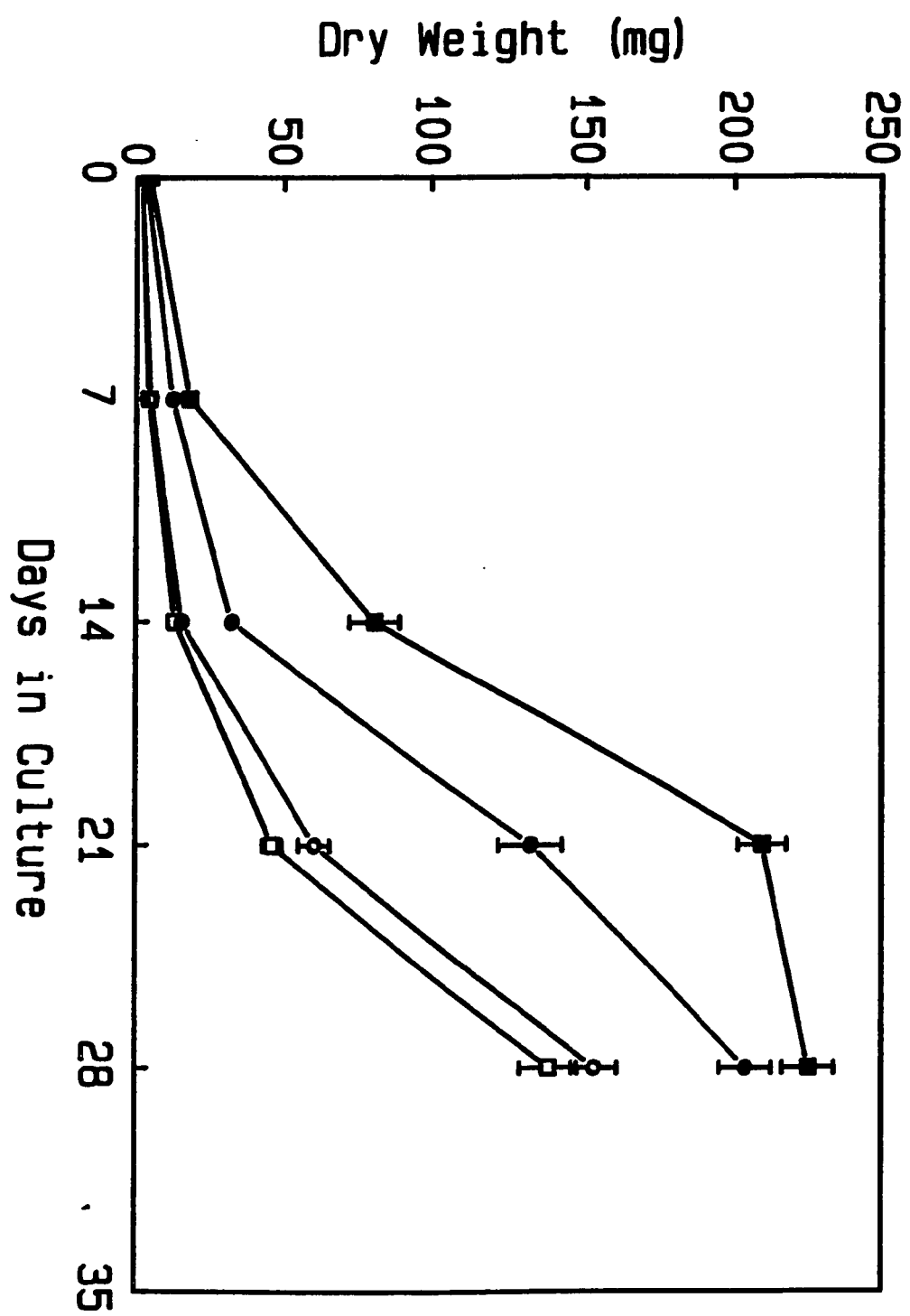


Figure 3-3. Dry weights of leaf disks at various times after initiation of cultures. Closed squares: disks from uninfected stem axis leaves; closed circles: disks from infected stem axis leaves; open squares: disks from uninfected rosette leaves; open circles: disks from infected rosette leaves. Data are presented as means $\pm$ standard errors of means.



CHAPTER IV

Accumulation of Necrotic Lesion Inducing Variants
in TMV Infected Plantlets Derived from
Leaf Disks of Nicotiana sylvestris

Abstract

In an isolate of TMV strain U1 there exists a small subpopulation containing a variant strain of the virus that induces the hypersensitive response on Nicotiana sylvestris Spegaz. & Comes. This type of variant (NL) is strongly selected for during the regeneration of plantlets from leaf disks of plants infected with TMV U1. Of 68 plants regenerated from infected leaf disks, 50 contained significant titers of NL-type virus. In these plants, relative titers of variant to wild type virus ranged from 0.1 to 1.5, in contrast with the original infecting U1 population, in which the relative titer of such variants is less than 0.0001.

When whole plants derived from disks were transferred into a glasshouse, those containing NL variants were severely stunted, showed mosaic symptoms, and most of them died. Some that had originally contained lower titers of NL-type virus survived to flower but produced only a few seeds. Plants that initially contained only wild type virus had high titers of TMV, survived and grew well, exhibited mosaic symptoms, and flowered and set seed normally. Repeated assays of virus in these plants revealed no detectable NL-type virus. Apparently, during callus development and organogenesis in culture, partial segregation of the mixed U1 population occurred, and NL variants preferentially infected the developing tissues.

This represents another situation in which dramatic change in the genetic structure of an RNA virus population occurs during development of the host plant.

Introduction

Selection for specific variants in an RNA virus population can be dramatic under certain conditions of replication. For example, Johnson (1947) found that passage of a severe strain of tobacco mosaic virus (TMV) through Eryngium aquaticum L. resulted in the selection and accumulation of a mild strain of the virus as infection progressed. Bald et al. (1974) showed that, after transfer of TMV strain U1 several times through Nicotiana glauca L., a strain indistinguishable from U5 became the dominant strain in the virus population. MacNeill and Boxall (1974) demonstrated that when genetically marked TMV strain 0 replicated in TMV susceptible tomato plants and moved across a graft boundary into TMV-resistant tissue, a new virus, which they called TMV 2, appeared in the resistant tissue; tomato plants that were resistant to TMV 0 were not resistant to TMV 2. Khan and Jones (1988) demonstrated that when leaves of N. sylvestris Spegazzini and Comes plants in the rosette form are inoculated with TMV U1 and then forced to bolt, strong selection for a variant that induces local lesions on N. sylvestris (NL variants), in which wild type TMV replicates systemically, occurs during replication of

the virus in the upper parts of the plants. Such effects collectively are called "host effects", and many other examples could be given (reviewed in Yarwood, 1979).

Mechanisms by which host selection effects occur are not understood. However, our previous work (Khan and Jones 1988) suggested that the selection for NL variants was related to developmental changes that take place when N. sylvestris plants initiate the flowering process. Although the detailed way in which this occurs is not known, the results clearly showed that strong "host effects" can occur even within one species of host plant when changes take place in the environment within which a virus replicates.

Because bolting of a plant involves rapid developmental changes in the plant, including cell division, expansion, and differentiation, it was of interest to determine whether accumulation of NL variants might occur in N. sylvestris under other conditions involving dramatic developmental changes. An obvious candidate is the regeneration of plantlets in vitro from TMV-infected leaf tissue. Tissue culture techniques have been used for many years to study plant virus biology, and they have been used successfully to obtain plants free of various viruses (e.g., Murakishi and Carlson 1976; Shepard 1975). It has also been reported that cultures of sugarcane tissues respond differently to different strains of sugarcane mosaic virus (Irvine and Benda 1985), which suggests that different variants of a

plant virus might behave differently during replication in cultured plant tissues. Beachy and Murakishi (1971) reported induction of local lesions in tobacco tissue culture with TMV infection.

We report here the results of experiments designed to determine whether the genetic structure of a TMV population is affected during the regeneration of N. sylvestris plantlets from infected leaf tissue placed into culture.

Materials and Methods

Plants

Three hosts were used, Nicotiana tabacum L. var. Xanthi, which carries the n allele (lack of hypersensitivity) (Holmes 1938); N. tabacum L. var. Xanthi-nc, which carries the N allele of this gene (hypersensitivity to TMV); and N. sylvestris Spegaz. and Comes, which carries the N' gene (hypersensitivity to some strains of TMV) (Valleau 1952; Valleau and Johnson 1943; Weber 1951). All plants initially were grown from seed under greenhouse conditions.

Virus

Virus strains used included TMV strain U1, a wild type strain that has been propagated for many years in Xanthi tobacco, and TMV204, a clonal population derived from transcripts of cDNA copies of the U1 genome (Dawson et al.

1986). Both these strains replicate systemically in N. sylvestris. Frequencies of NL variants in these stocks typically were less than 0.01 percent.

Virus was propagated in Xanthi tobacco for two to three weeks under greenhouse conditions. Leaves from infected plants were harvested, frozen at -20C, and used as a source of inoculum.

Purified virions (Gooding and Hebert 1967) were stored in 0.02 M sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.02 % sodium azide. Virus stocks were diluted in cold glycine -phosphate buffer just prior to use.

Plant material extracts were prepared in sterile mortars and pestles by grinding 1.0 g fresh weight tissue in 10 ml glycine phosphate buffer (50 mM glycine, 30 mM phosphate, pH 9.2) containing 1 percent (w/v) celite. TMV U1 infectivities were measured by local lesion assays on N. tabacum var. Xanthi-nc. Titers of variants that induce local lesions on N. sylvestris (NL variants) were determined by local lesion assays on at least three half -leaves each of Xanthi-nc tobacco and N. sylvestris. Infectivity assay plants were incubated at approximately 23C under fluorescent light.

