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CHARACTERIZATION OF RESISTANCE TO TOMATO MOSAIC VIRUS IN TOMATO SOMACLONES

By

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ABSTRACT

CHARACTERIZATION OF RESISTANCE TO TOMATO MOSAIC VIRUS IN TOMATO SOMACLONES

By

Sandra Lynn Schiller Smith

Six tomato somaclones from a tomato line that was fully susceptible to tomato mosaic virus (ToMV), were found to be resistant to ToMV multiplication. The purpose of the research of this dissertation was to characterize this resistance and determine its mode of inheritance. Screening for ToMV resistance was accomplished by inoculation of seedlings, incubation, and enzyme-linked immunosorbant assay (ELISA) for the detection of ToMV coat protein.

The resistance was shown to be stably inherited, with nearly complete penetrance through several generations. Based on evidence from crosses and backcrosses to the parental line, the trait appeared to involve nuclear genes(s) that were incompletely dominant and showed a gene dosage dependence. Screening of progeny from reciprocal crosses indicated there was also a maternal effect in the inheritance of the trait. Because of this, several cytoplasmic components were investigated, but chloroplast DNA did not differ between susceptible and somaclonal resistant plant lines according to limited restriction endonuclease analyses, and no endogenous double-stranded RNA was found.

In crosses with isogenic tomato lines expressing known resistance genes, it was shown that the somaclonal resistance was additive with *Tm-1* but not with *Tm-2*. The type of resistance seen in the somaclonal lines was similar to that of the gene *Tm-1* in that it suppressed

symptom formation, limited virus multiplication, was not temperature sensitive and had similar virus strain responses. There was no resistance to tobacco etch virus or cucumber mosaic virus, thus a specificity to tobamoviruses was evident.

Studies of virus movement in susceptible versus resistant plants indicated that transport of virus from the inoculated leaf was inhibited in the resistant plants. In grafting experiments, it was shown that virus multiplication was delayed in resistant scions grafted to susceptible, inoculated rootstocks. In the reciprocal graft, there was also a delay in virus multiplication in susceptible scions grafted onto resistant, inoculated rootstocks. In examining resistance at the cellular level, immunofluorescent staining of protoplasts released from inoculated resistant plants indicated restriction of virus titer and location.

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LIST OF ABBREVIATIONS

AU.....Absorbance units.

BSA.....Bovine serum albumin.

CMV.....Cucumber mosaic virus.

CP.....Coat protein.

cpDNA.....Chloroplast deoxyribonucleic acid.

CPW.....Cell protoplast wash.

DE-22.....DEAE-cellulose ion exchange material.

DNA.....Deoxyribonucleic acid

dsRNA.....Double-stranded ribonucleic acid.

EDTA.....Ethylenediamine tetraacetic acid.

ELISA.....Enzyme-linked immunosorbant assay.

EtOH.....Ethanol.

F_1.....First filial generation (after cross-pollination).

F_2.....Second filial generation (after cross-pollination).

FITC.....Fluoroisothiocyanate.

GCRI.....Glasshouse Crops Research Institute.

H:M:L.....ToMV resistance rating of high, medium or low for plants dependent on the amount of virus detected by ELISA in the plant after inoculation.

LSD.....Fisher's least significant difference multiple range test.

MS.....Murashige-Skoog plant cell growth medium.

PBS.....Phosphate-buffered saline.

PBS-Tween.....Phosphate-buffered saline + Tween 20 (a detergent)

PEG.....Polyethylene glycol.

pi.....post-inoculation.

PVX.....Potato virus X.

R_0.....Regenerated plant or somaclone.

R_1, R_2, *etc*.....Self-pollination generations of somaclone with subscript numbers designating the number of the generation.

RF.....Replicative form of virus.

RFLP.....Restriction fragment length polymorphism.

RI.....Replicative intermediate of virus.

RNase A.....Ribonuclease A.

Sc.....Somaclone.

SDS.....Sodium dodecyl sulphate.

S:I:R (or S:R).....ToMV resistance rating of susceptible, intermediate or resistant depending on the amount of virus detected in the plant by ELISA.

SNK.....Student-Newman-Keuls multiple range test.

STE.....Buffer composed of sodium chloride, Tris and EDTA.

TAE.....Buffer composed of Tris, acetate and EDTA.

TEV.....Tobacco etch virus.

Tm-1, *Tm-2*, *Tm-2*^{2}....ToMV-resistance genes described in tomato.

Tm-3.....Tentative name for somaclonal ToMV-resistance gene.

TMV.....Tobacco mosaic virus.

TMV-Fl.....Flavum strain of tobacco mosaic virus.

ToMV-O.....Common strain of tomato mosaic virus.

INTRODUCTORY SUMMARY

The goal of this dissertation research was to characterize tomato plants that had been selected for resistance to tobacco mosaic virus (TMV). These plants were recovered after regeneration from tissue culture of a fully susceptible tomato line. A new type of resistance was obtained, which appears to be encoded by a nuclear gene, with the addition of a maternal effect. The characterization of this resistance extends into several areas, including classical and molecular genetics, and virology, which will be treated in separate chapters.

Tomatoes were specifically chosen for this work because much is known about the genetics of the tomato. Two recent linkage maps are available (Mutschler *et al.*, 1987). The classical linkage map contains 327 loci defined by isozymes, morphological markers, resistance genes and physiological mutants, and the molecular linkage map which consists of 150 restriction fragment length polymorphisms (RFLPs) created by using random single copy DNA probes as well as clones of known genes. In addition, the chloroplast and mitochondrial genomes of tomato are characterized (Schiller *et al.*, 1982; Piechulla *et al.*, 1985; McClean and Hanson, 1986; Palmer and Zamir, 1982; Hause *et al.*, 1986).

Tomato mosaic virus (ToMV) is a strain of tobacco mosaic virus and one of the best characterized of plant viruses (CMI/AAB Description of Plant Viruses, 1977). These viruses, which infect a broad host range,

are closely related members of the tobamovirus group. They are composed of a single strand of RNA which is coated with protein subunits to make a straight rod ca.300 x 18 nm. Tomato mosaic virus is widespread and often epidemic in tomato, and is economically damaging in both glasshouse and outdoor tomato crops, with fruit yield reduced by 15 to 25% (Broadbent, 1964).

Virus diseases, in contrast to those caused by bacteria and fungi, cannot be controlled directly by chemicals or antibiotics. Resistant varieties have offered the most effective means of control (Gibbs and Harrison, 1976). There are three well-studied genes for resistance to ToMV in tomato: *Tm-1*, which slows virus replication and suppresses symptom formation, and *Tm-2* and *Tm-2*^{2} (which are allelic) effectively prevent infection of ToMV by a hypersensitive response. Near-isogenic tomato lines with these resistance genes are available.

Unfortunately, strains of ToMV have been isolated which overcome the resistance conferred by each of these alleles (Pelham, 1972; Rast, 1975). So far there are no strains which overcome the resistance of tomatoes which have both *Tm-1* and *Tm-2* or *Tm-2*^{2}. RNA genomes have a high rate of mutation (Reanney, 1982), thus it is only a matter of time before current resistant cultivars are overcome. Common ToMV field isolates, which may contain several virus strains, have been found to include strains able to overcome each type of resistance (Alexander, 1971; Rast, 1975). It has been demonstrated that a strain may change markedly on passage through some resistant tomato genotypes (Zitter and Murakishi, 1969; Pelham *et al.*, 1970), which seem to select for more virulent virus.

The resistances which are available in tomato have been crossed into the cultivated tomato from wild tomato species. Because these resistance genes are often linked with undesirable characteristics such as temperature sensitivity, severe stunting and chlorosis (Russell, 1978), conventional plant breeding can be more difficult and time consuming. In the search for new resistance genes, an alternative approach seemed desirable. It was demonstrated that it is possible to obtain ToMV resistance by regenerating tomato plants from tissue culture (Barden *et al.*, 1986). In this work, the tissue culture-derived resistance was characterized in order to determine the mechanism of resistance, and how it was generated.

ORIGIN OF SOMACLONES

The tomato somaclones were derived through tissue culture by Kristen Barden (1985). The description and selection of the six somaclones studied in this dissertation are described in detail in Appendix A (Barden *et al.*, 1986). The fully susceptible (+/+) line, GCRI-26, derived from the variety 'Craigella' (Glasshouse Crops Research Institute, Littlehampten, U.K) was chosen for the starting material because it is isogenic to a series of lines with the known ToMV resistance genes in tomato. This allowed direct comparison to the resistances conferred by these genes.

To regenerate plants, sections removed from fully-expanded leaves of GCRI-26 plants were placed on regeneration medium. Direct adventitious shoots formed after four weeks, were rooted and moved into soilless peat. The tomato somaclones were inoculated with TMV-Flavum, a yellowing strain of TMV. Visual mosaic symptoms were detectable in

susceptible plants 7-10 days post-inoculation. Symptomless plants were screened using enzyme-linked immunosorbant assay (ELISA) to determine the presence of viral coat protein in the plants. Local lesion assays were also done to determine the presence of infective virus.

Of 370 somaclones, 18 were virus-free after the initial screening. Records were not kept of the origin of each of these somaclones, but most of them were regenerated from different leaf sections. Twelve of the virus-free somaclones became infected with virus after re-inoculation. The remaining six virus-free somaclones (Table 1) were self-pollinated for seed collection. Seed was collected from each plant for evaluation of the resistance of the next generation, designated R_1. The R_1 plants were inoculated with TMV-Flavum and screened for resistance. Most plants were shown to be virus-free at 30 days post-inoculation (pi).

It was therefore shown that somaclones resistant to TMV could be obtained from a recognized fully susceptible source cultivar using ordinary tissue culture methods without the addition of a known mutagen. The type of resistance shown by the somaclones was a delay in symptom expression and virus multiplication. This dissertation includes the results of further studies to determine the nature of the resistance and its inheritance.

GENETICS

Evaluation of the plant material. The somaclonal plants were found to resemble the parental plant material in every way except for the resistance trait. This determination included comparisons of gross morphology (to screen for undesirable traits), chromosome number, seed

Table 1. Resistance of original somaclones (R) and progeny (R_1) to TMV-Flavum.

R Plant	ELISA values[a] (AU)	Duration of experiment (d)	R_1 virus-free/total	Days post-inoc.
Sc 12	0.00	51	15/15	30
	0.00		11/11	41
Sc 215	0.03	161	13/13	30
	0.04			
Sc 219	0.03	173	8/9	30
	0.02			
Sc 247	0.03	101	13/14	30
	0.04			
Sc 322	0.04	137	12/16	30
	0.04			
Sc 330	0.04	95	14/18	28
	0.04			
GCRI-26	>0.50	20	0/27	30
Uninoc. (range)	0.00-0.07	-	6/6	55

[a]ELISA to detect TMV-Flavum coat protein.
(Taken from Barden *et al.*, 1986.)

germination rate and pollen viability. The heritability of the viral resistance was then determined (to distinguish from epigenetic mechanisms), and the mode of inheritance was studied. Rating systems were devised to identify resistant phenotypes and were used to compare self-pollinated generations, crosses with the parental line (F_1) and their progeny (F_2) as well as backcrossed plant lines.

Because a complex genetic pattern was evident which included non-Mendelian inheritance, an effort was made to detect changes in the cytoplasm at the molecular level. To compare organelle genomes to the parental line, chloroplast DNA (cpDNA) was extracted and cut with four endonucleases for visualization of restriction fragment length polymorphisms (RFLPs). The same goal for mitochondrial DNA was not achieved. Also, screening for the presence of double-stranded RNA (dsRNA) was conducted to determine whether the presence of such a molecule might play a role in the viral resistance.

VIROLOGY

Evaluation of the plant-virus interaction. The somaclonally-derived resistance was compared to resistance conferred by other known TMV resistance genes. The plants were challenged by the common tomato virus strain (ToMV-0) under different conditions, by different ToMV strains and by other viruses. The systemic movement of the virus through susceptible tomato plants was compared to the movement in resistant somaclones. The movement of virus was also monitored in grafted resistant and susceptible plant material. Finally, the presence of virus was detected at the cellular level by releasing protoplasts from inoculated plants and immunofluorescent staining to detect virus coat protein.

CHAPTER 1

CHAPTER 1

GENETIC CHARACTERIZATION OF THE ToMV-RESISTANT SOMACLONES.

INTRODUCTION

In characterizing the genetics of resistance to ToMV in tomato somaclones, several questions were addressed. The somaclonal lines were compared to the parental line to determine if there were deleterious traits in the resistant somaclones, the heritability of the resistance, and the mode of inheritance. The type of resistance was also examined using virological assays and compared to other known types of TMV resistance in an effort to ascertain the origin of the somaclonal resistance.

LITERATURE REVIEW

Plant tissue culture is the *in vitro*, aseptic cultivation of plants, using plant organs, meristems, callus culture, protoplasts or single cells (Thorpe, 1981). Plants derived from many forms of tissue culture are called somaclones, and the variation seen in plants derived from tissue culture has been termed somaclonal variation (Larkin and Scowcroft, 1981). The genetic variability generated during plant cell culture has been reviewed extensively (Larkin and Scowcroft, 1981; Evans and Sharp, 1986; Evans *et al.*, 1984; Brettel and Ingram, 1979; Meins, 1983 and D'Amato, 1977) and evaluated for use in the selection of plants resistant to disease (Daub, 1986). Plant tissue culture has been proposed as a source of novel genetic variabiltiy to be exploited in selection programs (Buiatti *et al.*, 1985) and a review of the

literature supports the notion that plant cell culture itself generates genetic variability. It is still debated whether somaclonal variation is a result of genetic differences pre-existing in somatic cells of the explant or is induced during the tissue culture phase by components of the tissue culture medium (Evans and Sharp, 1986).

Some cases of genetic variability after plant tissue culture have been attributed to gross chromosomal changes such as altered chromosome number (D'Amato, 1978) and translocations (in oat, McCoy *et al.*, 1982; in celery, Orton, 1983). Many other cases of genetic variation have been characterized as single gene mutations which could be due to large or small gene rearrangements, gene amplification or deletion, transposable elements, sister chromatid exchange or cryptic virus elimination (Larkin and Scowcroft, 1981) as well as cytoplasmic changes in genomes of organelles (Gengenbach *et al.*, 1977). Single gene mutations have been detected in tomato (Evans and Sharp, 1983), maize (Edallo *et al.*, 1981), potato (Ramulu *et al.*, 1984), tobacco (Prat, 1983) and many more (Larkin and Scowcroft, 1981; Evans and Sharp, 1986). Multigenic inheritance has been established for a tissue-culture derived resistance to *Fusarium* wilt in celery (Heath-Pagliuso *et al.*, 1988). Cytoplasmic changes have been detected and confirmed by RFLP mapping of mitochondrial DNA in potato (Kemble and Shepard, 1984) and in maize (Gengenbach *et al.*, 1977).

In other cases, plant regeneration has resulted in transient variation, termed epigenetic, as well as genetic variation. Epigenetic variation involves selective gene expression that occurs with regularity in response to certain inducers present in the tissue culture system, and is not transmitted meiotically (Meins, 1983). Cytokinin habituation in tobacco is an example of epigenetic variation

(Meins and Lutz, 1980). Epigenetic inheritance must be tested by looking at the R_1 generation to determine whether the trait is expressed after meiosis.

Tomato has served as a useful model in studying somaclonal variation because the genome has been extensively mapped using morphological traits, isozymes and RFLPs. In one study of somaclonal variation in tomato, plants were regenerated and self-pollinated for screening of the R_1 progeny for morphological changes (Evans and Sharp, 1983). Seven recessive and three dominant traits (all showing normal single gene inheritance) were characterized (Evans *et al.*, 1984). In another approach, Sibi *et al.* (1984) studied the recombination between two linked genes on a chromosome in tomato plants regenerated from tissue culture, using two sets of nuclear gene markers. They found significant increases in the recombination rate in about half of the somaclones tested. Buiatti *et al.* (1985) self-pollinated 88 tomato somaclones to screen for chlorophyll deficiencies and other morphological abnormalities. About 17% of the R_1 progeny had deficiencies, all seemingly recessive. They also found chimeric variegation, suggesting that the regenerated plant originated from more than one cell in culture.

In another case, regenerated tomato plants with phenotypic variations were isolated and analyzed genetically (Sibi, 1986). Self-fertilization revealed that 56% of plants were mutant for five characters noted. Another 17% of the plants presented new characteristics but did not give segregating progeny, thus appearing to be homozygous. Of these, several showed differences between progeny of reciprocal crosses to the control line, mostly paternally and some

maternally oriented. Because of the stability of the effects after selfing the crosses between variants, as well as their asymmetrical behavior in reciprocal crosses, it was concluded that this phenomenon was not directly caused by heterozygous coding DNA, nor by cytoplasmic effects alone. The term 'epigenic modification' was used to describe this type of inheritance, and was attributed to modifications of heritable biological structures or dynamic systems, not involving expressed genes directly.

In an approach to assess genome stability in cultured tomatoes, O'Connell *et al.* (1986) analyzed isozyme patterns for eight enzymes and found no differences in patterns between regenerated plants and parental plant material. However, after callus culture (protoplast-derived callus grown for one month) or interspecific fusion with *L. pennellii*, they found variation in isozyme patterns. Similarly, Nagy *et al.* (1983) compared mitochondrial DNA RFLPs of tobacco before and after regeneration from tissue culture and found no differences, but could detect differences after interspecific protoplast fusion.

One of the limitations to utilizing somaclonal variation in a resistance breeding program is the selection and identification of desired mutant phenotypes. To recognize the desired resistance, an appropriate screening and selection scheme must be designed and utilized. For tomato, several approaches have been successful. In one example, tomato somaclones were selected for resistance to *Fusarium* wilt by including the toxin, fusaric acid, in the culture medium. After the successful selection of several lines and screening two generations of progeny, it was determined that a single gene for resistance was involved (Shahin and Spivey, 1986). In another approach, unselected somaclones of 'Bulgaria 12' (*esculentum x*

pimpinellifolium) were generated, self-pollinated and the progeny screened for novel resistance to *Clavibacter michiganense* subsp. *michiganense* (CMM) (R. DeVries, personal communication). Several resistant lines of tomatoes were recovered, but the mode of inheritance was not established.

Another strategy to generate resistance in cultured cells was to include a toxic molecule in the tissue culture medium to select for resistant individuals. In tomato, success was achieved in gaining glyphosate tolerance (Smith *et al.*, 1986), aluminum resistance (Meredith, 1978) and tolerance to paraquat (Thomas and Pratt, 1982). The paraquat-tolerant mutants were carried to the next generation to determine that the trait was heritable.

Tomatoes resistant to ToMV were recovered in this study. After determining the best system for rating resistance, the inheritance of this resistance was established by genetic analysis. Through self-pollinations, reciprocal crosses with the parental line, and F_1 backcrosses, as well as reciprocal crosses with another susceptible line of tomato, it was possible to estimate penetrance, expressivity, and the amount of cytoplasmic effect. Models to explain the mode of inheritance were discussed. It was also possible to compare somaclones to one another to establish their similarities. To assess the relationship of the somaclonal resistance to other ToMV resistance genes, reciprocal crosses were made with the near-isogenic tomato lines for *Tm-1*, *Tm-2* and *Tm-2*^{2}. This genetic characterization comprises the first part of this dissertation.

MATERIALS AND METHODS

PLANT AND VIRUS MATERIAL

Plants. The original source material for the somaclones was GCRI-26, a fully TMV susceptible line (+/+) of 'Craigella' (Glasshouse Crops Research Institute, Littlehampten, U.K.). Near-isogenic lines of 'Craigella' with the TMV resistance genes, *Tm-1* (GCRI-237), *Tm-2* (GCRI-236) and *Tm-2*^{2} (GCRI-267) were obtained from the same source for use in comparisons.

The seeds from GCRI lines and seeds collected from the tomato somaclone lines were sown in soilless peat (Baccto Professional Planting Mix, Michigan Peat Co., Houston, TX) that was moistened with distilled water and covered with plastic wrap. Germinating seeds were kept at room temperature (about 24^{o} C) under cool white fluorescent lights supplying 60 uEm^{-2}s^{-1} with a sixteen hour light cycle. Seeds usually germinated in five to seven days. Seedlings were transplanted into 1" x 2" pots at 9-12 d in preparation for screening. Plants were watered daily with distilled water containing fertilizer at one tablespoon of Peters 20-20-20 mix (Peters Fertilizer Products, Allentown, PA) per gallon.

Viruses. The common strain of Tomato mosaic virus (ToMV-O) was kindly provided by F. Motoyoshi of the National Institute of Agrobiological Research, Ibakari, Japan, and was increased in GCRI-26 tomato plants. The virus strain used in the early screening and selection of the original somaclones was TMV-Flavum (furnished by H. Jockusch of Max Planck Institut, Tübingen, Federal Republic of Germany, and increased in GCRI-26 tomato plants), a strain of TMV that causes bright yellow mosaic

symptoms on susceptible tomatoes. This strain is a tobacco strain, and presumably less virulent in tomato than ToMV-0. Therefore, further screening of the progeny of the somaclones was done using ToMV-0. Inoculum consisted of virus from the leaves of infected tomatoes that were collected, ground in 0.01 M sodium-potassium (NaK)-phosphate buffer, pH 7.0, at a dilution of 1 g:10 ml, filtered through four layers of cheesecloth, divided into aliquots and frozen at -20° C. Both strains of virus were preserved in this manner and used throughout all of the screening of the somaclones. This insured that the same virus strains were applied to all of the groups of tomatoes.

ARE SOMACLONES NORMAL PLANTS?

Gross morphology. Somaclonal R, R_1, R_2 and R_3 plants were observed for features that were different from the original source material, GCRI-26. Gross morphology of seedlings and mature, fruiting plants was noted.