Tissue Culture and Plant Regeneration

Disks were cut from infected rosette leaves with an 11-mm cork borer, disinfested, and placed directly onto

culture medium. Shoot-inducing medium contained MS salts (Murashige and Skoog 1962), 0.17 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3% sucrose, 1 mg/l thiamine HCl, 100 mg/l inositol, 1 mg/l each of naphthalene acetic acid (NAA) and benzyladenine, 80 mg/l adenine sulfate ($\cdot 2\text{H}_2\text{O}$), 100 mg/l L-tyrosine, and 0.8% Bacto-Agar (Difco). Root induction medium contained half strength MS salts, 1.5% sucrose, 100 mg/l inositol, 0.1 mg/l NAA, and 0.8% Bacto-Agar (Difco). Rooted plantlets were transplanted into perlite and placed under mist. After one week, plantlets were transferred to soil and left in mist for an additional week, after which they were moved to a greenhouse bench.

Results

Selection for NL Variants in Tissue Culture

As part of a preliminary experiment, disks from rosette leaves of nonbolting *N. sylvestris* plants infected with TMV U1 were cultured on shooting medium for 30-35 days, after which they contained large masses of callus and numerous small plantlets. Five such disks were chosen at random, 1.0 gram samples of the tissue masses were ground in 10 ml buffer, and the extracts were assayed for total infectivity and titer of NL variants. In all five, NL variants were present at frequencies much higher than in the initially cultured leaf tissue, in which they essentially were undetectable. In these five disks, relative virus titer

measured on Xanthi-nc tobacco was 189 ± 34 (mean $\pm$ SE) lesions per half leaf, while on N. sylvestris, titer at the same dilution was 64 ± 16 lesions per half leaf. Thus, NL variants apparently accumulated during the development of the callus and plantlet tissues in the leaf disks.

In another experiment, leaf disks from N. sylvestris plants infected with TMV U1 or TMV204 were placed onto shooting medium. After 3 weeks, 1.0 g samples from 10 randomly selected disks in each category were ground in 10 ml buffer and tested for presence of NL variants. All 10 disks from leaves of U1 infected N. sylvestris tested positive for NL variants. On Xanthi tobacco, the average titer was 118 ± 13 (mean $\pm$ SE) lesions per half leaf. On N. sylvestris, the average was 36 ± 9 . No NL virus was detected in disks infected with TMV204. A third experiment similar to these was performed, and the results were the same: NL variants accumulated only in tissues initiated from TMV U1-infected N. sylvestris leaves (data not presented).

We have previously shown that NL variants accumulate in leaves on the flowering axes of N. sylvestris plants infected with TMV U1 at the rosette stage and forced to bolt. The apparent accumulation of NL variants during the development of callus and the regeneration of plantlets from TMV U1 infected tissues from rosette leaves of the same species suggests that the responsible selective mechanism might be directly associated with the changes that occur

during the rapid development of plant tissues and organs both in vitro and in vivo. To further define the phenomenon, the fate of the virus populations in whole plants derived from the disk cultures was studied.

Nonbolting N. sylvestris plants were inoculated with TMV U1 and grown for 4 weeks. Disks were cut from systemically infected leaves of these plants and cultured on shooting medium. Parallel control cultures of disks from Xanthi tobacco plants infected with TMV U1, from N. sylvestris plants infected with TMV204, and from uninfected plants of both species also were established. When regenerated plantlets reached the 2-3 leaflet stage (approximately 5 weeks), 30 plantlets were removed from cultures of TMV U1 infected N. sylvestris tissue, and each was tested for relative concentrations of NL variants and wild type virus. Five of the 30 contained no detectable NL variants, but 25 contained variants in frequencies distributed over a broad range. On Xanthi-nc tobacco, relative titers ranged from 68 to 398 lesions per half-leaf (mean $\pm$ SE = 187 ± 15). On N. sylvestris, titers ranged from 0 to 132 lesions per half-leaf (mean $\pm$ SE = 67 ± 10). The ratio of infectivities on N. sylvestris to infectivities on Xanthi-nc ranged from 0 to 1.74, with mean and standard error of 0.54 ± 0.09 . In plantlets from control cultures, NL variants were not detected, and virus was not detected in plantlets from uninfected disks. These results confirmed

that NL variants accumulate during the regeneration and/or growth of plantlets from disks from TMV U1 infected N. sylvestris plants and showed that frequencies of NL variants in such plantlets can be highly variable, ranging from essentially zero to nearly 100 percent of the total virus titer.

A second set of 50 plantlets randomly selected from the TMV U1 infected N. sylvestris disks was transferred to rooting medium. Of these, 38 rooted and survived transfer to soil in pots in a glasshouse. Plants were transferred into the glasshouse in September and for the next six months remained in the vegetative state (rosette form). Subsequently, in March, in response to favorable light conditions, some of the plants bolted. During this time and afterwards, assays of relative total virus titer and titer of NL variants in the plants were performed.

Of the 38 plants that survived transfer to the glasshouse, 25 contained significant quantities of NL variants. These plants first were assayed when they had grown sufficiently that samples could be obtained without killing the plants. All 25 plants had high total titers of virus as assayed on Xanthi-nc tobacco at the standard dilution (mean $\pm$ SE = 45 ± 4 lesions per half leaf; range = 12 - 98 lesions per half leaf). On N. sylvestris, titers averaged 17 ± 2 lesions per half leaf, with a range of 7 -31 lesions per half leaf. In individual plants, the ratio of

titer on N. sylvestris to titer on Xanthi-nc ranged from 0.1 to 1.5 (mean $\pm$ SE = 0.5 ± 0.1). Thus, the distribution of relative frequencies of NL variants in these plants was not greatly different from that in the 30 plantlets described above. A plot of the distribution of NL variants in plantlets regenerated in both these experiments combined (68 total) is shown in Figure 4-1. These results show that selection for the NL variant type must occur during organogenesis and/or subsequent development of plantlets in vitro. Alternatively, random segregation of NL and wild type virus might occur during the initial stages of infection of the developing plantlets. However, the high frequency of occurrence of plantlets carrying NL variant virus favors the existence of an active selection process.

All the plants that were transferred into the glasshouse were allowed to continue growing. With time, plants that carried NL variants became stunted, some of them severely. The degree of stunting in individual plants was correlated approximately with the relative titer of NL variants in the plants. Nineteen of the plants containing NL variants died during the six months following transfer to the glasshouse. Six of them, although stunted, matured, bolted, and produced a few seeds.

In contrast, all 13 of the plants that carried no NL variants grew well, bolted in March, and produced abundant quantities of seed despite containing high titers of TMV

(mean $\pm$ SE = 35 $\pm$ 4 lesions per half leaf) and showing typical mosaic symptoms. After they bolted, no NL variant virus was detected in leaves on the stem axes of plants that earlier were shown to contain undetectable levels of NL variant virus, even though virus titers were high in them. Figure 4-2 shows the difference in symptoms exhibited by plants carrying NL variants compared with those that did not.

Effect of Leaf Age on NL Variant Accumulation

Previously, we showed that the selection and accumulation of NL variants during bolting of *N. sylvestris* plants lead to a gradient in frequency of variants in leaves along the developing stem axis; higher frequencies occurred in leaves higher on the axis. The selection process appeared to involve preferential replication and/or systemic movement of NL variants compared with wild type virus in the developing tissues. The situation described here is somewhat different, because bolting per se is not involved. The selection process occurs during organogenesis, perhaps at the cellular level, and virus moves systemically through the regenerated plants without the influence of bolting. The end result is rosette form plants that are not bolted but that contain high relative concentrations of NL variant virus. The question arises, then, as to whether younger leaves on these plants contain relatively higher frequencies

of variants, as is the case on bolted plants.

As stated above, these plants were severely stunted, and most of them died within a few weeks. But after four months in the glasshouse, 9 of the 25 were still alive and had periodically produced new leaves. Leaves of different ages in the rosettes of these plants (which had not bolted) were assayed for total virus titer and for titer of NL variants (Table 4-1). Total virus content of different aged leaves were not greatly different, but the relative content of NL variant virus increased in the younger leaves. Thus, even in the absence of bolting, NL variants accumulated during the development of younger leaves in N. sylvestris.

Segregation of Components of the Virus Population during Organogenesis

As discussed above, thirteen plants that originated from infected leaf disks and survived transplanting into the glasshouse carried undetectable amounts of NL variant virus. Two possibilities might explain this observation; these plants might be resistant or immune to NL variant type virus, or they might not initially have been infected with NL variant virus during organogenesis.

To determine whether the plants were resistant to this variant class, the following experiments were performed. First, the plants were inoculated with a purified NL variant (NL-18, Khan and Jones 1988) . No local lesions formed.

However wild type virus in the plants probably interfered with the expression of lesion formation under these conditions. In addition, leaf explants from these plants were placed in culture, plantlets were regenerated from them and tested for resistance to NL variant virus. No clear evidence of resistance was observed. Finally, seed progeny of several of the NL variant-free plants as well as those from the 6 NL-infected plants that survived to produce seed were tested for resistance. Again, no unequivocal indication of resistance to either wild-type or NL-variant virus was observed. These tests were not exhaustive, but, it is likely that the 13 plants containing no NL variants were infected solely with wild type virus during organogenesis in culture and thereafter acquired no NL variant virus either by infection or mutation. Thus, segregation of components of the original virus population in the cultured disks during development of individual plantlets occurred. That such is the case also is indicated by the fact that in one instance, two plantlets used in the study originated from the same leaf disk; one of them contained no NL variant virus, while the other was heavily infected with the variant type.

Green Leaf Phenomenon

As described earlier, plants systemically infected with NL-type virus during regeneration subsequently were severely

stunted when cultured in the greenhouse (Figure 4-2). Plants containing wild type virus were not stunted, but both types of plants showed extensive mosaic symptoms characteristic of TMV infection. Leaves on most of the plants containing NL-type virus subsequently developed severe necrosis, leading to the death of the plants. However, six plants survived and ultimately bolted, flowered, and produced a few seeds. On three of these plants, an occasional dark green leaf developed that did not exhibit mosaic symptoms (arrow, Figure 4-2). These leaves grew to be much larger than other leaves on the same plants, but they never developed mosaic symptoms, even though the majority of leaves on the same plants were heavily mosaiced and stunted. One of the three plants produced three dark green leaves intermittently along the developing stem axis.

Tissue samples from the dark green leaves were tested for the presence of TMV and found not to be free of the virus, although the total virus titer in them was lower than in younger or older (mosaiced) leaves on the stem axis (Table 4-2). Titers of NL-type virus were not greatly different in dark green or mosaiced leaf tissue; in both types of tissue, the percentage NL-type virus was less than five percent, which perhaps explains why these particular plants survived.

The question immediately arises as to whether the dark green leaf tissues are derived from cells that have

undergone somatic mutation leading to lack of development of symptoms (Avery, 1933; Stewart and Derman 1975), or whether the lack of symptom development is epigenetic. To generate plants with which to answer this question, disk explants from a dark green leaf were cultured on the standard medium used to produce regenerated shoots. Contrary to prior experience, these cultures failed to produce shoots; only a few roots developed. A second set of cultures was established that included leaf disks from a dark green leaf, from a mosaiced leaf, and from a leaf of an uninfected plant. The dark green leaf disks again produced roots, while shoots were produced on the other disk types. When disks from dark green leaves were placed onto medium in which the concentration of auxin was reduced by 50%, they produced both shoots and roots. These results suggest that the green leaf explants had altered hormonal metabolism compared with tissues in the other leaf types.

Shoots taken from each of three types of disks were transferred to a medium on which rooting occurred and finally into pots in a greenhouse. Thirty plants from the dark green leaf source and 10 each from the mosaiced and uninfected disks were used. All 30 plants from green leaf explants and the 10 from the mosaiced disks showed mosaic symptoms characteristic of TMV infection. On only two of the 30 plants derived from a dark green leaf was an occasional green leaf produced, which again contained

greatly reduced titers of virus. When challenged with a purified NL variant (NL-18; Khan and Jones 1988), local lesions formed on these green leaves as efficiently as on unmodified N. sylvestris leaves.

Discussion

In this report, we have shown that NL variants preferentially infect plantlets regenerated from cultured leaf disks from N. sylvestris plants infected with a wild type population of TMV U1. In some plantlets, nearly all the virus is of the variant type. Therefore, the selective pressure must be considerable, because in the original U1 population, the frequency of such variants is less than 0.01 percent. During callus and plantlet formation, competition between wild type and NL-type virus is heavily in favor of the variant, although most regenerated plants contain virus of both types.

This selective action leads to the generation of populations of plants many of which bear TMV populations with dramatically altered genetic substructures. The selective advantage of NL-type virus under these conditions is similar to that described in an earlier paper, in which we showed that NL virus preferentially infects the tissues of the flowering axis of N. sylvestris when they flower (Khan and Jones 1988). In both cases, NL-type virus is much more successful at invading rapidly developing tissues than

is wild type virus. In neither situation is the mechanism of selection known; neither is it yet established whether the variants that are selected for during bolting are genetically the same as those that are selected for during regeneration. It is possible that different genetic lesions are involved, both of which pleiotropically yield the lesion induction phenotype. Detailed comparisons of the genomes of such variants, especially of those constructed by directed mutagenesis, will provide insight into the relationships between viral and plant functions that lead to the phenomena described here.

In whole plants developed from the regenerants, NL virus replicates systemically, in contrast to the usual formation of lesions induced by NL virus if it is inoculated onto leaves of this species. Presumably, the action of the N' gene that leads to restriction of systemic movement of this type virus is bypassed when young plantlets are infected systemically during regeneration. Once the infection by NL virus is established, systemic necrosis and accelerated plant death frequently occur as the plants continue to grow. The presence of the variants in high frequency also leads to stunting of the host plants. Plants containing NL variants at a lower frequency survive to flower and produce seed, although seed set is severely reduced. Thus, the severity of effect of the virus infection appears to be correlated with the prevalence of NL

virus in the infecting population.

Some regenerated plantlets contained wild type TMV with undetectable quantities of NL-type virus. They grew normally, although they showed the mosaic symptoms typical of TMV infection. When they were induced to bolt, leaves on the flowering axes of these plants contained no detectable NL-type virus, in contrast to the accumulation of NL variants that occurred when rosette form plants inoculated with the initial stock of TMV U1 (Khan and Jones 1988) bolted. Thus, it appears that these plants become infected with a subpopulation of virus that contained no NL variants. All these results taken together show that dramatic changes in the structures of virus populations in individual plants can occur when the plants undergo developmental change.

Another unusual feature of this work was the occurrence of symptomless leaves on plants that were heavily infected with a mixture of wild type and NL-type virus. Virus titers in these leaves were substantially reduced, and exploratory experiments suggested that the phenomenon was epigenetic, perhaps involving altered hormone metabolism in the individual leaves. Certainly, the leaves had not escaped viral infection. In some respects, the phenomenon resembles the "green island" phenomenon described by Matthews and his coworkers (Atkinson and Matthews 1970; Chalcroft and Matthews 1967; Matthews 1973; Reid and Matthews 1966). Their work showed that interactions between different virus

genotypes and different host cell lineages can lead to segregation of viral genotypes and consequent differential effects on regions of the leaves of infected host plants. Whether the "green leaf" phenomenon is related to such an effect remains to be determined.

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Table 4-1. Relative titers of NL variants in leaves of different ages on plants infected with TMV during regeneration from infected leaf disks of Nicotiana sylvestris.

Leaf Number ^a	Assay Host ^b	
	Xanthi-nc	<u>N. sylvestris</u>
1	63 ± 5	11 ± 2
5	48 ± 4	16 ± 3
10	54 ± 4	29 ± 3

^a Titers were determined several months after plants were transferred into the glasshouse. Leaf 1 was the oldest living leaf on the plant.

^b Values are means and standard errors of triplicate assays of samples of leaves from 9 plants.

Table 4-2. Relative titers of wild type and NL-type virus in dark green leaves and in mosaiced leaves immediately adjacent.

Tissue ^a Source	Assay Host ^b	
	Xanthi-nc	<u>N. sylvestris</u>
Older leaf	43 ± 8	2 ± 0.7
Green leaf	13 ± 3	1 ± 0.2
Younger leaf	55 ± 13	1 ± 0.4

^a Titers were determined several months after plants were transferred into the glasshouse.

^b Values are means and standard errors of triplicate assays of samples from five dark green leaves and the immediately adjacent younger and older mosaiced leaves on three different plants.

Figure 4-1. Frequency of NL variants in regenerated plants from U1 infected leaf disks of N. sylvestris. Frequencies of NL variants were determined in a total of 68 plants. Vertical axis: percent of plants in each class of variant frequencies. Horizontal axis: classes of percentage NL variants, as follows. Class 1: 0-17%; class 2: 18-35%; class 3: 36-53%; class 4: 54-71%; class 5: 72-89%; class 6: 90-107%; class 7: 108-125%; class 8: 126-143%; class 9: 144-161%; class 10: 162-179%.

Frequency of Plants with NL Variants (%)