Determination of chromosome number. Root tip squashes were made from rapidly growing roots of transplanted tomatoes following procedures adapted from Krikorian *et al.* (1983). Sections (5-10 mm) were cut from exposed root tips and incubated at room temperature in 0.05% colchicine. After 5 h, the colchicine was replaced with a fixative (a 3:1 mixture of ethanol and acetic acid) and the tips were incubated overnight at room temperature. The tips were treated with 1 N HCl for ten min to soften the tissue, transferred to microscope slides and then wetted with additional fixative. The root tips were stained by the addition of several drops of acetocarmine stain (10% w/v acetocarmine in 45% acetic acid). The tip was excised at about 0.5 mm and pressed with the flat end of a glass rod to macerate, with more dye added as needed to keep

the tip moist. A coverslip was carefully added and gentle pressure was applied to squash the cells. Cells were viewed at 600x magnification using phase contrast and photographed using 160 ASA tungsten film, with 5.5 volt light source and exposures of 1/5, 1/10 and 1/25 s. For each line of tomato, several plants were sampled and from each plant, chromosomes from ten or more cells were counted.

Seed germination rate. Records were kept of all seeds planted as described earlier and the number of seeds that germinated. Percent germination was determined at 10 to 14 days after planting, though in a few cases it was as early as 8 days or as late as 28 days. For comparison of rates, control plants (GCRI-26) were included in every group, which was usually comprised of 20-30 seeds of each somaclonal line. Each group of seeds planted served as a replication (10-34 replications for each line), and from this, the mean and standard error were calculated. The germination rates of each somaclonal line were then tested using standard analysis of variance statistics and compared using several multiple range tests to determine whether the somaclonal lines differed significantly from the parental line (using the Number Cruncher Statistical System, or NCSS, software package).

Pollen viability. Pollen viability was determined using two different methods (Brewbaker, 1964). For each blossom examined, at least 500 pollen grains were scored. For each plant line, this was repeated several times using both methods. After collection of pollen from fully-opened flowers, pollen was released, placed on a microscope slide and stained with acetocarmine. Viable pollen grains excluded the dye, and looked intact and round, while those pollen grains which were stained by the dye often appeared shriveled. The second method was to

germinate the pollen *in vitro*. Again, pollen was collected from opened flowers but then released onto pollen-germination medium (20% w/v sucrose, 1 mg/l H_3BO_3 and 1.5% agar) in a petri plate. After 1-3 h, germinated and ungerminated pollen grains were counted. There was very good agreement in the results obtained by these two methods of determining pollen viability. Standard statistical methods (using NCSS software) were used to calculate means, standard errors, analysis of variance table and multiple range tests using the pollen germination data.

SCREENING FOR RESISTANCE TO TMV

Screening of tomato somaclones. For screening seedlings for resistance to TMV, plants which had been transplanted to 1" x 2" pots at 9-12 days were allowed to recover for at least one day and then inoculated with virus. Since the first true leaves were just beginning to expand and were not of uniform size, only the cotyledons were inoculated. For inoculation, frozen virus stock was thawed and diluted to 1:20 with 0.01 M NaK-phosphate buffer (pH 7). This was applied to carborundum-dusted cotyledons with a cotton swab. The concentration of ToMV was approximately 0.6 mg protein/ml, as determined by ELISA (using purified ToMV as a standard). Cotyledons were allowed to air dry and were then rinsed with distilled water to remove the excess virus and carborundum.

The inoculated tomato plants were placed on shelves in the laboratory, under cool-white fluorescent lights supplying 60 $uEm^{-2}s^{-1}$ with a 16-hour light period at room temperature (24° C). Plants were watered daily with distilled water containing 1 tablespoon Peters 20-20-

20 fertilizer per gallon. In earlier screening of tomatoes, plants were transplanted to larger pots at 30 d and moved to a controlled environmental chamber or to the greenhouse. Later, plants were kept in small pots on the lab shelf for 70 days unless they were needed for the production of seeds, in which case they were transplanted into large pots and kept in the greenhouse.

When it was time to assay the tomatoes for the presence of virus, small sections of leaf near the growing tip of the plant were removed. It was important to take the samples from the youngest, most rapidly dividing tissue because it had been established that the virus appears in that part of the plant very soon after infection, even if infection is initiated in other parts of the plant (Samuel, 1934). The leaf sections were weighed, and trimmed if necessary, to provide 20 mg fresh weight. Three types of virus assay were employed during different phases of the screening. Enzyme-linked immunosorbant assay (ELISA) for detection of ToMV coat protein was used in all screening of plants. Presence of viral coat protein has been shown to be closely correlated with infectivity, with the presence of ToMV-RNA in the GCRI tomato lines, and with ToMV (Fraser and Loughlin, 1980). ELISA data were supplemented with rub-inoculation to local lesion hosts or detection of viral inclusions using the light microscope.

Purification of virus. Virus was rub-inoculated onto leaves of 6-8 week-old Havana-38 tobacco plants and allowed to incubate for 2 to 3 weeks. Tobacco leaves were then removed and weighed. The leaf tissue was placed in the freezer and stored at -20^{o} C until use. For the purification, the method of Gooding and Herbert (1967) was followed with slight modifications. The frozen tissue was thawed and homogenized

(1 g tissue/2 ml 0.1 M NaK-phosphate buffer, pH 7.0) in a blender for three min. The homogenate was filtered through four layers of cheesecloth and the filtrate distributed into 250 ml centrifuge bottles. They were centrifuged at 10,000 rpm for 15 min at 4° C in a Sorvall GSA rotor (16,400 x g) to remove cellular debris. The supernatant was collected, adjusted to 8% (w/v) polyethylene glycol (PEG) MW 8000 and to 0.2 M NaCl. The final volume was noted and the preparation was stirred at room temperature for 30 min to precipitate the virus. The entire mixture was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor (9400 x g) for 10 min at 4° C. The virus precipitate was then resuspended in 0.01 M NaK-phosphate buffer, pH 7.0, at 1/5 the total volume used in the PEG precipitation.

Three repetitions of high (46,000 x g) and low (12,000 x g) speed centrifugations were performed to further purify the virus. The high speed ultracentrifugation of the virus solution was performed at 25,000 rpm for 2.5 h at 4° C in a Beckman Ti70 rotor to pellet the virus. The pellets were resuspended in 0.01 M NaK-phosphate buffer, pH 7.0 after overnight incubation at 4° C and then centrifuged for 10 min at 10,000 rpm (SS-34 rotor) to remove additional cell debris.

Concentration of virus was estimated based on absorbance at 260 nm. At this wavelength, the extinction coefficient for purified TMV absorbance (1 mg/ml) is 3.0 (Zaitlin and Israel, 1975). Purity of the preparation was measured by comparing the ratio of absorbance at 260 to 280 nm (a ratio of 1.17 is best). The purified virus was filtered through a Morton ultra-fine fritted disc filter (0.9-1.4 u pore diameter) to remove microorganisms, and stored in 0.01 M phosphate

buffer. After filtering, the absorbance at 260 nm was measured and the virus was diluted to a concentration of 1 mg/ml and stored in 1 ml aliquots at 4° C.

Enzyme-linked immunosorbant assay. The procedures used for ELISA were based on those of Clark and Adams (1977) and are detailed in Appendix B. Antisera against ToMV-0 and TMV-Fl were elicited in rabbits following three weekly intramuscular injections of 1 mg/ml purified virus emulsified in Freund's adjuvant (Difco Co., Detroit, MI). The rabbits were bled at the ear 10-14 days after the final injection. After allowing the blood to clot in 50 ml centrifuge tubes, the antiserum was collected following centrifugation in a Sorvall SS34 rotor at 5000 rpm for 10 minutes. The antiserum was then diluted 1:10 with distilled water, and precipitated by the addition of 10 volumes of saturated ammonium sulfate solution. After one hour of precipitation at room temperature, the antiserum was centrifuged at 6000 rpm for five minutes in a Sorvall type SS-34 rotor to collect the precipitated proteins. The protein pellet was resuspended in two volumes of 1/2 strength phosphate buffered saline (PBS) at pH 7.4. Phosphate buffered saline consisted of 0.1 M sodium-potassium phosphate and 0.85% NaCl, pH 7.4. The antibody solution was dialyzed against three changes of 1/2 strength PBS including once overnight to desalt. This solution was washed through a DEAE-cellulose ion exchange column (Whatman DE 23) using 1/2 strength PBS; the fractions which were collected were monitored at 280 nm on a Beckman DB spectrophotometer. The purified gamma-globulin was adjusted to have an absorbance reading of 1.4 units, which corresponds to a concentration of 1 mg/ml. Purified gamma-globulin was stored at -20° C in small aliquots.

Purified gamma-globulin of each virus was conjugated to the indicator enzyme, alkaline phosphatase. For each 1 mg of gamma-globulin, 2 mg of alkaline phosphatase type VII (Sigma P-5521) was used. These were mixed and dialyzed against three changes of normal strength PBS. Glutaraldehyde was added to the antibody-enzyme mixture to make a final concentration of 0.05% (v/v), to initiate crosslinking of the proteins. This reaction was allowed to proceed at room temperature for 4-5 hours. Glutaraldehyde was then removed by dialysis against three changes of PBS + 0.01% sodium azide. Bovine serum albumin was added to a concentration of 5 mg/ml to stabilize the conjugate. The conjugate was stored at 4° C for up to six months. The activity of the conjugated alkaline phosphatase diminished over time, but could be adjusted by increasing the concentration used in the assay.

Purified gamma-globulin and gamma-globulin-enzyme conjugate was diluted for use in the enzyme-linked immunosorbant assay. Tests were made to determine the optimum concentration, so that the assay was complete between 30-60 min. The resulting dilution was usually best between 1:1000 and 1:500.

Flat-bottomed microtiter plates (Dynatech Labs, Inc., Alexandria, Virginia) were coated with purified gamma-globulin for the appropriate virus. The gamma-globulin was diluted in a coating buffer comprised of 0.05 M sodium carbonate at pH 9.6. Each well was filled with 200 ul of buffer and plates were incubated for 2-4 hours at 37° C or overnight at 4° C. Plates were rinsed three times with PBS + 0.05% Tween-20 (PBS-Tween), then 200 ul of sample was loaded into each well. Samples were prepared at a 1:100 dilution by grinding 20 mg leaf slices in 2 ml grinding buffer (PBS-Tween with the addition of 2% w/v polyvinyl-

pyrrolidone, MW 10,000, and 0.2% w/v ovalbumin). Duplicate wells were used for each sample, and each plate contained four controls (in duplicate): a) grinding buffer alone, b) virus-free tomato, c) infected tomato and d) a standard amount of purified virus (100 ug ToMV/ml). Samples were incubated 4-6 hours at 37° C or overnight at 4° C. Plates were again rinsed three times with PBS-Tween. The gamma-globulin conjugate was then diluted appropriately in grinding buffer, and 200 ul was applied to each well, and incubated 3-4 hours at 37° C or overnight at 4° C. After rinsing three times with PBS-Tween, 300 ul of a reaction mixture containing 1 mg p-nitrophenyl phosphate/ml in 10% v/v diethanolamine, pH 9.8, was added. Absorbance readings were taken at 405 nm for each well using a Microelisa minireader II (Dynatech Labs, Alexandria, VA) and recorded on a printer (CIEX 80F/T, Components Express Incorporated, Santa Ana, CA 92705). Absorbance readings were taken between 30 minutes and 4 hours, determined by the time when the wells containing 100 ug/ml ToMV-0 reached an absorbance between 1.00 and 2.55 absorbance units (AU) for the best range of values.

Local lesion assay. Inoculated tomatoes were also assayed by inoculation to *Nicotiana glutinosa* or to *N. tabacum* xanthi-nc, which are indicator plants for both TMV-Flavum and for ToMV-0. If the tomato plant was infected with these viruses, then the sap would cause local lesions to appear on the tobacco several days after rub-inoculation. This assay was done to determine the presence of infective virus particles, since ELISA detects only the ToMV coat protein. A sample of tomato leaf from the newest growth was taken and ground in 0.01 M phosphate buffer at a dilution of 1:20. Carborundum (600 mesh) was

dusted on the tobacco leaf and the sample was applied using a cotton swab. After several minutes, the tobacco leaves were rinsed with distilled water. Local lesions developed after 4-5 days in the greenhouse, and the number of lesions roughly correlated to the amount of virus present. Later it was determined that the plant samples in the ELISA grinding buffer were suitable to inoculate the indicator tobacco plants provided the inoculated leaves were rinsed immediately after inoculation.

Detection of viral inclusions. A third assay to determine the presence of ToMV in the tomatoes was used on several occasions. Viral inclusions in the hair cells of the leaves could be seen in the susceptible tomatoes at 5 days after inoculation. Following the procedure described by McWhorter (1965), a thin section of rapidly expanding leaf tissue was excised, mounted in distilled water under a coverslip and viewed by bright field microscopy at 100-200x magnification. The inclusion bodies looked like crystals, often hexagonal (Figure 1), in the cytoplasm of the hair cells. The presence of inclusion bodies correlated very well with ELISA values above 0.50 AU.

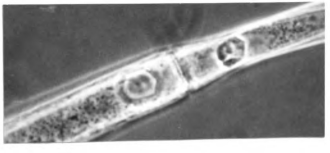


Figure 1. Inclusion bodies of ToMV-O in tomato leaf hair cells appear crystalline. (x500)

Analysis of ELISA values. After processing of the samples and the readings taken at 405 nm, the duplicate absorbance values were averaged and recorded in a notebook and entered in a computer database. If duplicate values did not agree, the sample was checked again in another pair of duplicate wells. Initially, if values were between 0.00 and 0.10 AU, the plant was considered to be disease-free. Values between 0.10 and 0.50 AU indicated that some virus was present, but often the plants recovered from this low titer of virus and were found to be free of virus at the next time of assay. Plants were always reassayed after having ELISA values in this range, since some were in the process of producing a high virus titer and others were able to overcome the infection. Plants which had ELISA values from 0.50 to 2.55 AU were considered to be infected with virus and were usually discarded after having high virus titer over a period of two time points, unless the plants were needed for breeding.

After the earliest screening of tomato somaclones, a regular schedule of ELISA was established so the results could be compared from group to group. Assays were taken at 10, 20, 30, 50, 70 and 150 days (plus or minus one day) until it was determined that the 150 day assay could be eliminated. The later groups (R_2-R_5 and crosses) were assayed until 70 days post-inoculation (pi) or 30 days pi.

Cross-pollination of tomatoes. Plants were maintained in 8-10" pots in the greenhouse with cool-white fluorescent light provided in a 16 h light cycle. Plants were watered daily with tap water containing Peters 20-20-20 mix fertilizer at the rate of one tablespoon per gallon. Using procedures from T. Kamps (personal communication), pollen from individual plants was collected and stored in petri dishes held at 4^{o} C

for two to three weeks. To prevent stray pollination, open blossoms were removed from plants to be pollinated. Flower buds that had not yet begun to open were used for pollination. First, the blossom was opened to expose the anthers, then using extra sharp forceps (Uni-fit size 3, Regine, Switzerland), the anthers were removed, leaving the stigma intact. At this time, the pollen was not yet mature and remained attached to the anthers. After one to two days, mature pollen from fully opened flowers of the desired plant was applied to the stigma. Pollen was applied by touching the stigma to freshly released pollen, three times in five days. Hands and tools were carefully washed in 95% ethanol after handling each kind of pollen to ensure that unintentional pollinations did not take place. Careful records of parents were kept in a notebook and by tags which were placed on each blossom. Fruit set could be observed about seven days after emasculation if the pollinations were successful. After fruit set was detected, fruit ripened in about 45 days.

Reciprocal crosses were made using different R_1 or R_2 plants of each somaclonal line and the susceptible parent line (GCRI-26) until there were 4-5 fruits produced for each of the desired crosses. For screening of the F_2 progeny, seed was collected from either uninoculated F_1 plants (unselected) or from previously inoculated and screened plants (selected for resistance or susceptibility). Sets of backcrosses were made between F_1 crosses and somaclone lines (plants of the R_1 or R_2 generation) or the susceptible GCRI-26. There was only one complete set of backcrosses, for somaclonal line 247.

Seed collection. Seeds were collected by cutting open tomato fruits and removing the gel layer from the seeds in one of three ways. Sometimes seeds were placed on a paper towel and scraped back and forth with a razor blade to mechanically remove the gel from the seeds. Most often, seeds were placed in a large test tube along with the juice of the fruit. Concentrated hydrochloric acid was added to about 12% and allowed to sit at room temperature for one to five hours. Seeds were then transferred to a sieve and rinsed in tap water. This acid treatment was also effective in destroying virus on the seed coat (Taylor *et al.*, 1961). The last method for removal of the seed gel was to place seeds in a test tube and allow the tomato juice to ferment over several days at room temperature. This was only used once, since it was unpleasant to handle the fermented juice. Seeds were moved to a sieve and rinsed in tap water. After the gel was removed, seeds were air dried on a laboratory table for several hours or overnight before packaging. Seed packets were placed in a dessicator for several days and then stored in the refrigerator until ready for use. Seeds collected from all self-pollinated fruits of each plant were pooled, but seeds from each cross-pollinated fruit were kept in a separate envelope.

GENETIC ANALYSIS

Rating of resistance in tomato somaclones. For proper classification of resistant phenotypes, it was essential to devise a rating system which reflected the predicted phenotypes for a given model. The assays employed in the study gave an estimate of virus concentration for each plant over a period of time, therefore the rating systems could incorporate virus titer and/or time. Symptomatology was not useful in rating for resistance for several reasons; the lack of clear mosaic

symptoms in susceptible leaves using ToMV-0, the time necessary for other symptoms to appear (such as stunting), and the lack of correlation between symptom formation and virus titer. The last reason can be exemplified by the resistance seen in plants expressing the *Tm-1* gene. This gene is dominant for symptom suppression but incompletely dominant for limitation of virus multiplication (Fraser and Loughlin, 1980). The following rating systems were evaluated.

At the time of screening, both virus titer and time were used in rating resistance because virus titer was measured in the plants at 10, 20, 30, 50 and 70 d pi. The earliest ratings of resistance in somaclones were very strict; plants were considered susceptible if there was detectable virus at any point in time. Later, the plants were grouped into three classes: S= susceptible (virus titer above 0.50 AU at 10 d pi), I= intermediate (virus titer above 0.50 AU at 20 d pi) and R= resistant (virus titer less than 0.50 AU at 10 and 20 d pi). This non-standardized rating system was the least refined since it was developed during the early part of screening. Using this rating system, there was unexplained variation between replicate groups screened (possibly due to variable expressivity caused by seasonal and environmental effects or horticultural practices). Since the ratings were somewhat arbitrarily defined by the choide of assay time (pi), it was felt that perhaps some sensitivity in the measure of resistance was lost.

Earlier investigators of TMV-resistance noted similar problems (Phillip *et al.*, 1965) and realized that the virus responded to resistance over time, with symptoms and virus titer dependent on age of

difficult. In another study, Holmes (1954) scored resistance five days after inoculation by examination of the inoculated leaf for symptoms caused by strain 1952D of TMV, which causes lesions on susceptible leaves. By limiting the resistance response to this early point in time, he was able to discern single gene inheritance of what later became known as the *Tm-1* gene for resistance.

In our study, because there was unexplained variation over time, it was decided that 10 d pi was most valid for describing resistance. Susceptible control plants consistently showed a high virus titer even by 7 d pi, so susceptibility to infection would be detectable at 10 d pi. This simplified system used only two classes: S= susceptible (virus titer above 0.50 AU) and R= resistant (virus titer below 0.50 AU) at 10 d pi. However, there was variation in the virus titer in plants rated S, so further classification was possible.

ELISA values from 10 d pi were standardized for each microtiter plate so that the range of absorbance values was from 0.00 to 2.00 AU (using purified ToMV-O or maximum control values to standardize). The distribution of these adjusted absorbance values was studied in the form of histograms, and cluster analysis (using NCSS software) was performed. Using data from the screening of each cross and backcross, a rating system was created to classify plants into three discrete groups. Plants with ELISA values between 0.0 and 0.5 AU were considered resistant (L= low), plants with values between 0.5 and 1.3 AU were intermediate (M= medium) and plants with values from 1.3 to 2.0 AU were considered susceptible (H= high). This classification accounted for all but 6.6% of the variation in the cluster analysis, and correlated visually with groupings from the histograms. Of the susceptible

controls, 89.2% were rated "H" and of the self-pollinated somaclonal lines, 97.4% of the plants were rated "L", thus giving confidence in this rating system.

As another measure of resistance, standardized ELISA absorbance values were used to calculate the mean, variance and standard error for each line and each cross or backcross. These values were also used for analysis of variance and multiple range tests. While these means correlated well with overall levels of resistance, they did not describe the discrete phenotypes seen. In the genetic analysis of a complex trait like viral resistance, it was important to consider all of the rating systems to evaluate proposed genetic models.

Crosses between somaclonal lines and susceptible tomato lines. To determine the mode of inheritance, resistant R_1 plants were used to make reciprocal crosses to the original parent line, GCRI-26. Screening of the progeny (F_1) would reveal either non-Mendelian inheritance or heterozygosity of resistance in the R_1. If the reciprocal progenies were not identical in the level of resistance seen, a cytoplasmic factor would be indicated. It is possible that the somaclones (R) were heterozygous for resistance gene(s), which would be seen as phenotypic variation between F_1 families in which different R_1 parent plants were used to obtain the F_1 and segregation within F_1 families.

The F_1 progeny were then self-pollinated to produce the F_2 generation. Because some F_2 were from uninoculated plants and others were from previously screened plants, the possibility of inadvertant selection for resistance was examined. A comparison between unselected and selected F_2 plants was made using analysis of variance and multiple range tests.

Outcrosses of the somaclonal lines were made to another susceptible tomato line, Bonny Best, to determine levels of expression in a different genetic background. Both F_1 and F_2 lines were screened and rated for resistance.

Reciprocal crosses were made of the F_1 plants back to both parents (somaclones and GCRI-26) to generate a set of backcrosses. There are eight possible backcross combinations, which were completed where possible and progeny were screened for viral resistance.