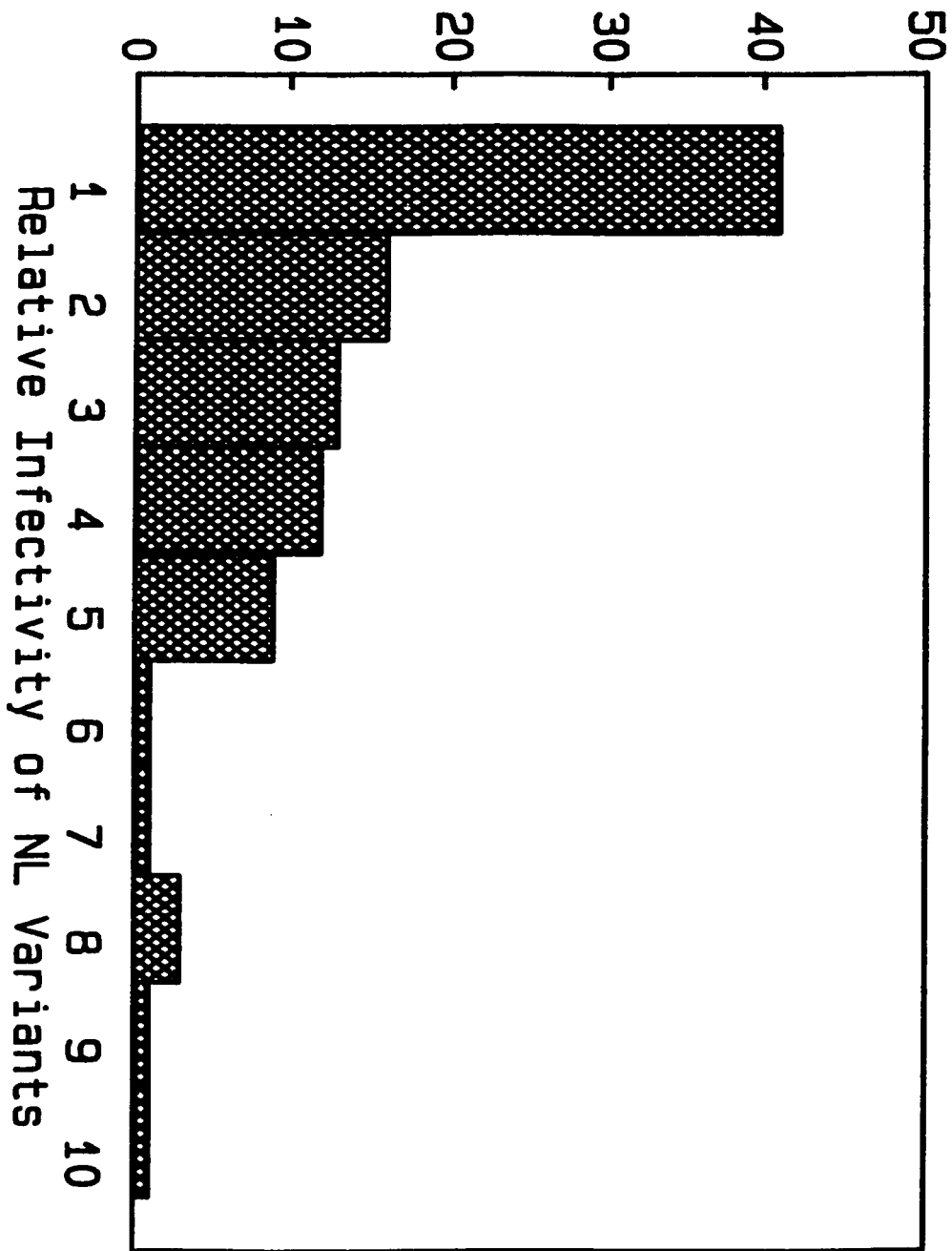
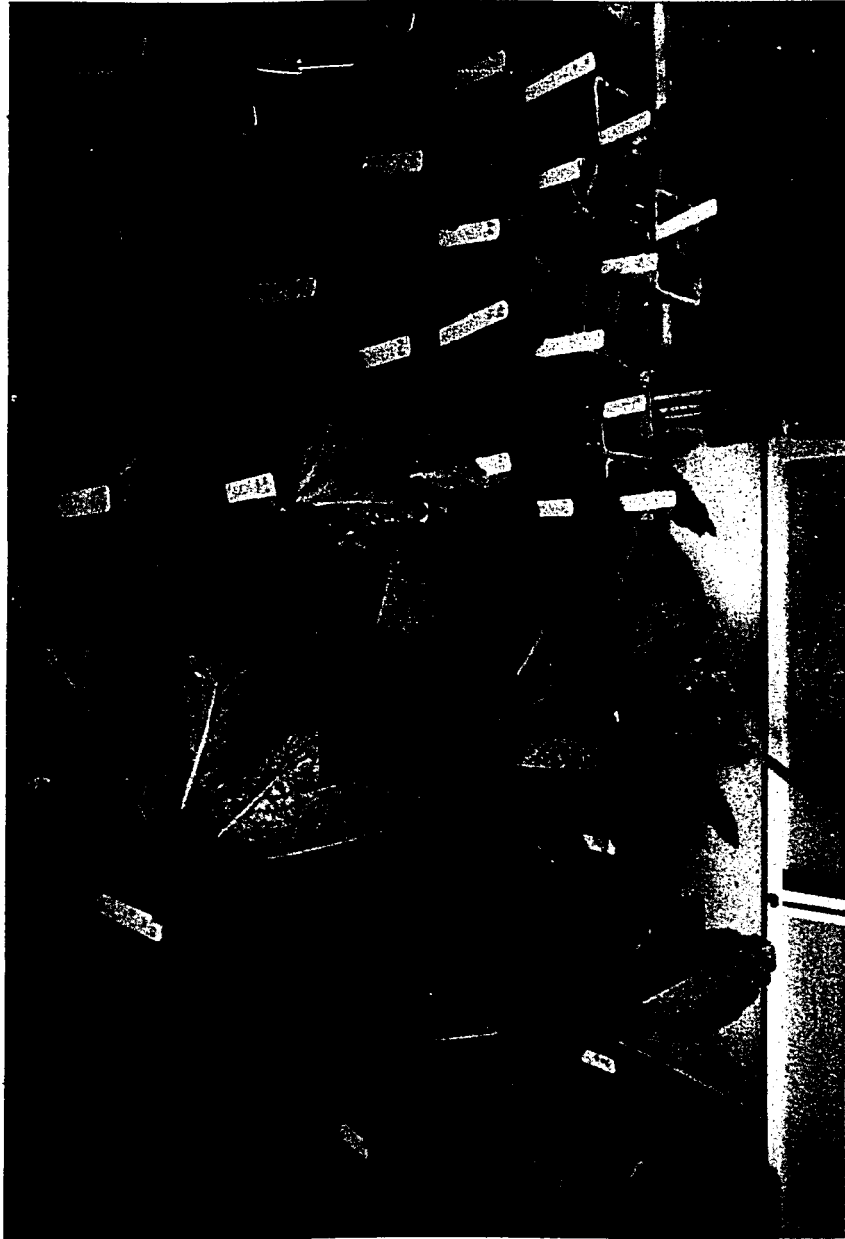


Figure 4-2. Two classes of *N. sylvestris* plants regenerated from U1 infected leaf disks. Mosaic symptoms are clearly visible in both classes. Plants on the left contain NL-type virus and are severely stunted. Plants on the right contain only wild type virus. The white arrow indicates a dark green leaf that developed on one of the plants that contained NL-type virus.



CHAPTER V

General Conclusion

General Conclusion

Protection of crop plants against diseases and pests is a significant problem. Virus diseases of plants are an especially serious threat to highly priced horticultural products. In the absence of chemical control, they can at best be managed by using genetic resistance and/or by cultural practices. High rates of success for such control measures can be assured if a precise understanding of the biology behind plant-virus interaction is developed.

One important issue is the emergence of new viruses and more virulent strains of existing viruses. The evolutionary genetics of this phenomenon is very poorly understood. A limitation in the study of viral genetics is the fact that viruses are obligate parasites and can only be studied in an appropriate host. A host-virus system is needed in which controlled experiments can be performed to investigate the evolution of viruses. Recent advances in the molecular biology of viruses (cloning and sequencing), particularly tobacco mosaic virus (TMV), offers an opportunity to genetically mark the virus and construct artificial populations. What is needed is a well defined system in which host effects are exerted predictably and with which the environment can be altered and controlled. Using such a system, the fate of genetically marked populations could be investigated.

In the present work, the interaction of TMV with one of

its hosts, Nicotiana sylvestris (which carries the N' gene) was investigated. This host allows systemic replication of wild type TMV strain U1 but shows the hypersensitive response to some mutants and strains of this virus. This response is manifested by the induction of local lesions, by which the virus is contained in the small leaf area defined by the lesion. The data presented in chapter II show that a class of variants (NL for necrotic lesions) is strongly selected for during the process of bolting. The variant is capable of inducing necrotic local lesions on the same host as long as plants are kept in the rosette form. The local lesion barrier appears to be broken by the developmental switch associated with bolting in this species. Also, selection leads to the development of a gradient in frequency of NL variants along the axis of bolted plants (Figure 2-1). The bolting process in N. sylvestris can be controlled by light and/or gibberellic acid application. The selection for NL-type virus occurred in a population of the U1 strain of the virus and dramatically altered the population structure of the virus.

A series of NL-type variants was isolated by seven sequential single lesion transfers through the differential host, each time selecting for lack of systemic spread in rosette leaves as a test for the absence of wild type virus. One variant of this class, NL-18, was thoroughly examined. NL-18 alone or in a mixture with U1 was shown to be selected

for during the process of bolting and yet produce local lesions on rosette leaves of plants. A critical host range survey (Table 2-3) revealed that it is distinct from other similar strains of TMV, in particular U2/U5, ToMV, and TMV-o. A dot-blot hybridization assay using a 1300 nucleotide long cDNA probe complementary to part of the 30K and coat protein genes of pTMV204, a clonal version of U1, indicated that NL-18, although related to U1, has undergone major genomic changes (Figure 2-2).

All selection experiments included two kinds of controls. First, pTMV204 was used to inoculate N. sylvestris, and second, U1 was inoculated onto N. tabacum. None of the control tests yielded selection for NL-type virus. This implies that the selection phenomenon reported here is specific to the U1 population of TMV replicating in N. sylvestris. The NL-type virus exists as a subpopulation in TMV U1, is initially present at low frequency in U1 populations (less than 0.01% by other estimates), but is strongly favored by replication and/or movement into the bolting axis. Since the bolting process is easily controlled, this system is one with which the evolutionary genetics of virus populations can be studied by using genetically marked virus populations.

In the second part of the study, leaf disk cultures were initiated from infected and uninfected N. sylvestris plants in a factorial design. Leaves from rosette and stem

axis were used as the source of explants. It is generally agreed that viral infection does not significantly reduce the growth of plant tissues in culture and does not affect the ability to regenerate plantlets from somatic tissues in culture. The results of the factorial experiment reported in chapter III indicate that the disks taken from uninfected leaves from the stem axis grew considerably faster than disks from virus -infected axis leaves for the first three weeks in culture. Disks from rosette leaves grew more slowly, and infection did not affect their growth. However, rosette leaf disks in both categories were still in exponential phase of growth at the end of four weeks, when these experiments were terminated (Figures 3-2, 3-3). The results reported in Table 3-1 show that the presence of the virus significantly lowered the number of plantlets regenerated on disks from axis leaves. This effect of the virus was not significant on rosette leaf disks. These results show that the developmental stage of plant tissues used in culture can have a significant effect on the outcome of experiments involving viruses; such effects might be exploited in further studies of host-virus interactions.

The third part of this research showed that the selection for specific virus variants under the influence of developmental changes taking place in the host plant can be studied in a tissue culture system. Rosette leaves of N. sylvestris are in a terminal stage of development. Explants

taken from this source can be induced to undergo a variety of developmental changes under precisely controlled conditions. Thus, a set of tissue culture experiments was initiated in which rosette leaves of U1-infected or TMV204-infected N. sylvestris and U1-infected N. tabacum plants were used as a source of disks along with healthy controls. The data in chapter IV show that NL variants preferentially infect plantlets regenerated from cultured disks of N. sylvestris plants infected with TMV U1. In some plantlets, nearly all the virus is of the variant type (Figure 4-1). During callus and plantlet formation, competition between wild type and NL-type virus is heavily in favor of the variant, although most regenerated plants contain virus of both types.

Two classes of regenerated plants emerged, one with and one without NL-type virus. Plants containing NL-type virus were severely stunted, and most of them died prematurely. Plants without nl-type virus grew well (Figure 4-2). Selection of nl variants under the influence of bolting did not take place in the second class of plants, even after extended period of growth. Thus, during infection of these plants while they were regenerating, no NL virus invaded them, and none arose afterwards. This is a case of genetic segregation of two subpopulations of the original U1 virus population. These results also support the original contention that U1 carries a subpopulation in very low

proportion that is selected for under certain conditions of replication and that this type variant does not result from a recent mutation.

In regenerated whole plants and in the flowering axes of bolting plants, the NL-type virus replicated systemically. Presumably, the action of the N' gene that leads to restriction of systemic movement of this type of virus is bypassed when young plantlets are inoculated systemically during regeneration or during the switch to the bolting phase of whole plants.

Another unusual feature of this work was the occurrence of symptomless leaves on plants that were heavily infected with a mixture of wild type and NL-type virus. Virus titers in these leaves were substantially reduced, and exploratory experiments suggested that the phenomenon was epigenetic, perhaps involving altered hormone metabolism in the individual leaves. In some respects, it resembles the "green island" phenomenon described by Matthews and his coworkers.

The selective advantage of NL-type virus under the influence of bolting and during the process of regeneration is similar. In both cases, NL-type virus is much more successful at invading rapidly developing tissues than is wild type virus. In neither situation is the mechanism of selection known; neither is it yet established whether the variants that are selected for during bolting are

genetically the same as those that are selected for during regeneration. It is possible that different genetic lesions are involved, both of which pleiotropically yield the lesion induction phenotype. Detailed comparison of the genomes of such variants, especially of those constructed by directed mutagenesis, will provide insight into the relationships between viral and plant functions that lead to the phenomenon described here.

APPENDICES

Appendix A

Tobacco Mosaic Virus

The electron micrograph shown in Figure A-1 is a 100,000-fold magnification of Tobacco Mosaic Virus (TMV), one of the most important and best studied plant viruses. It is the type member of the tobamo virus group. The TMV particle is rod shaped; has 6395 nucleotides and four major genes in its RNA genome; has a coat protein made up of 2130 subunits, each containing 158 amino acids; is 3000 A long; and has a diameter of 180 A. The assembly process of the particle is well studied. There is a 40 A central hollow core around which the RNA is helically wound.

TMV is a mechanically transmitted virus. It is highly stable at a wide range of pH (3-9) and temperature. It has an extremely wide host range, including several crop plants. The Solanaceae family is the primary host for this virus. Other important families include the Cucurbitaceae, the Brassicaceae, and the Rosaceae.

Figure A-1. An electron micrograph of TMV at 100,000x magnification. A U1 virus preparation was purified by high speed sucrose gradient centrifugation and then mounted onto an electron microscope grid to visualize and photograph.



Appendix B

Biological and Physical Characterization of NL-18

NL-18 was isolated from the U1 population on the basis of its accumulation along the axis of bolting Nicotiana sylvestris plants. It was purified through seven sequential single lesion transfers on rosette leaves of N. sylvestris plants. The critical test for purity was the nonsystemic spread of virus in rosette phase plants. The U1 strain of TMV replicates systemically in such plants. NL-18 is one of 18 such isolates. NL-18 was further propagated in N. tabacum L. var. Xanthi for 3 weeks in the green house, as was U1. The leaves were harvested and both types purified. Spectroscopic absorbance at 260 nm and 280 nm and the 260/280 ratio showed the preparations to be highly pure. (Table B-1). The yield of the two viruses was 118.3 mg and 117.8 mg per 100g of fresh weight for NL-18 and U1, respectively. To verify the physical nature of NL-18, the virus was visualized under an electron microscope. Typical TMV rods are unmistakable (Figure B-1).

A series of dilutions was run for both NL-18 and U1 to determine the relative infectivity and appropriate concentrations of virus for further experimentation. The data plotted in Figure B-2 indicate a lower relative infectivity for NL-18 compared to U1. However, the kinetics of infection for both are similar.

Additional biological characterization of NL-18 was

performed by carrying out a detailed host range survey. Plants for this series of tests were grown in the green house. The actual tests were performed at room temperature under fluorescent lights. Purified stocks of both viruses were used for infections, using 10 micrograms/ml of virus. This concentration of U1 was shown to be sufficient to produce 40-60 local lesions per half leaf of Xanthi-nc tobacco at room temperature under fluorescent lights. Table B-2 presents the results of a host range survey for both viruses and shows the close relationship between two.

Table B-1. Spectroscopic analysis of NL-18 and U1.

Virus	Dilution	A260	A280	260/280
NL-18	1/100	0.507	0.410	1.219
NL-18	1/50	0.997	0.815	1.222
U1	1/100	0.505	0.419	1.205
U1	1/50	0.935	0.774	1.208

Table B-2. A host range survey of NL-18 and U1.

Host	U1 ^a	NL-18	Remarks
<u>N. sylvestris</u>	SYS	LL	U2/U5, ToMV, and TMV-o induce LL as well.
<u>N. tabacum</u> Xanthi	SYS	SYS	U2/U5, ToMV, and TMV-o spread SYS as well.
<u>N. tabacum</u> Xanthi-nc	LL	LL	U2/U5, ToMV, and TMV-o induce LL as well.
<u>N. glutinosa</u>	LL	LL	U2/U5, ToMV, and TMV-o induce LL as well. The lesion morphology varies.
<u>Lycopersicon</u> <u>esculentum</u>	SYS	SYS	U2/U5 does not infect.
<u>N. glauca</u>	Infects poorly	Infects poorly	Natural host for U2/U5.
<u>N. benthimiana</u>	Lethal necrosis	Lethal necrosis	Natural host for TMV-o.
<u>Petunia</u> <u>hybrida</u>	SYS	SYS	ToMV induces LL.
<u>Phaseolus</u> <u>vulgaris</u> (Pinto)	LL	LL	U1 produces numerous LL faster.
<u>N. rustica</u>	LL	LL	Frequency of LL is less than for other LL hosts.

Table B-2 (continued).

<u>Datura</u>	LL	LL	Many LL.
<u>stramonium</u>			
<u>Capsicum</u>	LL	LL	-
<u>annuum</u>			
<u>Chenopodium</u>	LL	LL	Many LL.
<u>quinoa</u>			
<u>Chenopodium</u>	LL	LL	Few LL.
<u>amranticolor</u>			
<u>Phaseolus</u>	-	-	No symptoms.
<u>vulgaris</u> var.			
Lake Shasta			
<u>Physalis</u>	SYS	SYS	-
<u>floridana</u>			
<u>Solanum</u>	SYS	SYS	-
<u>nigrum</u>			

^a SYS = systemic spread. LL = Local lesions.

Figure B-1. An electron micrograph of NL-18
(100,000x magnification).