Seed transmission of virus. Because seed transmission of the virus could alter the results of the genetic screening, the amount of seed transmission of ToMV-0 was determined in GCRI-26 control plants and in some somaclonal crossing lines. Taylor *et al.* (1961) showed that seed transmission of ToMV in tomato occurred mainly by handling the seedlings during transplanting, with inadvertant contamination by virus on the seed coat. Only a small percentage of seeds were found with virus in the endosperm and none were detected with virus in the embryo. They found that the virus present on the seed coat was effectively removed by acid treatment of the seed (25 ml HCl per 5 lb fruit for 3 h, estimated to be about 1% v/v HCL).

To test virus transmission in susceptible tomatoes, 60 seeds of GCRI-26 were soaked overnight in ToMV inoculum (infected sap diluted 1:20 with 0.01 M phosphate buffer, pH 7.0). Seeds were planted (one per square) and allowed to germinate undisturbed. At 18 d the plants were sampled and ELISA values determined. As a control to detect other sources of virus contamination, 30 uninoculated seeds were similarly tested.

In another experiment, ELISA values were determined for virus-infected fruit-bearing plants, fruit juice and seed from the fruit after acid washing. These were compared with healthy plants, fruit and seed. Seedlings germinated from the seed of infected fruit were tested for virus using ELISA.

There was also indirect evidence from experiments designed to study virus movement in susceptible tomatoes. Seeds from infected plants were germinated and transplanted to 3" pots. After 25-30 d representative plants were sampled for ELISA to ensure that plants were virus-free before inoculation.

Crosses with known resistance genes. To test for allelism between the somaclones and other characterized resistance genes, reciprocal crosses were completed and the F_1 progeny were screened and rated for resistance. If high levels of resistance were maintained, with the genes being additive for resistance, this would serve as preliminary evidence for the genes being alleles of the same locus. If less resistance was observed, then the genes would not be considered additive or allelic. The plants used for these reciprocal crosses were near-isogenic lines of 'Craigella' which were homozygous for resistance genes: GCRI-237 (*Tm-1/Tm-1*), GCRI-236 (*Tm-2/Tm-2*) and GCRI-267 ($Tm\text{-}2^2/Tm\text{-}2^2$). As additional controls, reciprocal crosses were made between these homozygous lines to GCRI-26 (+/+).

To test for allelism between the different somaclones, crosses were made between them. Again, additivity of the genes to achieve a high level of resistance would indicate allelism, however, these plant lines have not been screened to date.

MOLECULAR GENETICS

Purification and restriction analysis of chloroplast DNA (cpDNA). Procedures were adapted from Kut and Flick (1986), for *in-organello* restriction digestion of DNA in purified chloroplasts. Leaves of plants grown under cool-white fluorescent light supplying 60 $uEm^{-2}s^{-1}$ with a 16 hour photoperiod were picked, weighed and refrigerated overnight in the dark. Five to ten g of young leaves were homogenized in a cold mini-blender in 40 ml isolation buffer which consisted of 0.35 M sorbitol, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.1% bovine serum albumin (BSA) (w/v), 15 mM 2-mercaptoethanol, 1 mM spermine and 1 mM spermidine (both from Sigma). Four bursts of 1-5 sec in the mini-blender were adequate to homogenize the leaf tissue. The homogenate was pressed through a double layer of cheesecloth and filtered through two layers of Miracloth into a 50 ml centrifuge tube on ice. All of the following steps were performed at 4^{o} C. To pellet nuclei and whole cells, the homogenate was centrifuged by bringing the speed up to 3000 rpm in a Sorvall type SS-34 rotor (1000 x g) and immediately allowing the rotor to slow down. The supernatant fluid was transferred to 50 ml tubes and centrifuged at 2500 rpm in SS-34 rotor (750 x g) for 10 min to pellet the chloroplasts. After discarding the supernatant, the pellet was gently resuspended with an artist's paintbrush in 2 ml isolation buffer.

The chloroplasts were further purified on a step gradient, using 12 ml 30% w/v sucrose layered onto 20 ml 60% w/v sucrose (both in isolation buffer). Each sample was divided into two fractions which were loaded onto two gradients. The gradients were centrifuged for 30 min at 26,000 rpm at 4^{o} C in a Sorvall AH-629 swinging bucket rotor (90,000 x g). The chloroplasts which banded at the 30%-60% sucrose interphase were removed

with a pasteur pipette and the two fractions of each sample were combined. Chloroplasts were washed by the addition of 15 ml isolation buffer followed by centrifugation at 2500 rpm in an SS-34 rotor (750 x g) for 10 min to collect the purified chloroplasts. The pellet was resuspended in 2.5 ml isolation buffer and divided between 5 Eppendorf tubes (0.5 ml each). These fractions were centrifuged at full speed on a Beckman Microfuge E (15,850 x g) for one min and the resulting supernatant was discarded. The pellet was saved.

The chloroplast envelopes were made more permeable by the addition of 100 ul swelling buffer (0.2 M NaCl, 20 mM $MgCl_2$, 10 mM Tris-HCl pH 7.8 and 0.01% nuclease-free BSA) per 5-10 g starting material and incubated at 37° C for 15 min. After swelling, 100 units (30-50 U/g fresh weight recommended) of the desired restriction enzyme was added to each tube and incubated for 1-2 hours at 37° C. The digestion was stopped by the addition of 20 ul 10% sodium dodecyl sulfate (SDS). To each tube, 0.18 g of CsCl was added and gently mixed, and warmed to 50° C for several min. The tubes were then centrifuged at full speed (15,850 x g) for 90 s at room temperature. The clear layer below the green pellicle was removed using a pasteur pipette and transferred to a microfuge tube containing 0.4 ml sterile water. This was centrifuged at full speed for 5 min at 4° C to remove insoluble material, and the supernatant was divided into two fractions and moved into new microfuge tubes. To each tube, 0.5 ml ice-cold 95% ethanol was added and the precipitation of the DNA was allowed to proceed at -20° C overnight. The samples were centrifuged at full speed for 10 min at 4° C and pellets were washed with 0.5 ml 80% ice-cold ethanol and centrifuged

again for 5 min at 4° C. Pellets were dried in a vacuum dessicator and resuspended in 10 ul TE (10 mM Tris, 1 mM EDTA). Duplicate samples were combined after resuspension.

Gel electrophoresis. Samples were prepared for electrophoresis by the addition of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 25% Ficoll, type 400 in H_2O) containing 1 ul RNase A (Sigma) (Maniatis *et al.*, 1982). Digestion of RNA was allowed to proceed for 10 min at room temperature. The samples were loaded in wells of a 0.6% agarose gel containing 0.5 ug/ml ethidium bromide in TAE buffer (0.04 M Tris-acetate pH 7.8 and 1 mM EDTA), and electrophoresed for 14 h at 30 mA constant current for a 25 cm gel, or 1-3 h at 50 mA for a 10 cm gel. Size markers consisting of lambda DNA restricted with *Hin*d III were included for each gel, with fragments that were 23.13 kb, 9.42, 6.7, 4.35, 2.32, 2.02 and 0.564 kb.

Extraction and analysis of double-stranded RNA (dsRNA). Procedures for the extraction of dsRNA were adapted from the protocol described by Fulbright *et al.* (1983) and modified for use in higher plants by Haufler and Fulbright (personal communication). Fully expanded leaves were picked from plants grown in the laboratory under cool-white fluorescent lights supplying 60 $uEm^{-2}s^{-1}$ with a 16 h photoperiod. For positive controls which served as size markers, samples from tomato plants infected with ToMV-O were extracted as well as samples from healthy pepper plants (Capsicum annuum cv. 'California Wonder') that have endogenous dsRNA (14 kd) as determined by A. Dodds (personal communication). The leaves were either used immediately or were wrapped in aluminum foil and stored at -20° C until use.

For each sample, 5-10 g of leaf tissue was placed in a chilled

mortar and was pulverized with a pestle in the presence of liquid nitrogen. The powder was transferred to a chilled centrifuge bottle or tube and four volumes extraction buffer (2 mM Tris-HCl pH 6.8, 20 mM NaCl, 0.2 mM EDTA, 3% SDS, 0.5% 2-mercaptoethanol and 0.5 mg/ml fractionated bentonite) was added. To this was added four volumes of STE-saturated phenol and two volumes of chloroform. (STE buffer consisted of 10 mM Tris-HCl pH 6.8, 100 mM NaCl and 1 mM EDTA.) This mixture was incubated in a shaking ice bath for 30 min. The mixture was then centrifuged at 7000 rpm in an SS-34 rotor (6000 x g) for 15 min. The upper, aqueous layer was taken by pipet and was filtered through two layers of cheesecloth. To adjust the ethanol content to 15-18%, 0.2 volumes of 95% ethanol was added.

Double-stranded RNA was selectively bound to a column consisting of Whatman CF-11 cellulose (Anspec Co., Ann Arbor, MI) in the presence of 15-18% ethanol. To prepare this column for samples of up to 20 g starting material, 2.5 g of CF-11 cellulose was mixed with 25 ml 85% STE:15% EtOH. The slurry was poured into a 50 ml syringe which was plugged with a disk of Miracloth. Samples (up to 160 ml each) were slowly added to the column down a glass rod, and were rinsed with a total of 120 ml 85% STE:15% EtOH added in 20 ml aliquots. Each aliquot was allowed to drain before adding the next. The dsRNA fraction was then eluted with 1x STE by adding 1 ml, 1 ml, 6 ml and 6 ml portions. The eluate was collected in a 50 ml centrifuge tube, and 28 ml 95% EtOH and 0.7 ml M 2.5 sodium acetate (pH 5.2) were added to precipitate the dsRNA. The extracted dsRNA was placed at -20^{o} C overnight to precipitate it. The extract was centrifuged at 9000 rpm in an SS-34

rotor (9,800 x g) for 30 minutes. The pellets were resuspended in 0.5 ml 1x STE and transferred to microfuge tubes. The nucleic acids were precipitated again by the addition of 0.5 ml 95% EtOH and 50 ul 2.5 M sodium acetate and cooled to -20° C overnight. The supernatant was removed after centrifuging for 15 min at 4° C at full speed (15,850 x g) in a Beckman microfuge. The pellet was dried and resuspended in 0.1 ml loading buffer (1x TAE, 0.25% bromophenol blue, 30% glycerol) and was electrophoresed on a 0.6% agarose gel (using 0.5 ug/ml ethidium bromide in 1x TAE) as described previously.

Photography. Photography of DNA or dsRNA gels was done using a Polaroid MP-4 Land camera (equipped with filters 2B and 23A) set up with a Foto UV 300 DNA Transilluminator (Fotodyne, Inc., New Berlin, WI). For routine records of gels, Polaroid type 57 sheet film was used with f5.6 and 1/2 s exposure. For higher quality photographs plus negatives, type 55 Polaroid sheet film was used, with a typical exposure of 2 min and an aperture setting of f5.6.

RESULTS AND DISCUSSION

ARE SOMACLONES NORMAL PLANTS?

Gross morphology. There were no notable differences in gross morphology between the somaclonal lines and the parental line, except after the plants had been inoculated with virus. After inoculation, the parental line became stunted and showed symptoms of virus infection, including mosaic and strapped leaves, but the resistant somaclonal lines did not have the same diseased appearance. However, dwarf plants were apparent among both the uninoculated parental line and the uninoculated somaclonal lines at the approximate occurrence of one in 16 plants.

Chromosome number. There was no difference in chromosome number between the somaclones and the parental line. All lines that were examined had the normal (2n=24) number of chromosomes (Rick, 1974). No determinations were done for somaclone lines 322 and 330.

Seed germination rate. Statistical comparison of seed germination rates in Table 2 showed that there was no significant difference in germination between somaclonal lines and the parental line, GCRI-26, when tested using the Student-Newman-Keuls (SNK) multiple range test ($p=0.05$).

Pollen viability. The results of pollen germination studies (Table 3) indicated that there was no significant difference between the somaclones and the parental line, GCRI-26, using the SNK multiple range test ($p=0.05$). Though pollen viability was normal, somaclone line 12 did not produce as many seeds as the other lines of tomatoes (quantitative data not collected).

Table 2. Comparisons of seed germination rates. Rates are compared among each generation of selfed tomato somaclones, the parental line, GCRI-26, and the isogenic line, GCRI-237, which is homozygous for the *Tm-1* gene.

Generations	Line	Seed % germination	S.E.	Replications (Environmental groups)
R_1-R_3	Sc 12	82.6	4.52	n=19
R_1-R_4	Sc 215	73.8	5.61	n=23
R_1-R_3	Sc 219	70.8	7.15	n=17
R_1-R_5	Sc 247	82.5	3.14	n=34
R_1-R_3	Sc 322	81.3	4.62	n=15
R_1-R_3	Sc 330	78.7	5.92	n=17
--	GCRI-26	88.4[a]	2.60	n=29
--	GCRI-237	84.3	7.68	n=10

[a]not significantly different from the other values (p=0.05) using the Student-Newman-Keuls multiple range test. No differences were seen among any of the lines.

Table 3. Comparison of pollen germination rates among pollen samples from somaclonal tomato lines and the parental line, GCRI-26.

Line	Pollen % germination	S.E.	# plants examined
Sc 12	74.2	2.66	n=3
Sc 215	66.5	7.43	n=9
Sc 219	77.8	5.68	n=5
Sc 247	68.3	6.33	n=8
Sc 322	88.6	6.18	n=3
Sc 330	58.5	6.58	n=8
GCRI-26	78.4[a]	3.47	n=16
F value	2.03 (n.s.)		

[a]not significantly different from the other values (p=0.05) using the Student-Newman-Keuls multiple range test. No differences were seen among any of the lines.

SCREENING FOR RESISTANCE TO TMV

Suitability of resistance ratings. Because of the complexity of the plant-virus interaction in studies of resistance, it is important to be accurate in the description of susceptible and resistant plant phenotypes. The three approaches to rating plants for resistance are compared in Table 4, for susceptible controls (+/+), resistant controls (*Tm-1/Tm-1*) and resistant somaclonal R_1 plants.

For the susceptible control line, GCRI-26, the S:I:R and S:R ratings indicated almost complete susceptibility of individuals, and

Table 4. Comparison of resistance rating systems, for susceptible and resistant control lines. S:I:R ratings are S=high virus titer at 10 d (pi), I=high virus titer at 20 d and R=no or low virus titer at 10 and 20 d. S:R ratings are S=high virus titer at 10 d and R=no or low virus titer at 10 d. H:M:L ratings are H=high virus titer (1.3-2.0 AU), M=medium virus titer (0.5-1.3 AU) and L=low virus titer (0.0-0.5 AU) all at 10 d. Mean ELISA value (AU) was calculated from standardized values.

Plant line Genotype	GCRI-26 +/+	GCRI-237 *Tm-1/Tm-1*	Pooled Somaclone R_1 unknown
S:I:R (%)	99:1:0	7:17:76	3:12:85
S:R (%)	99:1	7:93	3:97
H:M:L (%)	89:10:1	4:2:94	0:4:96
ELISA mean (10 d)	1.755	0.165	0.043
Standard error	0.015	0.050	0.011
Variance	0.097	0.178	0.026
Sample number	425	70	224

the H:M:L rating focused on the variation seen in levels of virus detected among the plants. With each rating system, 99% of the GCRI-26 plants had allowed virus to accumulate to levels above 0.50 AU by 10 d pi, indicating nearly complete penetrance of the susceptible trait. Figure 2A details the distribution of ELISA values of these susceptible plants. Most plants had a virus titer above 1.50 AU, but 11% of the plants accumulated less virus, indicating there was some variation in the expressivity of susceptibility, most probably reflecting subtle differences in the genetic backgrounds of the inbred GCRI-26 plants. The mean ELISA value is quite high (on a scale of 0.0 to 2.0 AU) with a fairly low standard error and variance.

The resistance trait seen in GCRI-237, the homozygous tomato line expressing the *Tm-1* gene, was not expressed with complete penetrance. The S:I:R and S:R ratings indicated 93% of the plants were resistant at 10 d pi, and that 17% more of the plants accumulated virus by 20 d pi. This is in agreement with the description of this resistance as causing a delay in virus multiplication and having a tolerance to low levels of virus (Fraser and Loughlin, 1980). The H:M:L rating also supports this, as does the ELISA mean. The ELISA mean indicates that there is a very low level of virus present in the population, with a larger variance and standard deviation seen than in the susceptible controls, reflecting the wider variation in expressivity of *Tm-1*. Figure 2B details the distribution of ELISA values seen among plants of this genotype.

Resistance in the somaclones was more consistent. Most of the R_1 somaclones screened (97%) were free of virus at 10 days pi and were often still free of virus at 150 d. In each rating system, there was 96-97% penetrance of the resistance trait seen in the somaclonal

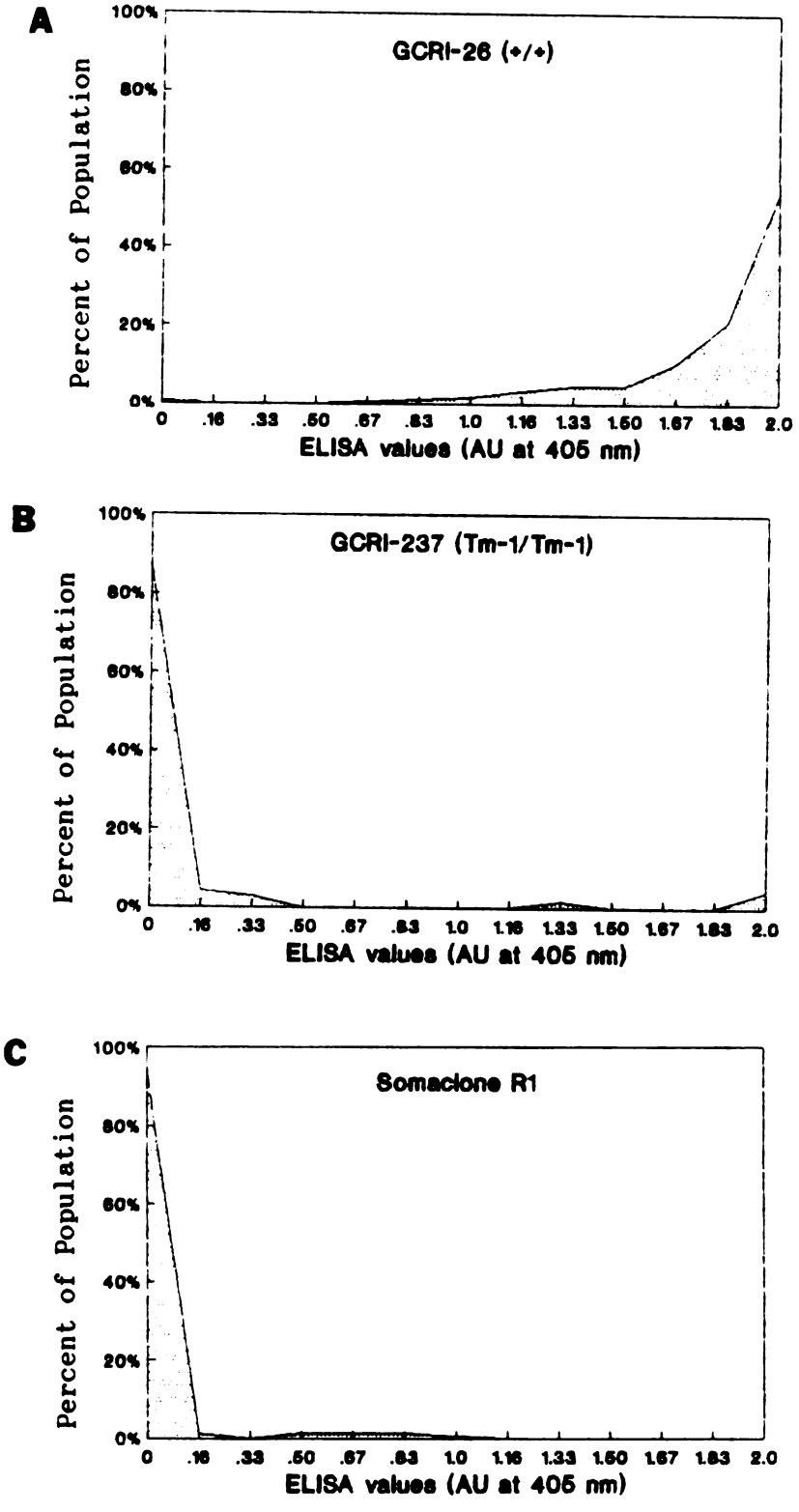


Figure 2. Distribution of ELISA values at 10 d pi for the following genotypes: A) susceptible *+/+* (GCRI-26) B) resistant *Tm-1/Tm-1* (GCRI-237) and C) resistant (somaclonal R_1).

progeny. The mean ELISA value was very low on the scale of 0.0 to 2.0 AU, with a low standard error and low variance. Examination of the distribution of ELISA values in Figure 2C allows one to conclude that there was little variation in expression of this resistance, and that the plants which were rated as being susceptible did not accumulate as much virus as did more than 90% of those which were susceptible controls. With this in mind, it can be stated that there was almost complete penetrance of this resistance trait and only 4% of the plants showed variation in the expressivity.

When compared to the resistance of the *Tm-1* gene, the somaclonal resistance was stronger in every rating system. When standard analysis of variance was tabulated for each somaclonal R_1 and GCRI-237, it was determined that there was a significant difference (5% level) between the mean ELISA values (F=3.83 with 6 and 287 df). Multiple range tests showed that GCRI-237 was significantly different from each of somaclonal R_1 lines (except Sc 12 which had fewer samples). Excluding Sc 12, there were no differences among the somaclonal R_1 lines.

Each of the rating systems accurately reflected the genotypes of the control lines. The S:I:R rating system, which incorporates a longer period of time, is useful as a more rigorous test of resistance. The H:M:L rating system is more fine-tuned, and very descriptive when paired with the mean of ELISA values. Later, its usefulness in describing intermediate phenotypes will be apparent.

Heritability of resistance. When the progeny generations of somaclones were inoculated and screened for the presence of ToMV-O, it was clear that the resistance was heritable. The results of screening for all somaclone generations are summarized and presented in Table 5 using the S:I:R (individuals) rating system. For each somaclonal line, at least

Table 5. Resistance ratings of selfed generations of somaclonal lines. Data were compiled for up to five generations for each somaclonal line using the S:I:R rating system which classified plants in three groups according to the length of time the plants remained free of detectable virus. S (susceptible) were plants with virus detected at 10 d pi, I (intermediate) were plants with virus detected at 20 d pi and R (resistant) were plants with no detectable virus at 20 d pi. Each entry represents plants which were screened in separate groups, which were totalled at the bottom. * indicates groups which were inoculated with TMV-F1 instead of ToMV-0.

Table 5.

	R_1	R_2	R_3	R_4	R_5	TOTAL
Sc 12	*0:0:11	0:0:12	0:2:10	--	--	5:17:183
	0:0:9	0:0:12	0:1:15			
	*0:0:5	0:0:16	0:0:12			
	*0:0:16	3:2:7	0:2:10			
	2:0:3	0:7:12	0:2:10			
	2:0:44	0:0:12	0:1:11			
		3:9:71	0:8:68			
Sc 215	*0:0:13	0:2:10	0:0:12	1:1:10	--	19:16:204
	0:0:4	0:0:18	0:1:4	1:1:10		
	*2:1:5	0:0:12	0:0:12	2:2:20		
	1:0:6	1:1:10	0:4:8			
	0:0:12	12:0:0	1:2:9			
	0:0:5	0:0:12	0:3:9			
	0:0:12	0:0:12	1:10:54			
	3:1:56	13:3:74				
Sc 219	*1:0:6	0:0:18	0:2:7	--	--	3:16:127
	1:5:2	0:0:9	0:2:13			
	*0:4:2	0:1:11	0:4:20			
	1:2:3	0:0:14				
	0:0:12	0:0:12				
	0:0:12	0:1:64				
	0:0:6					
	3:11:43					
Sc 247	*0:0:14	*0:0:8	0:0:12	0:0:12	0:1:11	4:17:446
	0:0:12	0:1:2	0:0:12	0:0:11	0:1:11	
	0:0:14	0:0:12	0:0:12	0:0:12	0:0:16	
	0:0:7	0:0:12	1:0:11	0:0:12	0:2:38	
	1:3:4	2:1:9	0:0:12	0:0:12		
	0:0:16	0:0:12	0:1:11	0:0:12		
	0:0:12	0:0:18	0:0:8	0:0:71		
	0:0:7	0:0:7	0:2:10			
	1:3:86	0:0:7	0:1:11			
		0:1:4	1:4:99			
		0:0:9				
		0:0:12				
		0:4:5				
		0:1:11				
		0:0:12				
		0:0:12				
		2:8:152				
Sc 322	*0:4:12	0:0:12	1:3:8	--	--	1:25:116
	0:3:7	0:0:2				
	0:7:1	0:0:12				
	*0:5:5	0:0:12				
	0:3:9	0:0:12				
	0:0:12	0:0:12				
	0:22:46	0:0:62				
Sc 330	*0:0:18	0:0:14	1:1:2	--	--	13:24:131
	4:3:5	0:0:12	1:1:10			
	*0:5:3	5:5:2	2:4:6			
	0:3:9	0:0:10	4:6:18			
	0:1:7	0:1:9				
	0:0:12	0:0:12				
	4:12:54	5:6:59				

three generations were screened for resistance. Examination of the data from each somaclonal line indicated that the virus resistance was consistently expressed in the progeny, with the conclusion that the resistance was genetically stable. Since the trait was heritable, this eliminated the possibility that the resistance trait of the somaclones was epigenetic.

The consistency of the resistance through three or more generations, averaging 97% penetrance, supports the idea that the resistance trait is true breeding. If the resistance was caused by a single gene, it would be homozygous. Segregation between resistant and susceptible phenotypes would be evident in the progeny if there was a single gene in the heterozygous state. If the resistance was inherited multigenically, it could be possible that the original somaclones were heterozygous for resistance genes, but only if each combination of genes was always expressed as a resistant phenotype in the subsequent generations. Further examination of this issue will be done as the crossing data are discussed.

Causes of incomplete penetrance. As noted earlier, only 97% of the somaclonal R_1 lines expressed full resistance. Highly resistant plants were selected to bear fruit in most cases, but when less resistant plants (in the I category) were used for seed production, their progeny were not less resistant, indicating that the I phenotype did not reflect the genotype in every case.

In these less resistant plants, resistance was lost at an early time (20 to 30 d pi), similar to loss of resistance conferred by the *Tm-1* gene. High virus titer was detected by ELISA and the virus was infective, causing local lesions when inoculated on *N. glutinosa*. To determine if the genotype was less resistant, seed were collected from

the plants and resulting progeny were screened by virus inoculation and ELISA. The progeny in each case were resistant. These results are presented in a detailed pedigree for each somaclonal line in Figure 3, using the S:I:R (individuals) rating system. Cases in which the progeny of infected plants were resistant are marked with #. The resistance trait was predominantly true-breeding.

The most striking exception can be seen when examining the pedigree for Sc 215 (Figure 3), which had one group of seedlings from one fruit that were all infected with virus at 10 d pi, yet siblings screened in another group were resistant (marked by #). When progeny of an infected plant from the first group were inoculated with ToMV and screened, they were virus-free and rated as resistant (marked by &). This phenotypic variability with seemingly constant genotype (incomplete penetrance) suggested that perhaps there were other conditions influencing phenotype expression. This could have been due to a less resistant genetic background, or to mutation of the virus to a strain which could overcome the resistance.

Influence of virus on phenotype. Evidence to support the theory that the virus had mutated to overcome the resistance was strong. Leaves from putatively resistant tomato plants that contained high virus titer were collected and examined. The symptoms were noticably different from the common strain, with a more yellow mosaic pattern. When the leaves were ground in phosphate buffer, and the sap was used to inoculate a group of resistant somaclonal seedlings, it was found that none of the resistant somaclones escaped infection at 10 d p.i. This was an indication that the virus had become more virulent with passage through the resistant somaclones. Zitter and Murakishi (1969) reported a

Figure 3. Pedigrees and resistance ratings of all generations of tomato somaclones screened. The S:I:R ratings classified plants in three groups according to the length of time the plants remained free of detectable virus. S (susceptible) were plants with virus detected at 10 d pi, I (intermediate) were plants with virus detected at 20 d pi and R (resistant) were plants with no detectable virus at 20 d. # indicates groups that were selected from seeds of parents with an S or I rating. * indicates groups which were inoculated with TMV-Flavum (all other plants were inoculated with ToMV-O). Upper- and lower-case letters indicate the groups of plants which were screened together. After the R_1 generation, each subsequent generation was labelled with a number indicating the plant which parented the generation. The numbers designating the plant lines are underlined. The S:I:R ratios (representing individuals) are given for each group screened, except in the cases marked with "un" which means the group was uninoculated.

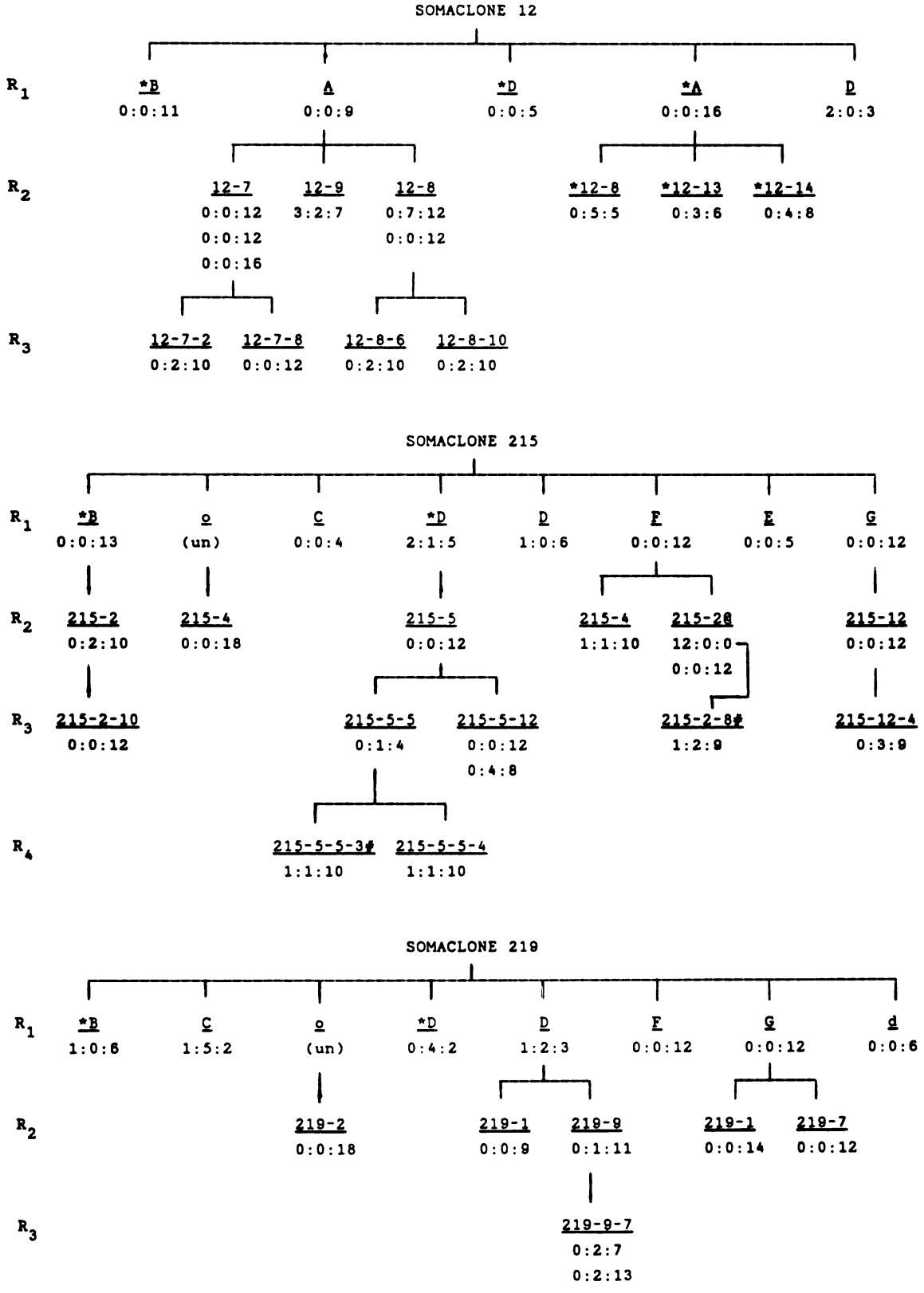

SOMACLONE 12
R1
*B
0:0:11
A
0:0:9
*D
0:0:5
*A
0:0:16
D
2:0:3
R2
12-7
0:0:12
0:0:12
0:0:16
12-9
3:2:7
12-8
0:7:12
0:0:12
*12-8
0:5:5
*12-13
0:3:6
*12-14
0:4:8
R3
12-7-2
0:2:10
12-7-8
0:0:12
12-8-6
0:2:10
12-8-10
0:2:10
SOMACLONE 215
R1
*B
0:0:13
o
(un)
C
0:0:4
*D
2:1:5
D
1:0:6
F
0:0:12
E
0:0:5
G
0:0:12
R2
215-2
0:2:10
215-4
0:0:18
215-5
0:0:12
215-4
1:1:10
215-2@
12:0:0
0:0:12
215-12
0:0:12
R3
215-2-10
0:0:12
215-5-5
0:1:4
215-5-12
0:0:12
0:4:8
215-2-8#
1:2:9
215-12-4
0:3:9
R4
215-5-5-3#
1:1:10
215-5-5-4
1:1:10
SOMACLONE 219
R1
*B
1:0:6
C
1:5:2
o
(un)
*D
0:4:2
D
1:2:3
F
0:0:12
G
0:0:12
d
0:0:6
R2
219-2
0:0:18
219-1
0:0:9
219-9
0:1:11
219-1
0:0:14
219-7
0:0:12
R3
219-9-7
0:2:7
0:2:13

Figure 3.

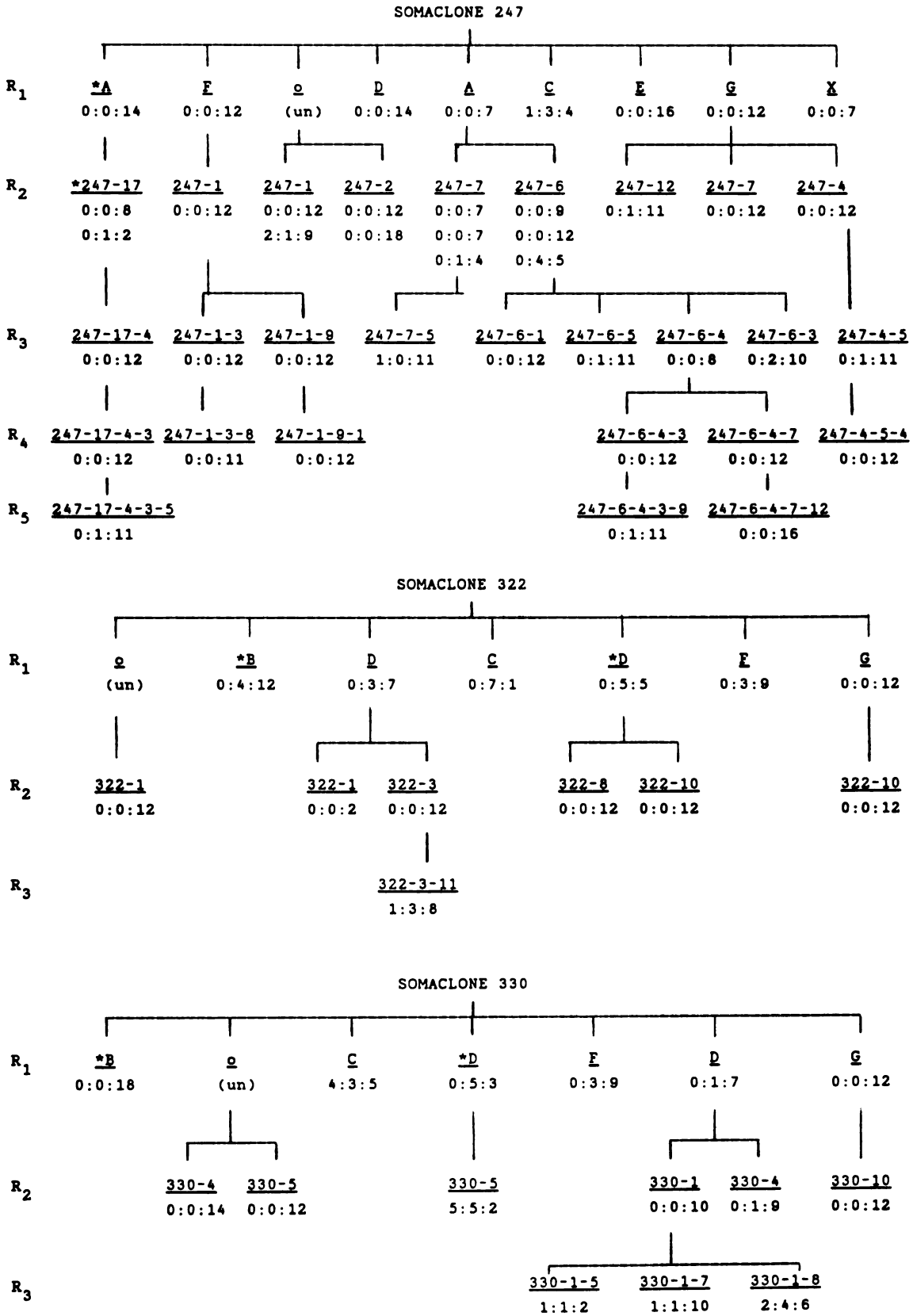


Figure 3. (continued)

similar occurance in resistant tomatoes. It would therefore be wise to consider virus mutation as a cause of some loss of resistance.

A second type of inconsistency in resistant plants was seen in occasional plants that would have a high virus titer at one time of assay (ie. 20 d), but not at the time of the next assay (ie. 30 d). Since only the growing tip of the plant was sampled at each time point, it appeared that the virus which was present at 20 d did not move into the newest leaf at 30 d. There were examples of this phenomenon among all the somaclonal lines, and especially among the F_1 lines which were cross-pollinated with GCRI-26. Because of this observation, a series of experiments was initiated to study the movement of the virus in the plants (included in the next chapter of the dissertation).

Chimerism. The possibility that the somaclones were chimeric plants was examined by looking at the R_1 progeny of each somaclone. For each somaclone, different replications to test R_1 plants were taken from different seed lots corresponding to different fruits. Seed lots were kept in separate envelopes. Accidental fire has since destroyed the envelopes and their contents so that identification of fruits and seed lots cannot be done. The possibility that the somaclones were chimeric cannot be completely eliminated, but it is doubtful that chimerism would play a role in the characterization of the somaclonal resistance, since there was no clear evidence of differences between replications of R_1 progeny.

Comparison of somaclonal lines. The S:R ratings at 10 d pi from each somaclonal line were summarized in Table 6 and converted into percentages for comparison between somaclones. The mean ELISA value for each somaclonal line and generation was also included, with results of analysis of variance and Fisher's least significant difference (LSD)

Table 6. Summary of responses of resistant somaclones in response to inoculation by TMV-O using A) the S:R rating at 10 d pi (S=virus titer greater than 0.50 AU and R=virus titer less than 0.50 AU) or B) the mean ELISA value (with results of LSD range test among somaclonal lines of each generation). F-values from the analysis of variance tables are listed below each generation.

A. Percent S:R at 10 days.

Line	R_1	R_2	R_3	R_4	R_5	MEAN
12	4:96	4:96	0:100			3:97
215	5:95	14:86	2:98	8:92		8:92
219	5:95	0:100	0:100			2:98
247	1:99	1:99	1:99	0:100	0:100	1:99
322	0:100	0:100	8:92			1:99
330	6:94	7:93	14:86			8:92
	3:97	4:91	2:98	2:98	0:95	3:97

B. Mean ELISA value and results of LSD (p=0.05) between each generation and between somaclones. Means with common letters were not significantly different from each other.

Line	R_1	R_2	R_3	R_4	R_5	MEAN*
12	0.134	0.104 (ab)	0.044			0.094 (bc)
215	0.038	0.267 (c)	0.089	0.114		0.131 (c)
219	0.025	0.021 (a)	0.055			0.034 (a)
247	0.036	0.027 (a)	0.040	0.017	0.038	0.035 (a)
322	0.027	0.009 (a)	0.090			0.042 (ab)
330	0.038	0.245 (bc)	0.090			0.124 (c)
F value	1.12 (ns)	6.87**	1.71 (ns)			5.13** (R_1-R_3)

*Total SE=0.006, variance=0.051
**significantly different from tabulated F value (p=0.01)

multiple comparison tests indicated. From these data it was possible to ask if the somaclones were similar to each other.

In the R_1 generation, the F value from analysis of variance was low, indicating that there were not significant differences. In the R_2 generation, Sc 215 and Sc 330 differed significantly from the other somaclonal lines, but relatively few plants caused this difference. There were no significant differences seen among somaclonal lines in the R_3 generation.

Because there were not consistent differences seen between somaclones in the first three generations, there was not conclusive proof that the resistance of the somaclones was the same or that it differed. Each line seemed to breed true for the resistance and showed a high level of penetrance, also indicating that there were not clear differences between the somaclonal lines. This evidence lends support to the bulking of data for selfed somaclonal lines. Further examination of this question was done in the analyses of the crosses.

RESULTS OF SCREENING CROSSES

The first step in the genetic analysis was to examine the resistance of the F_1 progeny of reciprocal crosses between the susceptible parent line, GCRI-26, and each of the somaclone lines. Since each of the somaclonal lines breeds true, that the resistance gene is homozygous is a reasonable assumption to make, although the trait might also be multigenic. When making reciprocal crosses with plants assumed to be homozygous for a trait, the nuclear backgrounds of the progeny will be identical. If differences were seen between progeny of the reciprocal crosses, it would indicate that the trait might be cytoplasmically encoded.

Other information that could be learned from the screening of F_1 progeny is the degree of dominance of a nuclear genetic trait. If all progeny displayed full resistance, the resistance gene would be dominant, and if none of the progeny displayed resistance, the resistance gene would be recessive. If intermediate levels of resistance were found in the progeny, then incomplete dominance of the gene could be possible, as well as more complex explanations.

Results of reciprocal crosses to parental line.

Detection of reciprocal differences. After inoculation and screening of the F_1 progeny, examination of early results indicated that there were differences among the F_1 of the reciprocal crosses for the somaclonal lines (Figure 4). When the somaclonal parent lines were used as the female parent (Sc x 26), the offspring showed a high level of resistance, although their resistance to ToMV infection was not as strong as that displayed by the offspring of self-crosses (Sc). In contrast, when the somaclonal plants were used as the male parent (26 x Sc), only a very low level of resistance was evident. But again, susceptibility to the virus was not as complete as that displayed by the susceptible control plants (26). When the reciprocal crosses were compared to each other using analysis of variance of the ELISA means, the F value was very high (33.73, with 1 and 279 df) confirming the reciprocal differences (using pooled data from all somaclone families).