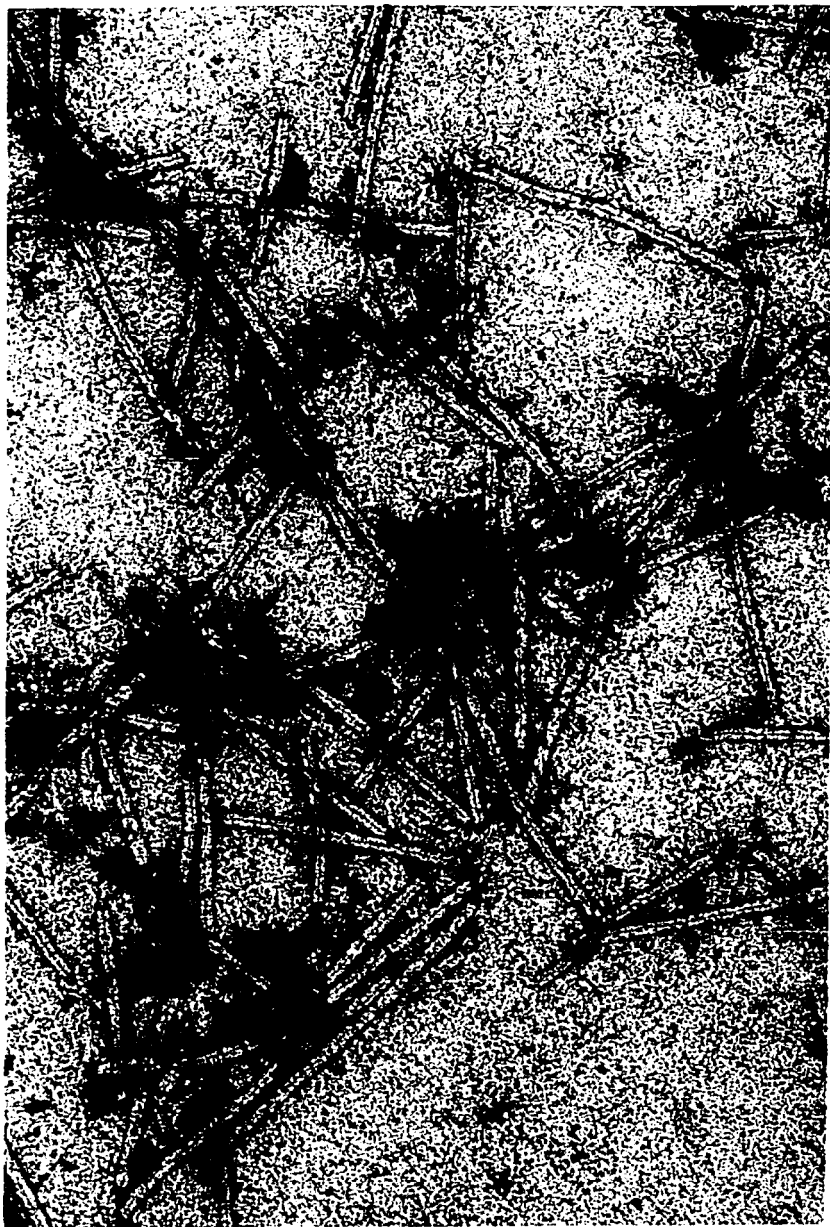
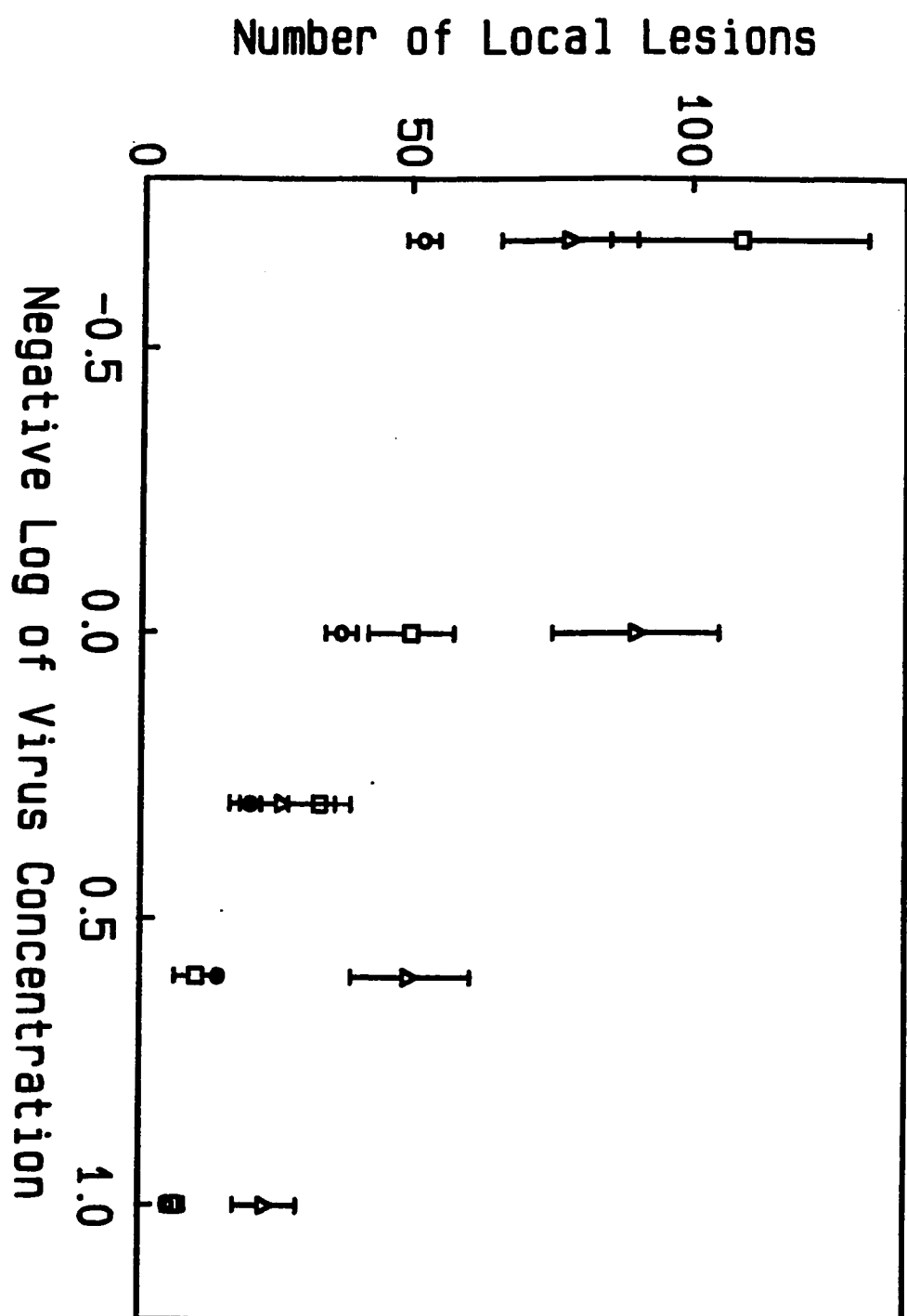


Figure B-2. Dilution curve for U1 and NL-18.
Triangles are U1 on Xanthi-nc; squares are NL-18 on
Xanthi-nc; circles are NL-18 on Nicotiana sylvestris.



Appendix C

Reconstruction of Virus Populations

The data presented in chapter 2 demonstrated that NL-type virus(es) exist as a subpopulation in the wild type stocks. Being at extremely low concentrations, such variants cannot be detected. It also has been shown that when, selection takes place, the population structure is radically altered. The availability of a pure NL-type population and clonal stocks of U1 offers an opportunity to construct synthetic virus populations and monitor changes caused by the selection process. It can be further used to study the evolutionary phenomenon under controlled conditions.

Two kinds of preliminary experiments were performed. The data in Figure C-1 represent the results of a series of differential inoculations. Nicotiana sylvestris plants were preinfected with NL-18 or U1, followed by infection with the other strain at different time intervals. The concentration of virus was sufficient to yield 40-60 lesions per half leaf on Xanthi-nc tobacco (1X concentration) in both cases. The local lesion response from NL-18 infection was quantified. Open circles are data from a preinfection with NL-18 and open squares the preinfection with U1. The slope of the regression line in the first case is $-.0287$, and the Y intercept is 30.26. A subsequent infection with U1 does not appear to inhibit the formation of local lesions by NL-18 (r

= -0.247). The lesions had already formed by 96 hours, when the experiment was stopped. However, in the second case, the influence of preinfection was significant. The presence of U1 in the host plant progressively inhibited lesion formation after NL-18 infection. The slope of the regression line is -0.0744 and the Y intercept is 23.98. The correlation coefficient (-0.886) is significant.

Another kind of reconstruction series was developed by mixing a range of NL-18 from 5X to 1/1000,000X with 1X U1 and testing for local lesions on Xanthi-nc and N. sylvestris. The data plotted in Figure C-2 show that the ability of NL-18 to induce lesions at or below 0.1X is severely inhibited.

Figure C-1. Differential inoculation. Several N. sylvestris plants were preinfected either with U1 or with NL-18 at time zero with a 1X concentration of each. Subsequently, inoculations with the other strain were made to sets of 3 plants each every 24 hours. Numbers of local lesions were counted and plotted as a function of hours after preinoculation. The open circles are data from preinfection with NL-18 and open squares represent preinfection with U1. The straight lines represent a linear regression curve.