Significance of reciprocal differences for each somaclonal line. It was evident after comparison of ELISA means of reciprocal crosses for each somaclonal line (Table 7) that there were maternally biased differences between the reciprocal crosses in most cases. Results of analysis of variance for reciprocal crosses within each somaclone family

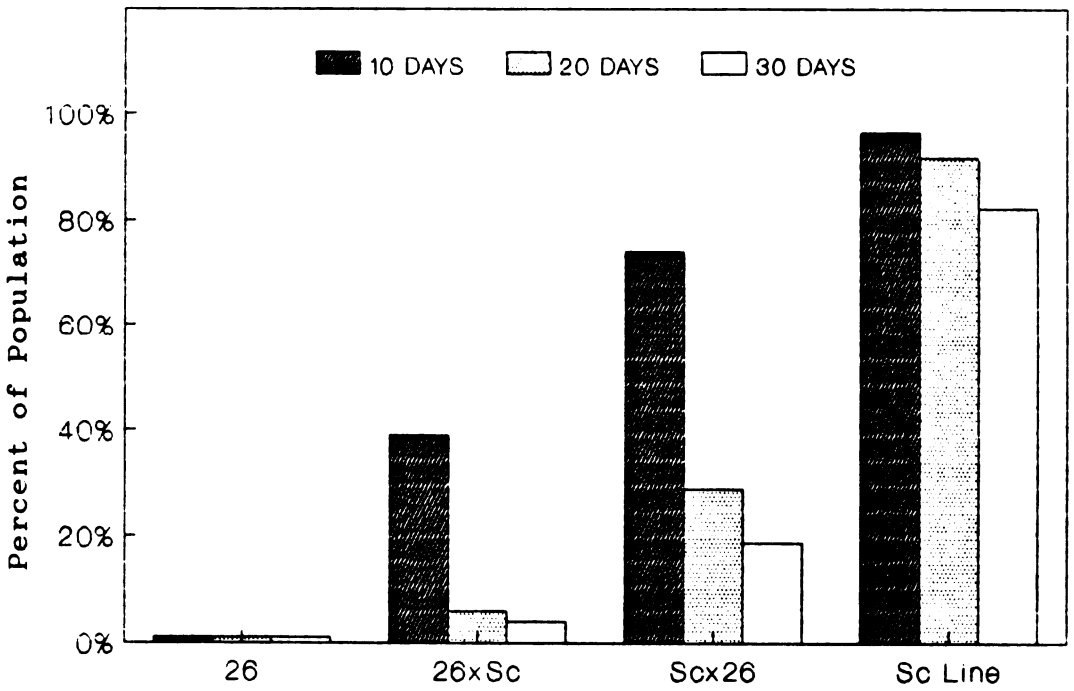


Figure 4. Comparison of virus resistance in F_1 of reciprocal crosses between somaclonal lines (pooled) and the susceptible parental line, GCRI-26, scored at 3 stages in their development: 10, 20, and 30 d pi.

Table 7. Testing for significant reciprocal differences for each somaclonal line. ELISA means for F_1 progeny of reciprocal crosses were used to calculate the analysis of variance.

	Sc x 26		26 x Sc		
Line	# plants	ELISA mean	# plants	ELISA mean	F value
Sc 12	32	0.868	80	0.778	0.47 n.s.
Sc 215	40	0.362	36	0.761	9.86**
Sc 219	33	0.538	14	0.866	3.66 n.s.
Sc 247	35	0.246	41	0.804	15.44**
Sc 322	10	0.059	24	0.812	20.41**
Sc 330	18	0.162	24	0.662	10.38**
Pooled	168	0.385	219	0.791	33.73**
GCRI-237 *(Tm-1/+)*	12	0.821	12	0.832	0.00 n.s.

**Highly significantly different ($p=0.01$).

indicated that the differences were highly significant ($p=0.01$), except for reciprocal crosses of somaclones 12 and 219. It is also helpful to evaluate the same plants using the S:I:R ratings (Table 8A). Notably, the distribution of plants in the S:I:R ratings showed a considerable maternal bias toward higher resistance among each somaclone family. When ELISA values were used for statistical analysis, the reciprocal differences were not as consistent. Reciprocal crosses made between GCRI-26 and GCRI-237 did not show differences using either rating.

Are there differences among the somaclonal lines? It was possible to examine the similarity of inheritance of resistance among the somaclonal lines by comparing the S:I:R ratings and ELISA values of F_1 progeny, and by calculating the F value from the analysis of variance of ELISA values (Table 8). Evaluation of S:I:R ratings for differences among somaclone families was difficult, so initial comparisons were made using ELISA values.

Of the maternal somaclone crosses (Sc x 26), the F value was 5.57 (with 6 and 156 df), indicating very significant differences among the somaclone families ($p=0.01$). Results of the LSD multiple range test showed that this was due to F_1 progeny of somaclonal line 12, which differed significantly from the other somaclonal lines. There was variation between F_1 progeny of somaclonal line 219 and lines 322 and 330, and progeny of lines 215 and 247 were intermediate. Overall, these differences were not as great as that of line 12. Line GCRI-237 (*Tm-1* gene) was included in the comparison and was shown to be significantly different from all somaclonal lines except line 12. However, when the S:I:R ratings were compared between 12 and 237, different distributions were apparent, thus weakening the significance of this result.

Table 8. Summary of results after screening F_1 tomato plants for resistance to ToMV (at 10 d pi). A) S:I:R ratings. B) Mean ELISA values. Lower case letters in parentheses following each ELISA mean indicate the results of LSD multiple range testing among families, where means with the same letter are not significantly different from each other ($p=0.05$). C) Result of analysis of variance among somaclone families.

A) S:I:R ratings at 10 d pi.

	Sc x 26		26 x Sc	
Line	# plants	(S:I:R)	# plants	(S:I:R)
Sc 12	32	53:38:9	80	61:31:8
Sc 215	40	23:45:32	36	50:50:0
Sc 219	33	30:45:25	14	57:29:14
Sc 247	35	17:40:43	41	68:29:3
Sc 322	10	0:60:40	24	63:33:4
Sc 330	18	11:61:28	24	63:25:12
GCRI-237 (*Tm-1/+*)	12	75:25:0	12	75:25:0

B) Mean ELISA values at 10 d pi.

Line	ELISA mean	ELISA mean
Sc 12	0.868 (c)	0.778
Sc 215	0.362 (ab)	0.761
Sc 219	0.538 (b)	0.866
Sc 247	0.246 (ab)	0.804
Sc 322	0.059 (a)	0.812
Sc 330	0.162 (a)	0.662
GCRI-237 (*Tm-1*)	0.821 (c)	0.832

C) Results of analysis of variance among somaclone families.

F value	5.57**	0.32 (n.s.)

**Highly significant difference ($p=0.01$).

Of the crosses in which somaclonal lines were the pollen parent (26 x Sc), no significant differences were found among somaclonal lines and GCRI-237, with a very low F value (0.32, with 6 and 211 df). That there was not a significant difference among somaclonal lines and GCRI-237 when these were used as the pollen parent was of interest, perhaps indicating that the mode of inheritance of somaclonal and *Tm-1* resistance genes is similar.

Analysis of ELISA values indicated that, unlike the other somaclonal families, somaclone 12 did not show a maternal effect. But because this was not substantiated by the S:I:R ratings, evidence provided by analysis of further screening is necessary before resistance of somaclone line 12 can be proven to be different from the other lines. In conclusion, there was no clear evidence in the F_1 cross data to suggest that the somaclonal lines differ in their inheritance of resistance. However, as a precaution, F_1 data from crosses with somaclonal line 12 were not bulked in further analysis.

Estimation of resistance due to the maternal effect. To estimate the maternal effect, the distributions of ELISA values were compared for reciprocal F_1 generations (Figure 5). As an example of resistance without cytoplasmic inheritance, progeny of crosses between GCRI-26 and GCRI-237 (*Tm-1/Tm-1*) were screened for resistance and compared to the somaclonal lines. Plants with the hybrid genotype *Tm-1/+* showed intermediate virus titer (Figure 5A), with some variation in expressivity that resulted in a normal distribution of ELISA values, slightly favoring plants with lower titers of virus. Results of the two reciprocal crosses were combined for GCRI-237, since there was no difference observed between them. However, the distribution of ELISA

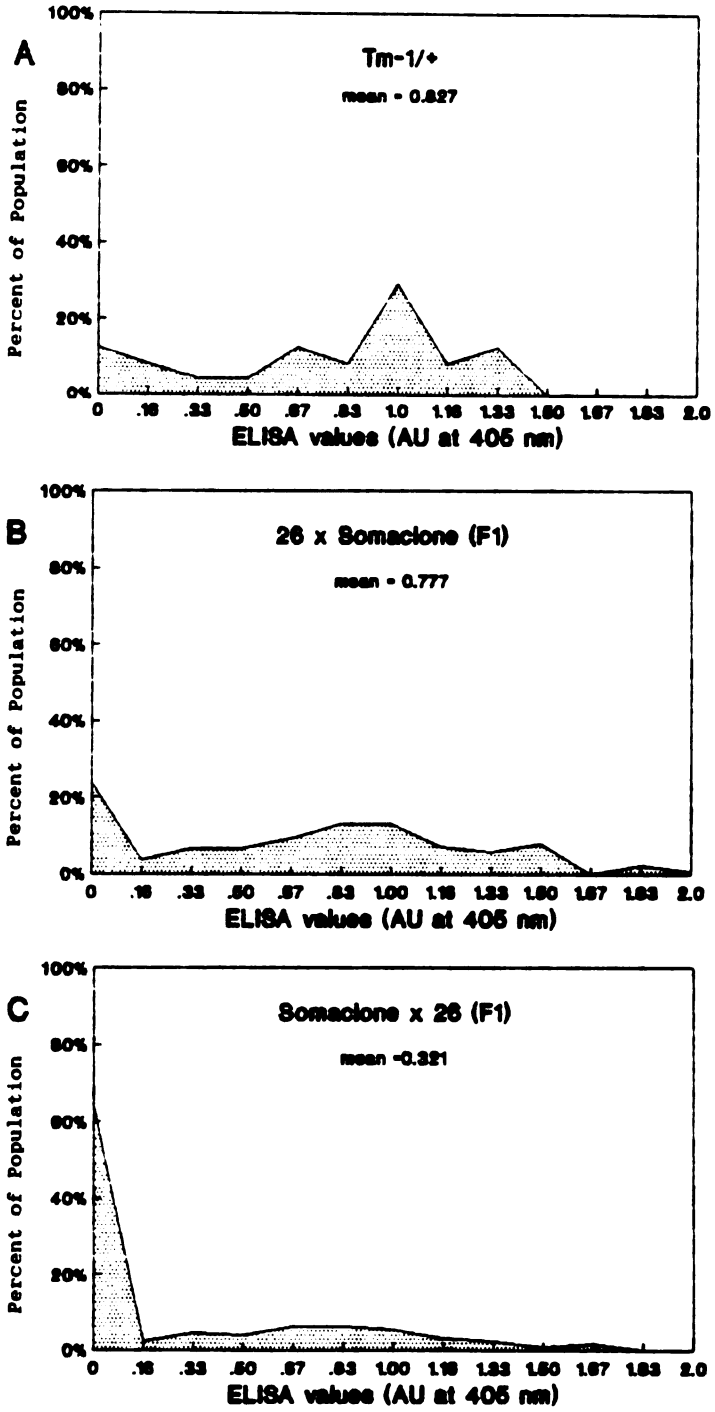


Figure 5. The distribution of ELISA values for virus detected in plants at 10 d pi, from the crosses A) between GCRI-26 (+/+) and GCRI-237 (*Tm-1/Tm-1*), B) GCRI-26 x somaclone lines 215, 219, 247, 322 and 330, and C) the same somaclone lines x GCRI-26.

values from progeny of reciprocal crosses using the somaclonal lines (excluding Sc 12) differed significantly. The distribution of ELISA values from F_1 progeny of crosses using the somaclonal lines as the pollen parent (26 x Sc)(Figure 5B) resembled that of *Tm-1/+* (Figure 5A), with the majority of plants having intermediate ELISA values, but again favoring lower virus titers. In the progeny of the reciprocal cross (Sc x 26) much less virus multiplied, with the distribution skewed toward less virus (Figure 5C). This was also reflected in the ELISA means, of 0.777 AU for 26 x Sc compared to 0.321 AU for Sc x 26. In the more resistant progeny of Sc x 26, 71.2% of the plants displayed a high level of resistance, with ELISA titers of less than 0.50 AU, compared to only 34.1% of the progeny of 26 x Sc, a distribution shift, with 37.1% of the plants with more resistance to virus multiplication.

These differences in progeny phenotypes from reciprocal crosses suggested either maternal inheritance or a maternal effect. Maternal inheritance usually means that a trait is encoded by organelle DNA. In tomato the transmission of cytoplasmic genes is usually uniparental maternal (Kirk and Tilney-Bassett, 1978), however, there may be incompletely dominant or multigenic nuclear genes, or other types of cytoplasmic factors responsible for the intermediate levels of resistance seen in the somaclonal lines. For example, an extra-chromosomal plasmid or a cryptic virus could be involved. In this regard, a number of plants have been shown to contain double-stranded RNAs (dsRNA) of no known function or phenotype, including beans (Wakarchuk and Hamilton, 1985) and some varieties of solanaceous plants; pepper, tomato and tobacco (A. Dodds, personal communication; Ikegami and Fraenkel-Conrat, 1979). Because a maternal bias in inheritance was observed in most of the somaclonal lines, it was logical to examine them

for the presence of any such unusual nucleic acids which could be correlated to the resistance trait.

There was less resistance to virus replication seen in the F_1 progeny compared to the somaclonal parent line. Therefore, if there was also a nuclear gene for the resistance trait, resistance was not a dominant allele, rather it was incompletely dominant or additive. If there had been complete dominance, there would not be the notable reduction in resistance in the F_1 plants relative to the resistant somaclone lines, unless it was heterozygous.

Were the original somaclones homozygous for the resistance trait? If the original somaclones were homozygous for the resistance trait, then variation among groups of F_1 progeny should mostly be due to environmental differences. Because there seemed to be variation among groups of siblings screened at different times (termed environmental groups), the variation attributed to environment needed to be estimated and compared to the variation among somaclonal families.

Detailed results of screening the F_1 seedlings from cross-pollination can be studied by referring to Table 9. Close examination of these data allowed identification of variation among environmental groups with the same parents, which are marked by a (+) to the left of the rating. These replicates were not necessarily from seed of the same fruit. Results of screening are presented using the 10 d pi S:R rating system and the ELISA means. Using the ELISA values, LSD range tests were employed to identify ELISA means of environmental groups which were significantly different from each other within somaclonal families ($p=0.05$), and are identified by the lower case letters to the right of the ELISA means. ELISA means with letters in common were not significantly different. It was clear that there was much variation due

Table 9. Summary of S:R resistance ratings and mean ELISA values (from 10 d pi) of F_1 progeny of reciprocal crosses between each somaclonal line and the parental line, GCRI-26. The letters in parentheses denote the groups of plants that were screened in the same environmental group. Brackets and (+) indicate that the crosses were made using the same R_1 parent plants. Within each environmental group, F_1 plants were siblings. Small letters to the right indicate groups determined by LSD multiple range tests ($p=0.05$).

Line	Sc x 26 S:R (10 d) rating	Sc x 26 ELISA mean	26 x Sc S:R (10 d) rating	26 x Sc ELISA mean
Somaclone 12	3:3(I)	0.908 a	+10:2(I)	0.659 a
	6:8(I)	0.738 a	+ 7:5(Z)	0.659 a
	8:4(BB)	0.764 a	+10:2(N)	1.069 b
	17:15	0.868	+ 9:3(I)	1.284 b
	(53:47%)		+ 3:5(Q)	0.500 a
			5:7(H)	0.641 a
			5:7(BB)	0.414 a
			49:31	0.778
		n.s.	(61:39%)	**sig.
Somaclone 215	0:12(I)	0.000 a	+ 5:7(H)	0.570 a
	+3:3(Q)	0.507 b	+ 9:3(I)	1.156 b
	+2:4(Q)	0.493 b	+ 1:5(Q)	0.439 a
	4:8(Z)	0.585 b	+ 3:3(Q)	0.673 ab
	9:31	0.362	18:18	0.761
	(23:77%)	**sig.	(50:50%)	*sig.
Somaclone 219	3:3(N)	0.923 b	+ 8:4(N)	1.006 a
	2:4(Q)	0.343 ab	+ 0:2(dl)	0.026 b
	+1:5(Q)	0.286 a	8:6	0.516
	+1:7(dl)	0.308 a	(57:43%)	
	+3:4(d2)	0.853 ab		
	10:23	0.543		
	(30:70%)	n.s.		**sig.
Somaclone 247	0:12(Q)	0.000 a	+ 4:5(H)	0.328 a
	0:5(T)	0.000 ab	+ 3:5(I)	0.500 a
	4:2(Z)	0.749 c	11:1(N)	0.963 b
	2:10(BB)	0.344 b	10:2(BB)	1.204 b
	6:29	0.246	28:13	0.804
	(17:83%)	**sig.	(68:32%)	**sig.
Somaclone 322	0:3(Q)	0.193 a	+ 5:1(N)	0.911 b
	0:7(Q)	0.002 a	+ 4:8(Z)	0.490 a
	0:10	0.059	6:0(N)	1.356 b
	(0:100%)		15:9	0.812
		n.s.	(63:37%)	**sig.
Somaclone 330	+0:9(S)	0.043 a	11:1(N)	0.977 a
	+2:4(Z)	0.427 a	4:8(Z)	0.372 b
	0:3(S)	0.000 a	15:9	0.662
	2:11:5	0.162	(63:37%)	
	(11:89%)	n.s.		**sig.

to environmental group, but results were inconclusive when assessing variation among somaclonal families. There was no clear evidence to determine whether or not the original somaclones were heterozygous.

If the somaclones were heterozygous, the R_1 progeny would segregate into different genotypes (ie: RR, Rr and rr), thus giving F_1 progeny that would show differing segregation patterns when homozygous or heterozygous R_1 parents were used in crosses. Families from one parent should not show differing segregation patterns, but progeny from differing parents might. In Table 9 there were as many differences among groups of siblings as there were among groups that were not siblings, regardless of environmental group.

Estimation of environmental effects. The results of LSD multiple range testing ($p=0.05$) are presented in Table 10A for comparisons between environmental groups screened. Results of multiple range tests of groups (designated by lower case letters in parentheses) show that there was significant variation among ELISA means. This environmental variation is probably refected in the broad distribution of ELISA values seen in Figure 5.

Because the experimental design gave an unbalanced case, analysis of variance for interactions between environmental group and somaclone families could not be calculated. Instead, the data were analyzed for variation of pooled somaclone families within each environmental group (Table 10B) and for variation of environmental groups within each somaclone family (Table 10C).

The results of these analyses of variance suggested that of the large variation between environmental groups seen in Table 10A was responsible for much of the total variation. For the Sc x 26 families, the significant variation was due to Sc 215 in Group I, and Sc 247 in

Table 10. Results of analysis of variance and LSD multiple range tests ($p=0.05$) of ELISA means of F_1 progeny of pooled somaclone families. A) Among environmental groups. Groups were identified by upper and lower case letters which were assigned in alphabetical order at the time of screening. B) Among somaclone F_1 families within environmental groups. C) Among somaclone F_1 families.

A. Variation in pooled somaclone families among environmental groups.

Sc x 26			26 x Sc		
Environmental Group	# plants	ELISA mean (LSD)	Environmental Group	# plants	ELISA mean (LSD)
T	5	0.000 (a)	d	2	0.026 (a)
S	12	0.030 (a)	Z	36	0.507 (a)
Q	46	0.226 (a)	H	33	0.530 (a)
BB	12	0.344 (ab)	Q	20	0.534 (a)
I	32	0.493 (bc)	I	44	0.936 (b)
d	14	0.564 (bc)	N	59	1.031 (b)
Z	24	0.586 (bc)	BB	12	1.204 (b)
N	6	0.923 (c)			
F value between environmental groups		3.71*			9.54**

B. Variation among pooled somaclone F_1 families within environmental groups (in groups where comparisons can be made).

Sc x 26				26 x Sc			
Group	Sc lines	# plants	F value	Group	Sc lines	# plants	F value
I	12	20	11.20**	I	12	24	
	215	12			215	12	2.68
					247	8	(n.s.)
N	219	6	---	N	12	12	
					219	12	
					247	12	0.41
					322	12	(n.s.)
					330	12	
Q	215	12		Q	12	12	0.04
	219	12	3.18*		215	12	(n.s.)
	247	12					
	322	10					
Z	215	12	0.98	Z	12	12	1.46
	247	6	(n.s.)		322	12	(n.s.)
	330	6			330	12	

C. Variation among somaclone F_1 families among different environmental groups.

	Sc x 26			26 x Sc		
Line	# plants	# groups	F value	# plants	# groups	F value
Sc 12	32	2	0.18	80	6	4.11**
Sc 215	40	3	9.01**	36	3	2.99*
Sc 219	33	3	2.52	14	2	12.52**
Sc 247	35	4	6.15**	41	4	6.81**
Sc 332	10	2	3.86	24	2	10.94**
Sc 330	18	2	2.28	24	2	18.32**

* Significant differences ($p=0.05$).
**Highly significant differences ($p=0.01$).