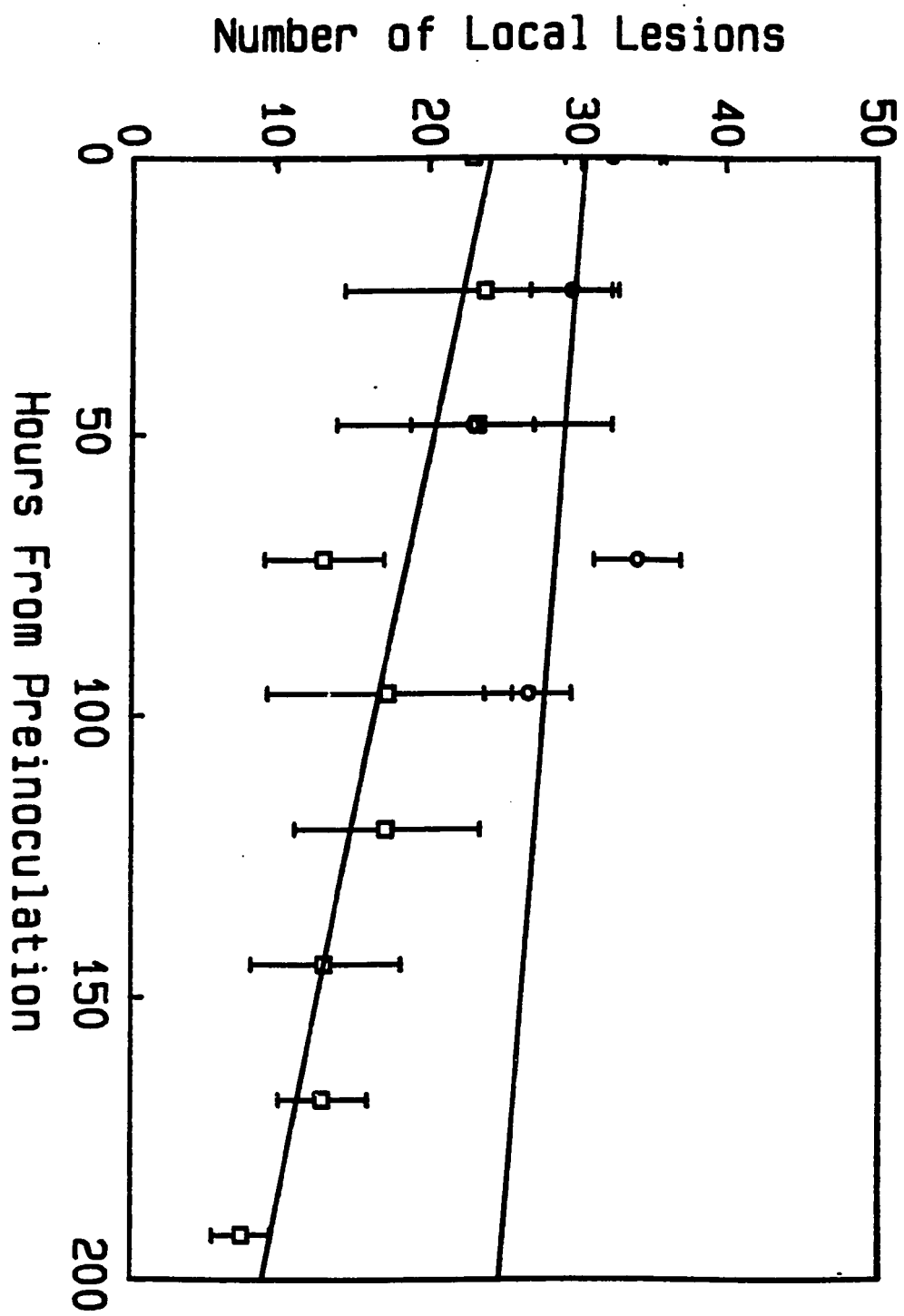
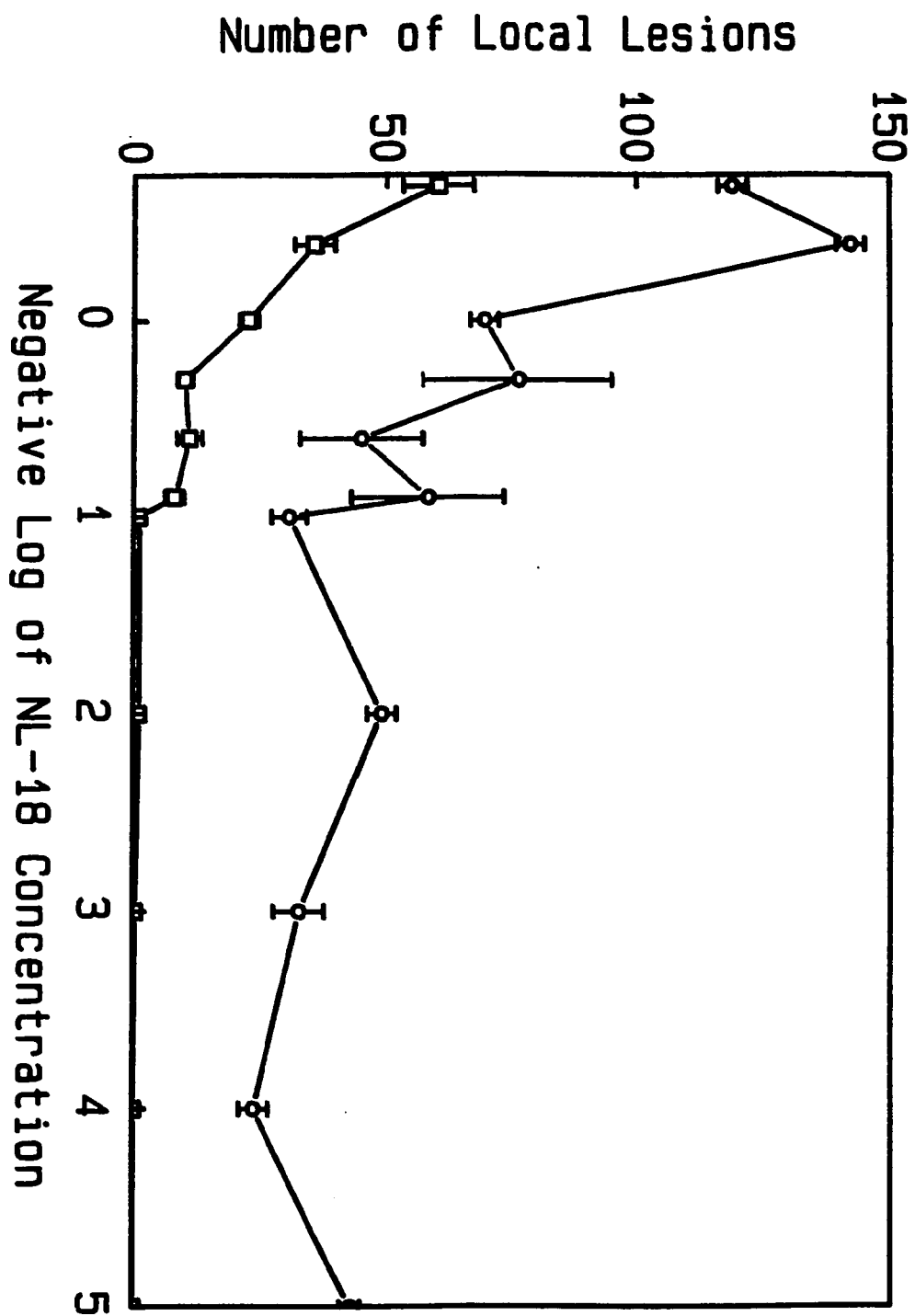


Figure C-2. Mixed inoculation. A constant 1X quantity of U1 was mixed with serially diluted NL-18. N. sylvestris and Xanthi-nc plants were infected in replicates of 3 plants for each combination. Number of local lesions was plotted as a function of the negative logarithm of NL-18 concentration. The zero on the x axis represent the 1X concentration of NL-18. Open circles represent local lesion on Xanthi-nc and open squares represent local lesions on N. sylvestris.



Appendix D

Accumulation of NL-type viruses in Tissue culture

As reported in chapter IV, NL-type TMV can accumulate in plantlets derived from tissue culture. Most such plants died. The surviving plants showed an interesting aspect of plant-virus interaction. As shown in Figure D-1, a gradient for accumulation of NL-type virus developed along the axes of surviving plants. The wild type virus content, as reflected here in terms of local lesions formed on Xanthi-nc tobacco, are relatively constant along the axis. Another interesting feature shown by the surviving plants was their ability to select against the accumulation of NL-type virus as they aged. The data plotted in Figure D-2 shows a significant decline in NL-type virus when the plants were assayed at 40 weeks of age.

Figure D-1. The gradient of NL-type virus along the axis of surviving regenerated plants of N. sylvestris. Leaves were sampled at positions 1, 5, and 10 and assayed on Xanthi-nc tobacco for total infectivity and on N. sylvestris for relative concentration of NL-type virus. The checked bars represent local lesions on N. sylvestris, and the slashed bars represent local lesions on Xanthi-nc tobacco. Error bars represent standard errors of means.