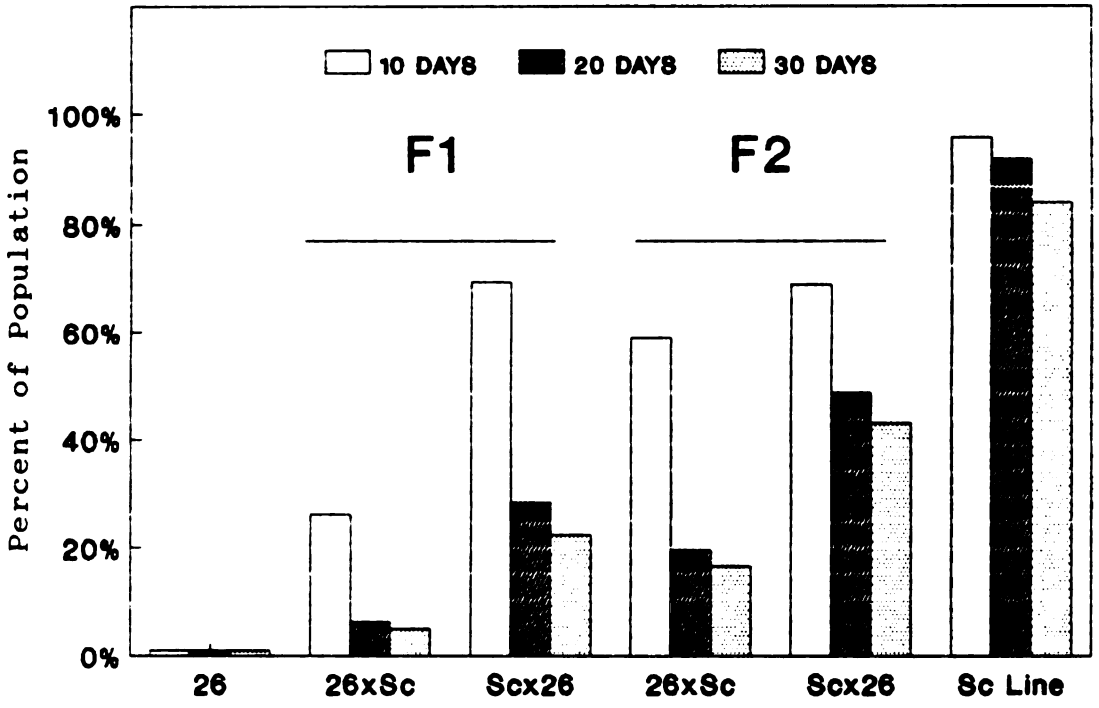
Group Q. In each case, only one set of progeny differed from the others, possibly displaying differences due to heterozygous somaclones. However, this was not seen consistently in all somaclone families (possibly because of the relatively low sample numbers).

Among the 26 x Sc families, there was not much variation among the families within environmental groups (Table 10B), rather the variation seemed to be among F_1 families (Table 10C). That there were not differences among the somaclone families, but there were differences within the somaclonal lines indicates one of two things. One possibility is that each somaclone was heterozygous for resistance and produced different F_1 genotypes, but this seems unlikely considering that the somaclonal lines breed true. Another possibility is that most of the variation was due to environmental effects, and the somaclonal lines can each be considered homozygous and similar to one another. Analysis of more crossing data may help to discern between these possibilities.

Results of screening the segregating generation.

Detection of reciprocal differences. The next part of the genetic analysis was to screen the F_2, or segregating generation of the reciprocal crosses. These plants were obtained by self-pollinating several F_1 plants from each somaclone family to obtain seeds which were germinated, inoculated with ToMV and screened for virus using ELISA. A general presentation of these results is made in Figure 6A, comparing the percentage of virus-free F_1 and F_2 progeny from the reciprocal crosses. When the less resistant F_1 (26 x Sc) were self-pollinated, the F_2 progeny showed more resistance than the F_1. In the reciprocal cross (Sc x 26), the resistance of the F_2 progeny was about the same as in the F_1. For further analysis, the mean ELISA values were listed (Figure 6B).

A. Resistance response of reciprocal crosses.



B. ELISA values (from 10 d pi) for F_2 progeny of reciprocal crosses.

	Sc x 26		26 x Sc		
Line	# plants	ELISA mean	# plants	ELISA mean	F value
Sc 12	36	0.356	58	0.626	3.95 n.s.
Sc 215	44	0.386	28	0.588	1.82 n.s.
Sc 219	29	0.491	34	0.774	2.80 n.s.
Sc 247	107	0.400	74	0.441	0.20 n.s.
Sc 322	32	0.623	44	0.658	0.06 n.s.
Sc 330	37	0.665	56	0.782	0.52 n.s.
Pooled	285	0.461	294	0.628	9.26**

**Highly significant difference ($p=0.01$).

Figure 6. Resistance to ToMV in progeny of reciprocal crosses of tomato somaclonal lines with the parent susceptible line, GCRI-26, for both the F_1 and F_2. A) Bars represent the percentage of virus-free plants at 10, 20 and 30 d. B) Testing for significant reciprocal differences for each somaclonal line.

Examination of the mean ELISA values of the F_2 progeny resulted in the recognition of a slight maternal bias. Though progeny plants in each somaclone family had less virus accumulation for the Sc x 26 cross than for the the reciprocal cross, none of the differences were significant unless the data from somaclone families were pooled. Evaluation of S:I:R ratings was needed to supplement these observations.

The detailed pedigrees of all the F_1 and F_2 plants which were screened are presented in Appendix C. From these pedigrees, 10 d pi S:I:R ratings of the F_2 progeny were compiled (Table 11) and supplemented with the ELISA means for analysis of variance and LSD multiple range testing. After determining whether inadvertent selection for resistance occurred in the choice of F_2 progeny, further analysis could be done.

Was there unintentional selection of resistant F_2 plants? To ensure that the results obtained were not skewed by possible selection of F_2 progeny of plants that were more resistant, a comparison was made between F_2 progeny that were taken from fruit of unscreened plants (unselected) and from plants that had been screened for resistance and were rated either susceptible (S) or resistant (R). The ELISA values for each plant were used for analysis of variance between selected and unselected F_2 progeny, and demonstrated that there were not significant differences. F values were 3.18 (with 1 and 292 df) for selected versus unselected 26 x Sc F_2 and 4.94 (with 1 and 283 df) for selected versus unselected Sc x 26 F_2, both were below the value of 5.02 for significance ($p=0.05$). Because there were not significant differences between selected and unselected F_2 plants, all F_2 data were used in further analysis.

Table 11. Summary of results after screening F_1 and F_2 tomato plants for resistance to ToMV (at 10 d pi). A) % S:I:R ratings. B) mean ELISA values. Lower case letters in parentheses following each ELISA mean indicate results of LSD multiple range testing among families.
C) Result of analysis of variance among somaclone families.
D) Comparison of reciprocal F_1 and F_2 families.

A. % S:I:R ratings at 10 d pi.

Line		Sc x 26 F_1	Sc x 26 F_2	26 x Sc F_1	26 x Sc F_2
12	(%)	40:46:14	16:24:60	61:31:8	42:24:34
215	(%)	23:45:32	20:18:62	50:50:0	43:21:36
219	(%)	30:45:25	37:19:44	57:29:14	50:13:37
247	(%)	17:40:43	26:9:65	68:29:3	28:15:57
322	(%)	0:60:40	41:18:41	63:33:4	39:27:34
330	(%)	11:61:28	35:23:42	63:25:12	45:17:38

B. Mean ELISA values at 10 d pi (AU at 405 nm).

12	0.868(c)	0.356	0.778	0.626
215	0.362(ab)	0.386	0.761	0.588
219	0.538(b)	0.491	0.866	0.774
247	0.246(ab)	0.400	0.804	0.442
322	0.059(a)	0.623	0.812	0.658
330	0.162(a)	0.665	0.662	0.782

C. Results of analysis of variance among somaclone families.

F value	5.57**	1.80	0.32	2.00
(df)	(6,156)	(5,279)	(6,211)	(5,288)

D. Comparison of reciprocal F_1 and F_2 families (pooled).

Family	ELISA mean	(LSD)	F value (df)
F_1 Sc x 26	0.385	(a)	
F_1 26 x Sc	0.791	(c)	
			16.92** (3 and 932)
F_2 Sc x 26	0.461	(a)	
F_2 26 x Sc	0.628	(b)	

**Highly significant difference ($p=0.01$).

Significance of reciprocal differences. It was evident after comparison of mean ELISA values for F_2 progeny (Figure 6B) that there was a slight maternal bias, which was not significant within crosses from each somaclonal line. However, when the data were bulked, the reciprocal differences were highly significant. Examination of the S:I:R ratings for the same plants (Table 11A) indicated that there were consistently small differences between the F_2 progeny of reciprocal crosses. In comparison to the considerable differences between the F_1 progeny, it became clear that the maternal bias was greatly reduced. This unusual transmission pattern for resistance will be important in consideration of a model to explain the inheritance of the resistance.

Are there differences among the somaclonal lines? It was possible to examine the similarity of the somaclonal lines to each other by comparing the results of screening the F_1 and F_2 generations. The S:I:R ratings and mean ELISA values at 10 d pi (Table 11) were available for direct comparisons. The differences among the ratings were difficult to interpret, so ELISA values were employed to calculate the analysis of variance among somaclonal lines for each of the reciprocal crosses for each generation. Analysis of variance indicated that there were not significant differences among the somaclonal lines in the F_2 generation (including Sc 12). The F values were low for F_2 progeny of both of the reciprocal crosses, with 1.80 for Sc x 26 F_2 and 2.00 for 26 x Sc F_2. There was no clear evidence for differences among the somaclonal lines, which again helps to justify the pooling of data from the lines.

When testing for differences between F_1 and F_2 progeny of the reciprocal crosses (Table 11D), the F value was 16.92, indicating that there were very significant differences among the four ELISA means

($p=0.01$). Results of LSD multiple range testing ($p=0.05$) indicated that there was not a significant difference between the F_1 and F_2 of the cross Sc x 26, but there were differences between these and the F_1 and F_2 of the cross 26 x Sc. Also, there was a considerable difference between the F_1 and F_2 generations of the cross 26 x Sc. These comparisons substantiate the initial observations seen in Figure 6, which showed differences in F_1 progeny of reciprocal crosses biased for maternal inheritance of the resistance, with the F_2 generations showing similarly high levels of resistance, with an unusual increase of resistance seen from the F_1 to the F_2 generation of the 26 x Sc cross.

Estimation of resistance due to the maternal effect. The distribution of ELISA values for all F_2 plants screened was examined in Figure 7. The distributions were similar for the F_2 progeny of reciprocal crosses, except that a greater portion of plants fell into the range of 0.00-0.16 AU for F_2 progeny of the Sc x 26 cross. There were only 42.5% of plants in this range for the 26 x Sc cross (Figure 7A) compared to 58.2% of the Sc x 26 cross (Figure 7B), a difference of 15.7% between the reciprocal crosses. This was only about half the difference seen between reciprocal crosses in the F_1 generation. There were more 26 x Sc F_2 plants in the very high ELISA range (between 1.33-2.0 AU) than there were Sc x 26 F_2, an increase of only 5.6% of the plants in this range. It was notable that there were not discrete groups seen in the segregating F_2 generation, possibly due to environmental effects, thus making it more difficult to make genetic models to explain the inheritance of the resistance.

Were the original somaclones homozygous for the resistance trait? If original somaclones were homozygous for the resistance trait, the R_1 plants used in cross-pollination would all be homozygous, and the F_1

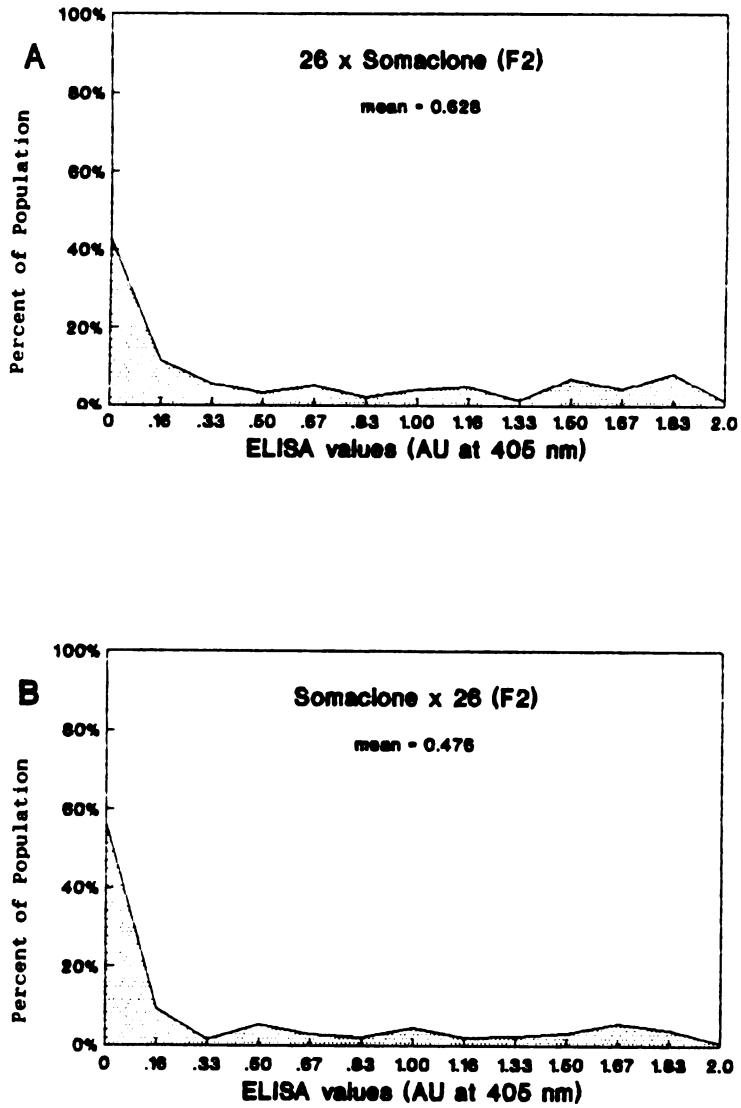


Figure 7. The distribution of ELISA values for virus detected in F_2 progeny at 10 d pi from crosses between A) GCRI-26 and somaclonal lines and B) the reciprocal cross, of somaclonal lines and GCRI-26.

progeny of crosses using different R_1 parents from the same somaclonal line would not show segregation. The F_2 progeny would segregate into different resistance phenotypes, but there would not be variation in segregation patterns among sets of F_2 progeny from different F_1 parents. Therefore, there should not be more variation among distantly related replications than among sibling replications. In replications among cousins, significant variation in segregation could occur only if the original somaclone was heterozygous for resistance.

To examine the data for evidence of heterozygous parentage, both the 10 d pi S:R ratings and ELISA means were used for each replication of each somaclonal line (listed in Table 12). In this table, siblings screened in different environmental groups are marked with (+) and other data sets are from more distantly related plants within the somaclonal family. The results of LSD multiple range tests for differences in replications of each reciprocal cross for each somaclone are included to the right of each ELISA mean.

It was evident that somaclone lines 12, 215 and 219 did not show significant differences among the data sets from environmental groups, so that these lines did not provide evidence to support the theory that the original plants were heterozygous. It must be noted that the theory was not disproven; perhaps more replications of these lines might allow detection of differences. Similarly, in somaclonal lines 322 and 330, more replications would have been useful to clarify the ambiguous results. In each of these lines, there were differences between data sets for the F_2 progeny of one cross (Sc x 26), but not in the reciprocal cross (26 x Sc).

Enough data sets of somaclone family 247 were screened to provide evidence to support the possibility that the original somaclone was

Table 12. Summary of resistance ratings at 10 d pi of F_2 progeny of reciprocal crosses between somaclonal lines and the susceptible line, GCRI-26. (+) means groups are siblings. The letters in parentheses denote the environmental group. Plants within each group were siblings.

Plant line	Sc x 26 S:R (10 d) rating	Sc x 26 ELISA mean	26 x Sc S:R (10 d) rating	26 x Sc ELISA mean
Sc 12	2:10(T)	0.279 a	5:11(R)	0.368 a
	2:12(T)	0.445 a	6:8(z)	0.856 b
	2:9(n)	0.318 a	3:4(z)	1.005 ab
	6:31	0.356	4:8(W)	0.462 ab
	(16:84%)		8:5(R)	0.734 ab
			26:36	0.626
		n.s.	(42:58%)	n.s.
Sc 215	2:14(R)	0.143 a	9:7(R)	0.779 a
	+ 5:11(i)	0.685 b	3:9(n)	0.334 a
	+ 2:11(n)	0.309 ab	12:16	0.588
	9:35	0.386	(43:57%)	
	(20:80%)	n.s.		n.s.
Sc 219	8:8(W)	0.151 a	+ 4:9(W)	0.482 a
	+ 1:5(d1)	0.247 a	+ 3:2(d1)	0.840 a
	+ 3:7(d2)	0.271 a	+ 8:4(d2)	1.028 a
	0:2(n)	0.428 a	2:2(n)	0.928 a
	12:20	0.491	15:15	0.774
	(38:62%)	n.s.	(50:50%)	n.s.
Sc 247	+ 0:16(c)	0.053 a	11:5(R)	0.740 c
	+ 1:15(u)	0.236 ab	+ 4:12(u)	0.484 bc
	0:16(s)	0.032 a	+ 6:10(x)	0.725 c
	0:14(x)	0.102 a	+ 0:13(s)	0.091 ab
	12:4(i)	0.927 d	+ 0:14(u)	0.024 a
	+ 11:5(i)	0.888 cd	21:54	0.442
	+ 4:9(s)	0.550 bc	(28:72%)	
	27:79	0.400		
	(25:75%)	**sig.		**sig.
Sc 322	11:5(c)	0.909 a	10:6(i)	0.945 b
	2:14(x)	0.336 b	+ 4:8(W)	0.448 a
	13:19	0.623	+ 3:13(x)	0.528 ab
	(41:59%)		17:27	0.658
		**sig.	(39:61%)	n.s.
Sc 330	11:5(c)	1.011 b	+ 5:8(W)	0.472 a
	4:12(y)	0.565 ab	+10:6(y)	1.186 b
	2:14(x)	0.069 a	5:11(x)	0.718 ab
	17:31	0.665	6:7(i)	0.736 ab
	(45:55%)		26:32	0.782
		*sig.	(46:54%)	n.s.

heterozygous for resistance. The data from both the F_1 and F_2 generations were compiled to make the pedigree presented in Figure 8. Of the 247 x 26 crosses, the first example was consistently highly resistant in the F_1 and each of the four F_2 families, but not as high as the self-pollinated somaclonal line. In the second example, the F_1 plants were very resistant but their F_2 progeny (taken from a single fruit) were not, indicating genetic and/or environmental variation between generations. The third and fourth examples each displayed intermediate levels of virus accumulation in the F_1 and F_2 generations. When the different crosses were compared to each other, it seemed clear that there were consistent differences between them, thus supporting the idea that there were different R_1 genotypes used for the crosses. These results are confounded because data sets were from different environmental groups.

In the reciprocal (26 x 247) crosses, differences were also observed between crosses. The first example showed an intermediate level of virus accumulation in the F_1 progeny and more virus accumulation in the F_2 generation. The second and third examples were similar in that the F_1 allowed somewhat high levels of virus to accumulate. The F_2 progeny of the second cross gave strong evidence for genetic differences in the progeny of different F_1 plants. Two sets of data (from different environmental groups) of F_2 progeny were screened from two different F_1 plants, resulting in convincing differences. The two data sets of F_2 screened from one F_1 plant showed an intermediate level of virus present, but the data sets of F_2 progeny from another F_1 plant showed a consistent resistance to virus accumulation. These differences were evident in both the S:R rating system and the

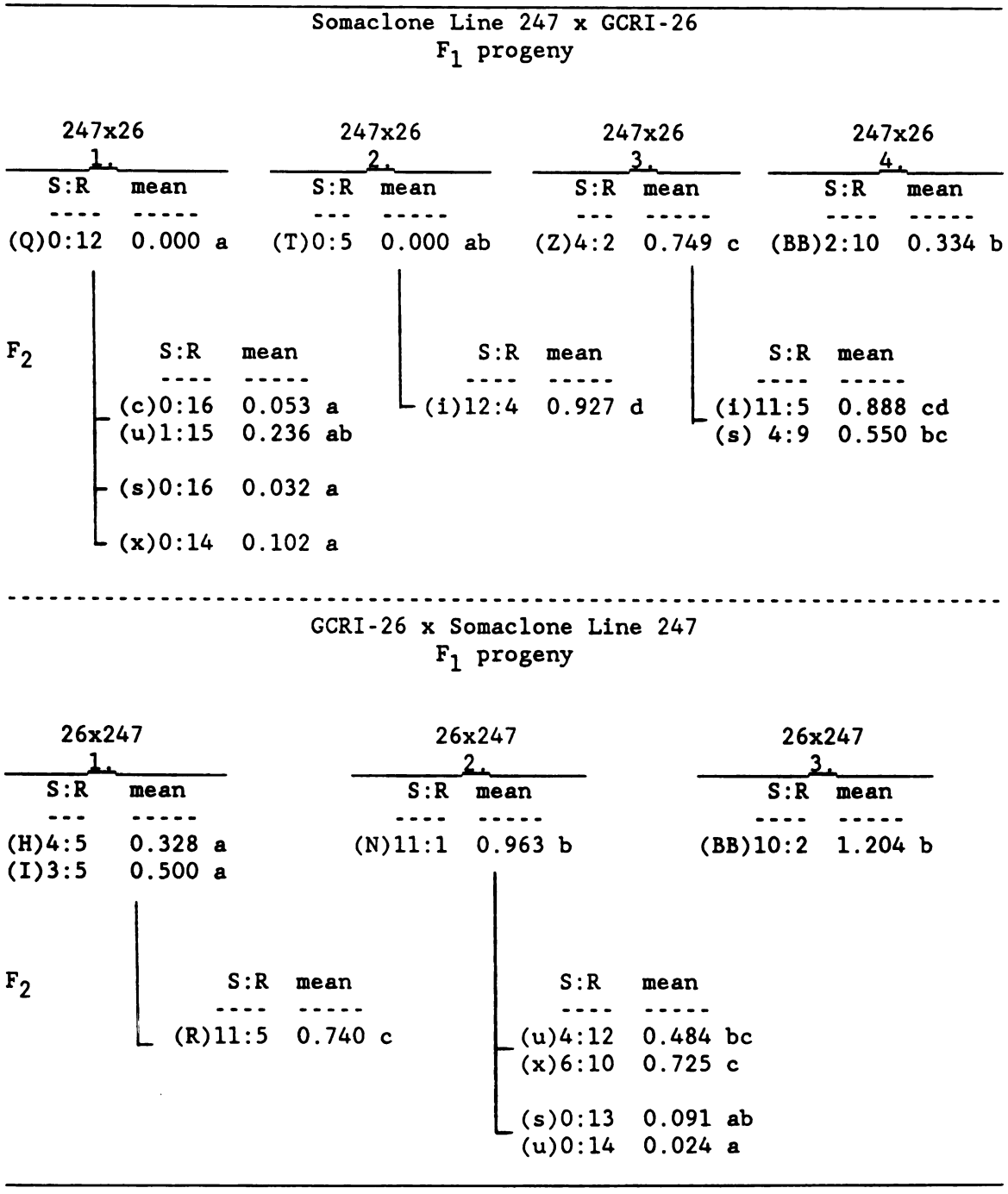


Figure 8. Pedigree of reciprocal crosses of somaclonal line 247. Different R_1 plants were used for the different numbered crosses shown. For each family of progeny screened, the environmental group letter (in parentheses) is given, followed by the S:R rating at 10 d pi and the mean ELISA value. Results of LSD tests were included for comparison of means of each generation of each particular cross.

comparisons of ELISA means, and differences between environmental groups were not large, indicating that there may be genetic differences.