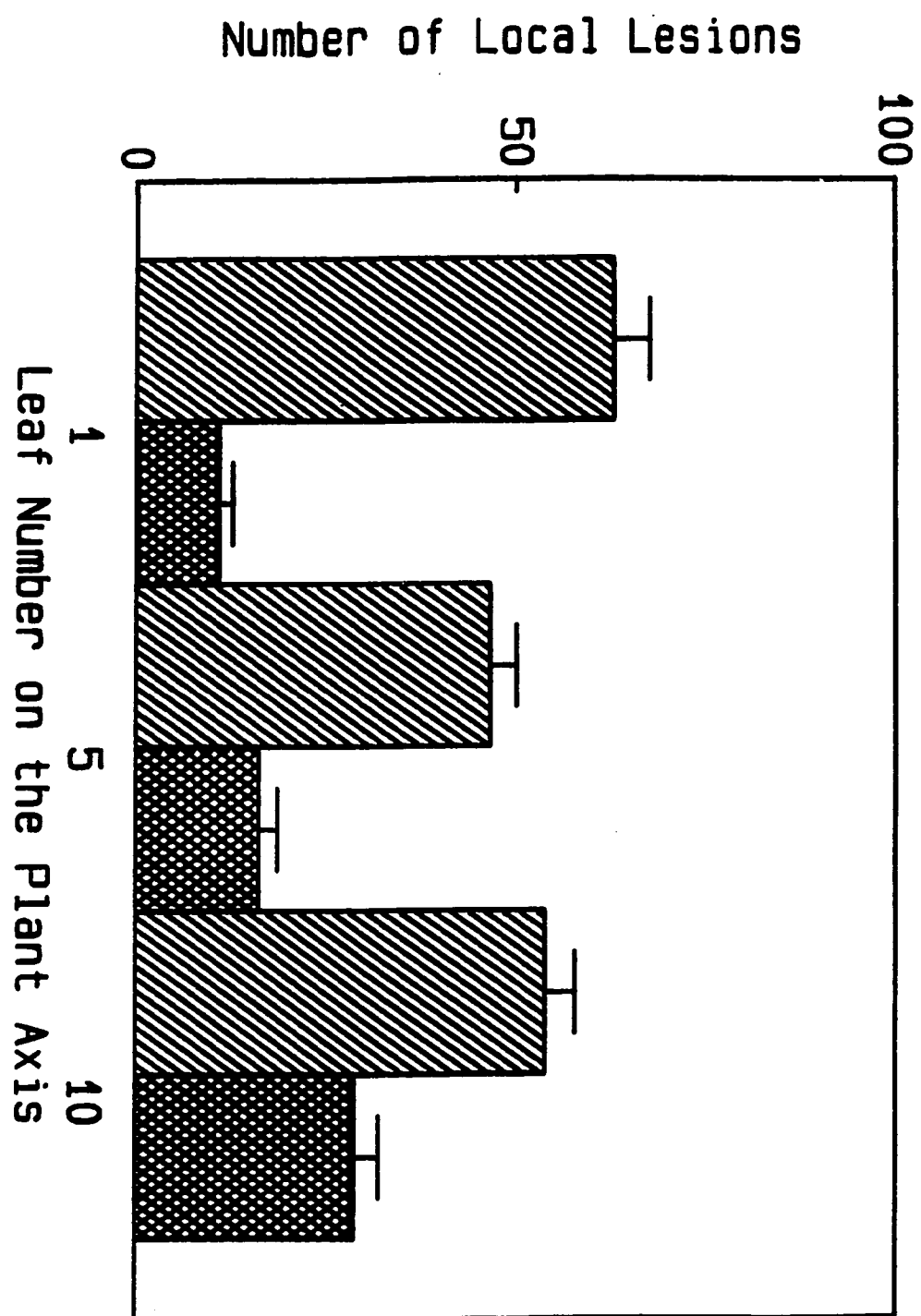
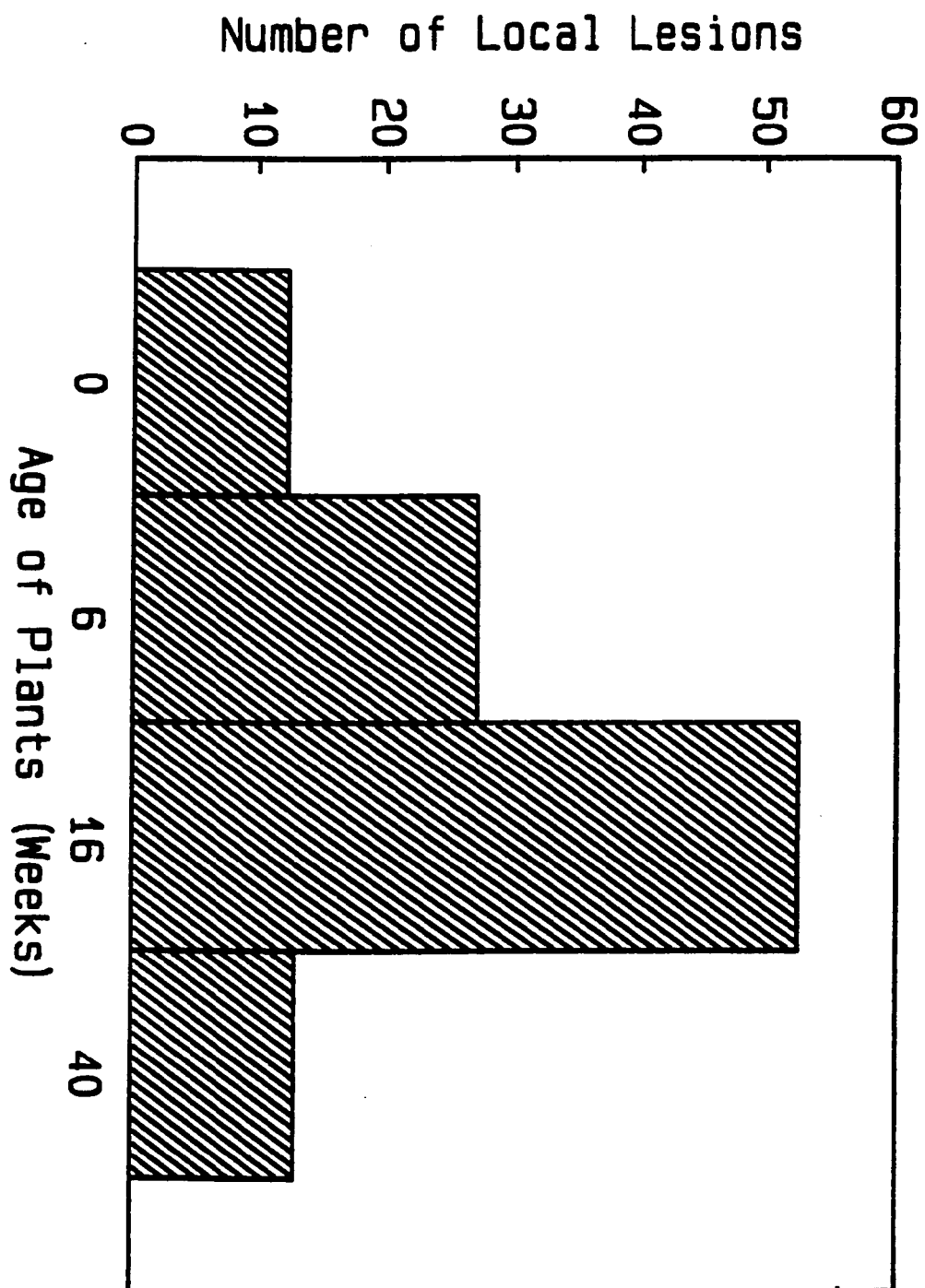


Figure D-2. The relative concentration of NL-type virus in regenerated plants at different ages. The surviving plants were tested at 0, 6, 16, and 40 weeks of age for NL-type virus by local lesion assay.



Appendix E

Control of Bolting Process in *Nicotiana sylvestris*

The selection for NL-type virus, including NL-18, has been shown to occur during the process of bolting. As shown in Table E-1, a detailed study of light conditions for inducing bolting was performed. Application of gibberellic acid (GA) also was used. A range of GA concentrations was applied either as a foliar spray or as local application on shoot tips. Triton-X100 was used as a surfactant in all applications. The bolting response, as seen by the extension of internodes (height) due to GA application was quantitative regardless of the light conditions (Table E-2). It was further shown that GA-induced bolting is equally effective in terms of selection for NL-type virus, including DK-24, a molecularly constructed strain with a single amino acid change in its coat protein. (Table E-3).

Table E-1. Control of bolting in N. sylvestris.

Growth conditions	Time taken to bolt ^a	Plant characteristics
Green house		
<u>Summer</u>	2-3 weeks	6-8 leaves before bolting and
<u>Winter</u>	No bolting	Numerous leaves in rosette
Growth chamber		
<u>Fluorescent light</u>		Numerous leaves in
210 $\mu\text{E m}^{-2} \text{ sec}^{-1}$	No bolting	rosette
<u>Switch to tungsten</u>		
30 $\mu\text{E m}^{-2} \text{ sec}^{-1}$	1-2 weeks	Bolting initiates
<u>Switch back</u>	Bolting stops	Rosette forms
<u>Switch to tungsten</u>	Bolting resumes	Stem axis elongates
<u>GA application</u>		Quantitative
(any light cond.)	2-3 weeks	response

^a Time taken to bolt is measured from time of transplanting seedlings into individual pots. During summer once bolting is initiated in the greenhouse, it will continue under artificial light conditions.

Table E-2. GA application and the bolting response in N. sylvestris.

0		5		25		50		100		500	
N	H	N	H	N	H	N	H	N	H	N	H
12	5.5	16	7.0	13	4.8	13	7.0	14	8.7	15	10.2
10	3.0	14	8.6	12	4.6	13	6.4	11	6.0	14	10.0
10	3.4	15	6.0	8	4.2	12	6.7	11	4.9	14	13.5
Means											
11	3.9	15	7.2	11	4.5	12.6	6.7	12	6.5	14	11.2

^a GA application in ppm; N is # of nodes; H is height in cm.

Table E-3. Accumulation of NL-type virus in response to GA induced bolting in N. sylvestris.

Virus	Frequency	No. lesions/half leaf
U1 (Wild type)	5/8	8.0
NL-18	4/4	29.5
DK24	5/8	29.3

Appendix F

Partial Cloning of NL-18 Genome

The selection of NL-type variants such as NL-18 is a fascinating aspect of plant biology. This is a unique system which offers a way to ask questions about population and evolutionary genetics of viruses. Hybridization data indicate that there are gross changes in the genome of NL-18 as compared to U1 in the 30K and coat protein genes. A partial cloning of the putatively mutant region of NL-18 into pTMV204 would be a useful tool. Figure F-1 is a flow diagram of the cloning procedure used in a preliminary attempt to clone this region of the genome of NL-18. The availability of a series of pTMV204 derivatives from Professor W. O. Dawson's laboratory provided an easy solution to the cloning problem.

Crucial steps in cDNA cloning are making of first and second cDNA strands using appropriate oligonucleotide primers complementary to the region of interest and then translating them upstream using appropriate polymerases. Two different primers were tested as possible sites to begin cloning. The data from these preliminary experiments showed that this step in the process was successful. This cloning was not within the scope of the dissertation and was not carried to completion.

Figure F-1. A Flow Diagram for partial cDNA
cloning of NL-18.

1. Isolate RNA from virions.
2. Reverse transcribe the region of interest.
3. Cut cDNA with NcoI/Nsi and clone into pDK4.
This plasmid has only a small piece of
pTMV204 and bears a unique Nsi
site in the region of interest.
4. Recover pIKI.
5. Cut pIKI with NcoI/SalI and clone into
pS3-28. Plasmid S3-28 has the entire coat
protein gene deleted. A SalI site is unique
to tetracycline resistance gene and
is a positive selection marker.
6. Recover pIKII and test for biological
activity, the induction of local
lesions on *Nicotiana sylvestris*.