Overall, these data from both generations of the reciprocal crosses support the hypothesis that the original somaclone 247 might have been heterozygous for resistance, however, the mode of inheritance for the resistance would need to be complex to explain why the self-pollinated generations did not display segregation into different phenotypes. A genetic model which incorporated heterozygosity was considered and will be discussed in a later section. However, a closer examination of the effect of environment was important.

Estimation of environmental effects. Just as was seen in the screening of the F_1 progeny, there was notable variation among environmental groups of the F_2 progeny. The results of testing for significant differences among groups are presented in Table 13A. The F values were very significant ($p=0.01$) for F_2 progeny of both reciprocal crosses, and multiple range testing showed where the differences occurred among environmental groups. These differences among groups represent a considerable environmental influence on screening. Again, because experimental design gave an unbalanced case, variance due to interactions among environmental group and genotype could not be calculated.

Data were analyzed for variation among somaclone families within each environmental group (Table 13B) and for variation of environmental groups within each somaclone family (Table 13C). Variation among progeny of each somaclonal line was not significant, except in the case of Group c (due to progeny of Sc 247 x 26, which is also reflected in

Table 13. Results of analysis of variance and LSD multiple range tests ($p=0.05$) of ELISA means of F_2 progeny of pooled somaclone families. A) Among environmental groups. B) Among somaclone F_2 families within environmental groups. C) Among somaclone F_2 families.

A. Variation among F_2 environmental groups.

Sc x 26			26 x Sc		
Environmental Group	# plants	ELISA mean (LSD)	Environmental Group	# plants	ELISA mean (LSD)
R	16	0.143 (a)	s	13	0.091 (a)
x	37	0.197 (a)	u	29	0.278 (a)
u	16	0.236 (ab)	W	50	0.467 (ab)
d	11	0.244 (ab)	n	16	0.483 (ab)
s	29	0.264 (ab)	R	60	0.656 (bc)
n	25	0.331 (ab)	x	48	0.657 (bc)
T	25	0.372 (abc)	i	29	0.852 (cd)
y	14	0.565 (bcd)	z	18	0.873 (cd)
W	16	0.657 (cd)	d	17	0.962 (d)
c	48	0.658 (d)	y	14	1.186 (d)
i	48	0.833 (d)			
F value between environmental groups		4.81**			4.57**

B. Variation among pooled somaclone F_2 families within environmental groups (in groups where comparisons can be made).

Sc x 26				26 x Sc			
Group	Sc lines	# plants	F value	Group	Sc lines	# plants	F value
R	215	16	----	R	12	16	
					215	16	1.83
					247	16	(n.s.)
W	219	16	----	W	12	12	
					219	13	0.01
					322	12	(n.s.)
					330	13	
i	215	16	1.16	i	322	16	0.72
	247	32	(n.s.)		330	13	(n.s.)
n	12	11	0.16 (n.s.)	n	215	12	3.28
	215	13			219	4	(n.s.)
	219	2					
c	247	16	15.97**	c	--	--	----
	322	16					
	330	16					

C. Variation among somaclone F_2 families among environmental groups.

	Sc x 26			26 x Sc		
Line	# plants	# groups	F value	# plants	# groups	F value
Sc 12			0.29		1.42	
Sc 215			3.83		3.47	
Sc 219			1.32		1.31	
Sc 247			9.74**		5.07**	
Sc 322			8.89**		2.72	
Sc 330			5.06*		2.09	

* Significant differences ($p=0.05$).
**Highly significant differences ($p=0.01$).

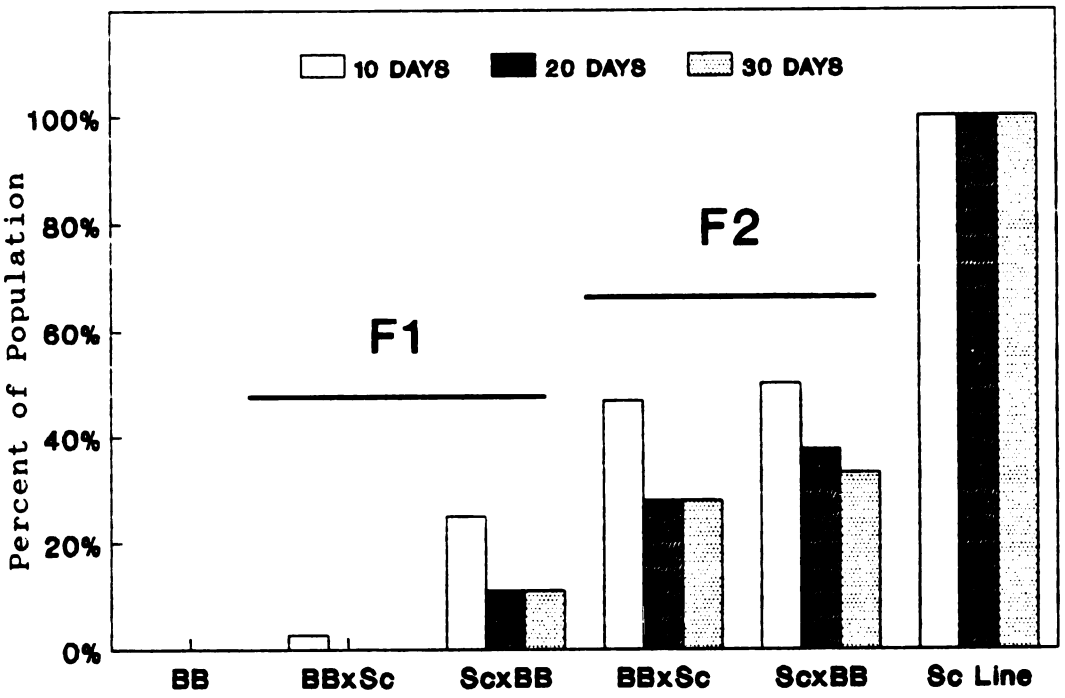
Table 13C). Otherwise notable differences were not seen among somaclone families when examined within the environmental groups.

Consistent variation among data sets within each somaclonal family was not observed (Table 13C). Only among the progeny families of somaclone 247 was there significant variation, but it was not possible to determine the source. In general, because most testing did not attribute variation to differences among somaclone families or within somaclone families, it is likely that most of the variation is due to environmental factors.

Reciprocal crosses with Bonny Best. To test the level of somaclonal resistance when moved into another susceptible cultivar, several somaclonal lines were outcrossed to Bonny Best tomatoes. This step was taken in order to determine the general level of resistance with another genetic background. It was also important to confirm that there was a difference in progeny phenotypes of reciprocal crosses, thus eliminating the possibility that the line GCRI-26 was the cause of the previously detected differences.

Results of screening the F_1 and F_2 of reciprocal crosses with Bonny Best are presented graphically in Figure 9. In the F_1 progeny, there appeared to be some maternal effect in the inheritance of the resistance, and in the F_2 generation, there was increased resistance (greater number of resistant plants), and the loss of the maternal effect. Overall, there was less resistance expressed in progeny of these crosses than progeny of crosses with GCRI-26. This type of change in expressivity was expected, since it was reported previously in studies with the *Tm-1* gene (Fraser, 1985). A more detailed inspection of the resistance ratings and mean ELISA values was required for further analysis.

A.



B.

ELISA mean (AU at 10 d pi)	1.730	1.209	0.732	0.825	0.875	0.043
S:R rating (10 d pi)	24:0	35:1	24:12	15:14	12:12	0:24

Figure 9. A) graph of resistance to ToMV in reciprocal crosses of tomato somaclones with the susceptible line, Bonny Best for both F_1 and F_2 progeny, scored at 3 stages in their development: 10, 20 and 30 d pi. B) mean ELISA values.

The pedigrees of the crosses are listed in Table 14, with both S:I:R ratings and mean ELISA values (10 d pi). The crosses for somaclone lines 215 and 330 were done reciprocally with individual plants, thus eliminating the possibility that there would be complications due to differing nuclear backgrounds of the somaclonal parents when the progeny were examined for any differences between the crosses. The F_1 plants were screened in one set and F_2 plants were screened in another set, eliminating as much variation due to environment as possible.

The results of screening the respective F_1 and F_2 progeny for each somaclone were similar, using both S:I:R ratings (Table 14A) and mean ELISA values (Table 14B). Analysis of variance confirmed that there were not significant differences among somaclonal families. Pooling of the data then allowed greater precision in comparisons between the F_1 and F_2 progeny by providing a larger sample number.

The differences seen in ELISA means between reciprocal crosses were statistically significant using the pooled data. The results of LCD multiple range testing are presented in Table 14B. These differences were consistent in the F_1 progeny for each somaclonal line, though were not always significantly different when an analysis of variance was done, again due to small sample number. Differences between the ELISA means of the F_2 progeny were not evident in individual somaclonal lines, confirming the same result as when the somaclonal lines were pooled. All F_1 and F_2 progeny were significantly different from the control Bonny Best plants, showing less virus accumulation when the somaclonal resistance genes were present.

Again to estimate the amount of resistance due to cytoplasmic inheritance, it was useful to look at the distribution of ELISA values

Table 14. Results of screening of cross-pollinated plants from reciprocal crosses of tomato somaclone R_2 with the susceptible cultivar, Bonny Best. A) S:I:R ratings and B) Mean ELISA values, with LSD multiple range tests ($p=0.05$) to compare ELISA means between crosses within each somaclonal line.

A. S:I:R ratings at 10 d pi.

Line	Sc x BB		BB x Sc	
	F1	F_2	F_1	F_2
12-2	8:1:3	5:1:6	-	-
12-9	-	-	12:0:0	9:5:2
215-6	7:5:0	7:2:3	12:0:0	6:2:4
330-5	9:2:1	-	11:1:0	-
Pooled	24:8:4	12:3:9	35:1:0	15:7:6
Control Bonny Best	24:0:0			

B. Mean ELISA values (AU at 405 nm) at 10 d pi.

Sc R_2 Parent	Sc x BB		BB x Sc		F value
	F1	F_2	F_1	F_2	
12-2	0.682(a)	0.637(a)	-	-	
12-9	-	-	1.334(b)	0.942(ab)	3.03*
215-6	0.706(a)	1.111(ab)	1.207(b)	0.707(ab)	2.20
330-5	0.803(a)	-	1.088(a)	-	3.38
Pooled	0.732(a)	0.875(a)	1.209(b)	0.825(a)	14.40**
Control Bonnie Best	1.730(c)				

*Significant differences ($p=0.05$).
**Highly significant differences ($p=0.01$).

for the F_1 and F_2 plants screened. In Figure 10A, the control parental lines were graphed to show that there was a sharp difference between the two genotypes. The somaclonal lines were always in the range less than 0.50 AU and the susceptible Bonny Best plants were always much above 0.50 AU. It appeared that there was variation in the expressivity of susceptiblity in Bonny Best that almost looked like two discrete groupings.

In both of the F_1 progenies from the reciprocal crosses, there were intermediate levels of virus accumulation seen (Figure 10B and C), but there were 22% more plants in the resistant range of 0.00-0.50 AU when the somaclonal lines were used as the seed parent in the cross than when used as the pollen parent. As in crosses of somaclones with GCRI-26, the F_1 resistance levels are intermediate relative to the parents, thus looking much like an incompletely dominant trait.

When the ELISA distributions of the F_2 progeny from the reciprocal crosses were examined (Figure 11), there seemed to be clear segregation into classes having high or low virus titers, but lacking plants with mid-point values. This distribution is similar to that of crosses to GCRI-26 (Figure 7), but because of the elimination of environmental effects, the peaks are more discrete in the crosses with Bonny Best.

When the F_2 progeny of the reciprocal crosses were compared for differences indicating maternal inheritance of the resistance, there were no differences seen. The F_2 progeny of Sc x Bonny Best had only 3% more plants in the range of 0.0-0.5 AU than the reciprocal Bonny Best x Sc F_2. This supported the other evidence for the absence of the maternal inheritance of resistance in the F_2 generation.

Backcrosses. Completion of backcrosses of the F_1 plants to the somaclone R_1 or R_2 and susceptible GCRI-26 parents was accomplished for

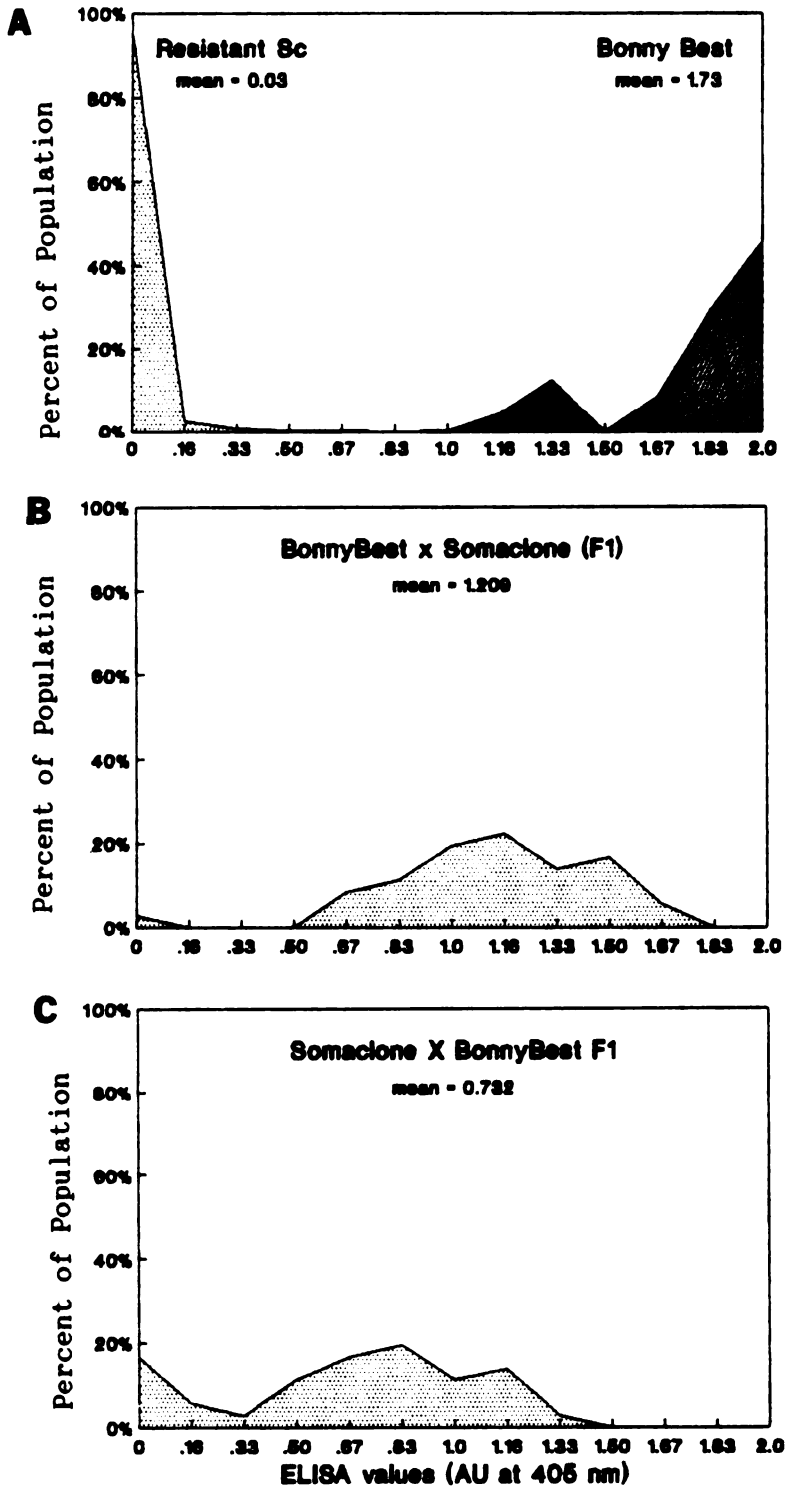


Figure 10. The distribution of ELISA values for virus detected in plants from A) resistant somaclonal lines and susceptible Bonny Best, B) the F_1 progeny of the cross Bonny Best x Sc and C) the F_1 progeny of the reciprocal cross, Sc x Bonny Best.

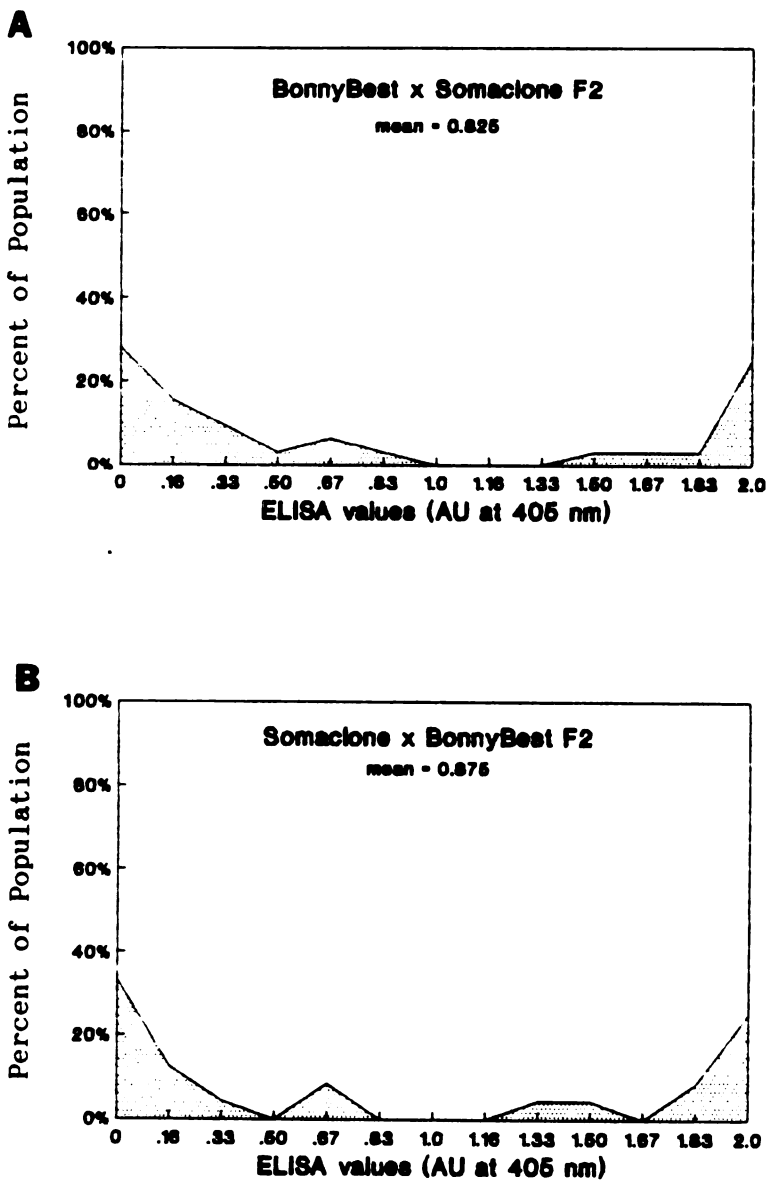


Figure 11. The distribution of ELISA values for virus detected in F_2 progeny plants from the crosses A) Bonny Best x Sc and B) Sc x Bonny Best.

many of the possible crosses, but only somaclonal line 247 had a complete set of the eight backcrosses. In initial comparisons, ELISA means from the resistance screenings were combined for all somaclonal lines and are presented graphically in Figure 12. The corresponding ELISA means and standard error of the mean are listed in Table 15.

Detection of differences among backcrosses. When the susceptible line, GCRI-26, was used twice in the crossing scheme, high levels of virus were allowed to accumulate in the progeny. However, when the somaclone lines contributed twice to the pedigree, less virus accumulated in the progeny plants, to show that a higher level of resistance was expressed, though not as high as in the self-crossed somaclones. This provided evidence for an additive effect that could be due to incomplete dominance of the resistance gene(s). More detailed analysis was necessary to establish the significance of the differences seen.

The results of analysis of variance and LSD multiple range tests ($p=0.05$) in Table 15 confirmed that the differences in ELISA means were very significant among the backcrosses and controls. The very large F value of 657.9 (with 13 and 1970 df) provided strong evidence for differences, which were then identified using the range test. Both resistant and susceptible controls were significantly different from the backcrosses, and among the backcrosses there were differences.

Backcrosses A-D were clearly different from backcrosses E-H, which confirmed the gene dosage effect. For direct comparisons of selected backcrosses, orthogonal contrasts were done (using NCSS Software). When backcrosses ABCD were contrasted to EFGH for the combined somaclones (Table 16), the T value was 9.71 (395 df), confirming highly significant differences ($p=0.01$) due to gene dosage.

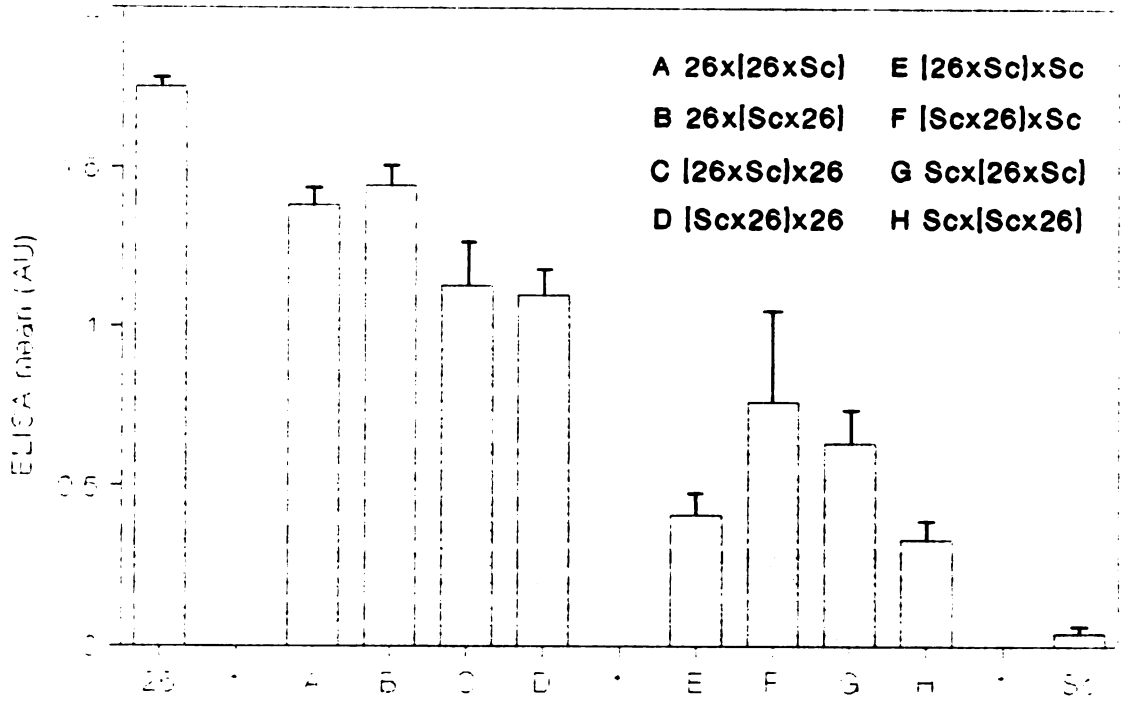


Figure 12. Results of screening backcrosses for resistance to ToMV. Bars represent the ELISA mean for each backcross, with lines indicating standard error of the mean (using pooled data from all somaclonal lines).

Table 15. Mean ELISA values for each backcross shown in Figure 12. The results of analysis of variance and LSD multiple range tests ($p=0.05$) were included.

Backcross	# plants	ELISA mean (AU)	S.E.	LSD
Control 26	425	1.755	0.0151	a
A. 26x[26xSc]	83	1.384	0.0624	b
B. 26x[Scx26]	70	1.448	0.0634	b
C. [26xSc]x26	28	1.133	0.1387	c
D. [Scx26]x26	66	1.104	0.0841	c
E. [26xSc]xSc	78	0.413	0.0710	e
F. [Scx26]xSc	10	0.758	0.2965	d
G. Scx[26xSc]	36	0.636	0.1148	d
H. Scx[Scx26]	46	0.332	0.0740	e
Control Sc	224	0.0436	0.0108	f
F value		---	----	657.9**

**Highly significant differences ($p=0.01$).

Table 16. Orthogonal comparisons between backcrosses using ELISA values at 10 d pi from the pooled somaclone families.

Backcross	# plants	ELISA mean	ORTHOGONAL COMPARISONS				
A. 26x[26xSc]	83	1.384	-1	-1			
B. 26x[Scx26]	70	1.448	-1		-1		
C. [26xSc]x26	28	1.133	-1	1			
D. [Scx26]x26	66	1.104	-1		1		
E. [26xSc]xSc	78	0.413	1			-1	
F. [Scx26]xSc	10	0.758	1				-1
G. Scx[26xSc]	36	0.636	1			1	
H. Scx[Scx26]	46	0.332	1				1
T value: (395 df)			9.71**	2.27*	2.69**	0.56 (n.s.)	0.71 (n.s.)

*Significant difference ($p=0.05$).
**Highly significant difference ($p=0.01$).

When looking for maternally biased reciprocal differences, backcross B was contrasted to D, and backcross E was contrasted to G. In both cases, differences were seen, but only in backcross B versus D was there significantly less virus accumulation in progeny with the somaclonal cytoplasm; in backcross E versus G, the differences in virus accumulation were not significant. Therefore, the maternal bias seen between F_1 reciprocal crosses did not consistently appear in the progeny of backcrosses. When the other reciprocal pairs were contrasted, backcrosses A and C showed significant differences even with the same cytoplasmic backgrounds. Backcrosses F and H were not significantly different. Analysis of ELISA data from backcrosses of somaclone line 247 were nearly identical to the pooled results. A larger sample would help in discerning the true differences among the backcrosses.

Are there differences among the somaclonal lines? To look at variation among somaclone families, the S:R ratings (at 10 d pi) were listed for data sets from each environmental group (Table 17). There was consistency among the somaclone families in which similar crosses were duplicated. For a closer look, the results were converted to percentage S:R in Table 18A, with the corresponding ELISA means found in Table 18B. When differences among somaclone families were tested for each backcross using analysis of variance of ELISA values, these differences were not significant for each case. Thus, the pooling of the data from different somaclonal lines was valid.

Distribution of ELISA values for backcrosses. In Figure 13, it was evident that backcrosses A-D showed fewer plants with low virus titer (upper graph) compared to backcrosses E-H (lower graph). The biggest differences seen in the distribution of ELISA values seemed to be between these two groups, which were divided into those backcrosses which used the somaclonal lines once (A-D) or twice (E-H). Looking at the reciprocal backcrosses B and D, it was noted that backcross D, which had the cytoplasm of the somaclonal line, had more progeny in the low ELISA value range. Of the other reciprocal pair which had differing cytoplasmic backgrounds (E and G), there was not a striking difference between the distributions of ELISA values. As an added note, there were not discrete phenotype groups evident in the distribution of ELISA values, which suggests variation due to environmental effects.

Summary of results of screening somaclonal line 247 for resistance to ToMV. The next step in the genetic analysis was to look at all of the data from one somaclonal line. Somaclonal line 247 was chosen for this analysis for two reasons: it displayed both a high level of resistance

Table 17. S:R ratings (10 d pi) of backcrosses which were screened with ToMV. Results of each environmental group were shown for each somaclone family.

Backcross	12	215	219	247	322	330	SUM
A. 26x[26xSc]	10:0 12:0 10:1 32:1	11:1 7:0 18:1	-	12:0 10:0 22:0	-	-	81:2 (%) 98:2
B. 26x[Scx26]	10:0	12:0 10:0 22:0	-	7:9 9:1 16:10	-	12:0	60:10 (%) 86:14
C. [26xSc]x26	-	-	-	9:3 8:2 17:5	-	4:2	21:7 (%) 75:25
D. [Scx26]x26	-	-	8:2	9:1 7:5 8:2 24:8	9:3	10:2	51:15 (%) 77:23
E. [26xSc]xSc	5:7 2:8 5:7 0:17 12:39	0:5	-	0:6 4:3 4:9	-	4:5	20:58 (%) 26:24
F. [Scx26]xSc	-	-	-	4:6	-	-	4:6 (%) 40:60
G. Scx[26xSc]	6:6 4:4 2:4 12:14	-	-	3:7	-	-	15:21 (%) 42:58
H. Scx[Scx26]	-	3:9	3:9	0:10	-	3:9	9:37 (%) 20:80

Table 18. Results of screening of backcrosses with ToMV. A) Expressed as percentages (S:R rating at 10 d pi). B) Mean ELISA values at 10 d pi.

A. Percent S:R rating for each somaclonal line.

Backcross	12	215	219	247	322	330	SUM
A. 26x[26xSc]	97:3	95:5	--	100:0	--	--	98:2
B. 26x[Scx26]	100:0	100:0	--	62:38	--	100:0	86:14
C. [26xSc]x26	--	--	--	77:23	--	67:33	75:25
D. [Scx26]x26	--	--	80:20	75:25	75:25	84:16	77:23
E. [26xSc]xSc	23:77	0:100	--	31:69	--	44:56	26:74
F. [Scx26]xSc	--	--	--	40:60	--	--	40:60
G. Scx[26xSc]	46:54	--	--	30:70	--	--	42:58
H. Scx[Scx26]	--	25:75	25:75	0:100	--	25:75	20:80

B. ELISA means for each somaclonal line.

Backcross	12	215	219	247	322	330	SUM
A. 26x[26xSc]	1.427	0.971	--	1.673	--	--	1.384
B. 26x[Scx26]	1.568	1.354	--	1.447	--	1.520	1.448
C. [26xSc]x26	--	--	--	1.180	--	0.963	1.133
D. [Scx26]x26	--	--	1.304	1.018	1.136	1.138	1.105
E. [26xSc]xSc	0.377	0.007	--	0.548	--	0.645	0.413
F. [Scx26]xSc	--	--	--	0.758	--	--	0.758
G. Scx[26xSc]	0.682	--	--	0.516	--	--	0.636
H. Scx[Scx26]	--	0.401	0.488	0.009	--	0.376	0.332

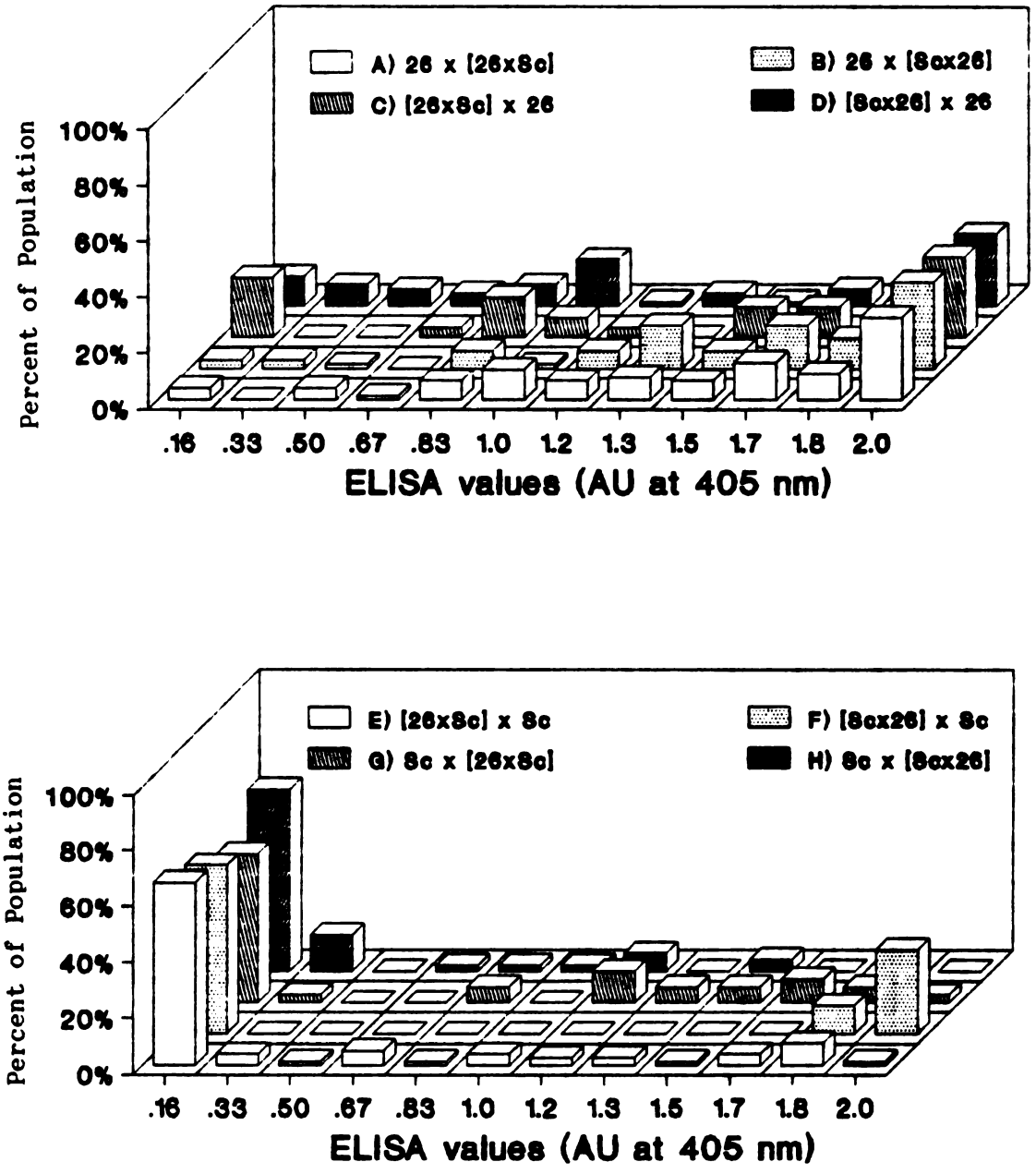


Figure 13. The distribution of ELISA values for virus detected in plants from the backcrosses made between susceptible GCRI-26, resistant somaclonal lines and the F_1 progeny of these. Upper graph) Backcrosses A-D used the somaclonal lines once in the pedigree and Lower graph) backcrosses E-H used the somaclonal lines twice in the pedigree.

and a clear maternal effect. Also, extra plants of line 247 were screened at each level to provide larger numbers for the statistical work.

A summary of results from screening somaclone line 247 (using three rating systems) is listed in Table 19. Somaclone line 247 was found to breed true for resistance after self-pollination. There were clear differences in reciprocal crosses in the F_1 favoring a maternal effect in the inheritance of resistance, but not in the F_2. As noted in Figure 8, there was weak evidence for heterozygosity in the original somaclone, which must be considered when genetic models for the inheritance of resistance are conceived. For the backcrosses, more resistance was seen in the crosses which used somaclone line 247 twice compared to the crosses in which GCRI-26 was used twice, indicating the additive resistance trait is dependent on gene dosage.

Summary of results of screening somaclonal line 12 for resistance to ToMV. Somaclonal line 12 was examined separately since a maternal bias was not detected between F_1 progeny of reciprocal crosses to GCRI-26. Data from this line also incorporates results of crosses with Bonny Best. A summary of results for this somaclonal line is presented in Table 20, using three rating systems. In the R_1 generation only 14 plants were assayed. After self-pollination, resistance ratings improved in each generation, and selection for resistance may have been an important factor in this notable improvement. Because so few R_1 plants were assayed, the possibility that the original somaclone was heterozygous for resistance is impossible to explore and cannot be eliminated. However, there was no evidence of segregation of self-pollinated progeny into different phenotypes, so that it was likely that the resistance was true breeding.

Table 19. Summary of results from screening somaclonal line 247, using the percent S:R at 10 d pi, the ELISA mean and the H:M:L ratings (high = 1.33-2.0 AU:medium = 0.51-1.32 AU:low = 0.00-0.50 AU) for virus titer.

Cross	Sample size	% S:R	ELISA mean	S.E.	% H:M:L
247 R_1	50	1:99	0.04	0.023	0:4:96
247 R_2	122	1:99	0.03	0.006	0:1:99
247 R_3	132	1:99	0.04	0.006	0:0:100
247 R_4	71	0:100	0.02	0.004	0:0:100
247 R_5	40	0:100	0.04	0.009	0:0:100
R_1-R_5	415	1:99	0.03	0.004	0:1:99
26 x 247 F_1	41	68:32	0.80	0.092	19:44:37
247 x 26 F_1	35	17:83	0.25	0.077	9:9:82
(26 x 247) F_2	74	28:72	0.44	0.069	13:13:74
(247 x 26) F_2	107	26:74	0.40	0.057	15:11:74
Backcrosses					
A) 26x[26x247]	22	100:0	1.67	0.074	82:18:0
B) 26x[247x26]	21	62:38	1.45	0.152	71:10:19
C) [26x247]x26	22	77:23	1.18	0.162	54:23:23
D) [247x26]x26	32	75:25	1.02	0.120	31:44:25
E) [26x247]x247	13	31:69	0.55	0.232	31:0:69
F) [247x26]x247	10	40:60	0.76	0.286	40:0:60
G) 247x[26x247]	10	30:70	0.52	0.262	20:10:70
H) 247x[247x26]	10	0:100	0.01	0.003	0:0:100

Table 20. Summary of results from screening somaclonal line 12, using the percent S:R at 10 d pi, the ELISA mean and the H:M:L ratings (high = 1.33-2.0 AU: medium = 0.51-1.32 AU: low = 0.00-0.50 AU) for virus titer.

Cross	Sample size	% S:R	ELISA mean	S.E.	% H:M:L
12 R_1	14	4:96	0.134	0.090	0:14:86
12 R_2	83	4:96	0.104	0.054	5:0:95
12 R_3	76	0:100	0.044	0.010	0:0:100
R_1-R_3	156	3:97	0.094	0.022	2:1:97
26 x 12 F_1	80	61:39	0.778	0.064	18:46:36
12 x 26 F_1	32	53:47	0.868	0.133	37:19:44
(26 x 12) F_2	63	42:58	0.626	0.090	21:19:60
(12 x 26) F_2	36	16:84	0.356	0.093	14:11:75
BonBest x 12 F_1	12	100:0	1.334	0.087	50:50:0
12 x BonBest F_1	12	75:25	0.682	0.126	8:59:33
(BonBest x 12) F_2	16	56:44	0.942	0.213	44:12:44
(12 x BonBest) F_2	12	42:58	0.637	0.203	25:17:58
Backcrosses (incomplete set)					
A) 26x[26x12]	34	97:3	1.427	0.096	62:32:6
B) 26x[12x26]	10	100:0	1.568	0.111	60:40:0
C) [26x12]x26	0	-	-	-	-
D) [12x26]x26	0	-	-	-	-
E) [26x12]x12	50	23:77	0.377	0.078	10:20:70
F) [12x26]x12	0	-	-	-	-
G) 12x[26x12]	26	46:54	0.682	0.126	23:27:50
H) 12x[12x26]	0	-	-	-	-

The F_1 progeny of reciprocal crosses made with somaclonal line 12 and GCRI-26 did not differ significantly from each other. Though the ELISA means of the F_2 generation did not differ significantly when tested statistically, the S:R and H:M:L ratings were not similar between the reciprocal crosses, indicating that the reciprocal differences might be real.

The results of screening outcrosses made to Bonny Best were not consistent with the reciprocal crosses with GCRI-26, because reciprocal differences were seen in the inheritance of resistance between the crosses with Bonny Best. The differences between F_2 progeny were not as strong.

The ratings of backcrosses for somaclonal line 12 were consistent with the pattern of gene dosage dependence seen with the other somaclone families. When the somaclonal line was used only once in the pedigree, there was less resistance than when the somaclonal line was used twice. When reciprocal backcrosses were compared (E versus G), the progeny with the somaclonal cytoplasm allowed more virus accumulation, so that again there was no maternally biased transmission of the resistance.

Seed transmission of TMV. Some plant-virus combinations allow seed transmission of the virus (Gibbs and Harrison, 1976), which can resemble cytoplasmic inheritance of infection. To determine the role of endogenous ToMV in the differences in virus titer seen between reciprocal crosses, the seed-transmissibility of ToMV was assessed. The results provided evidence to confirm that ToMV was not seed transmitted.

Of 60 GCRI-26 seeds which were soaked in virus inoculum and then allowed to germinate undisturbed, no virus was detected by ELISA at 18 d pi (Murakishi, personal communication). In the same experiment, of the 30 GCRI-26 seeds which were not treated with the virus before

germination, none were found to be infected by virus at 18 d. This evidence indirectly supported that of the previous reports that ToMV was not transmitted through the seed (Taylor *et al.*, 1961).

A stronger line of evidence was provided with the results of screening uninoculated seedlings of GCRI-26 which were produced on infected parent plants. The absence of ToMV after 25-30 d, in these susceptible GCRI-26 plants (which were used in studies of the movement of virus) was consistent for all plants screened. This was an important result in eliminating seed transmission of virus as part of the 'resistance' mechanism..

In another experiment, ELISA values were determined for virus-infected fruit-bearing plants, fruit juice and seed from the fruit after acid washing (Table 21). From these data, it was clear that there was virus present at high levels in the fruit of infected plants, but that the acid washing treatment commonly used in this work effectively destroyed the virus (coat protein) on the seed coat.

Table 21. Results of testing for the presence of ToMV using ELISA in different parts of infected or healthy plants.

	Plant ELISA mean	Fruit ELISA mean	Seed ELISA mean
Infected plants	1.48 AU	1.61 AU	0.00 AU
Healthy plants	0.03 AU	n.d.	0.00 AU

Results of crosses with known resistance genes. To test for allelism between the somaclones and the other characterized resistance genes, fresh reciprocal crosses were completed and the F_1 progeny were

inoculated with ToMV and screened using ELISA. Somaclone lines 12 and 247 were used in crosses with the isogenic lines GCRI-237 (*Tm-1* gene), GCRI-236 (*Tm-2* gene) and GCRI-267 ($Tm\text{-}2^2$ gene). The *Tm-1* gene is incompletely dominant for limitation of virus multiplication (Fraser and Loughlin, 1980), and the genes of the *Tm-2* locus are incompletely dominant for the hypersensitive response to ToMV (Hall, 1980). When the *Tm-2* genes are heterozygous, temperature dependent systemic necrosis occurs, which is considered to be incomplete dominance of the hypersensitive response. The *Tm-2* gene is less effective than the $Tm\text{-}2^2$ gene against most strains of ToMV.

For incompletely dominant genes, if the F_1 progeny were to show high levels of resistance, then the genes being tested could be considered to be additive in nature, and might be allelic to each other. The same results might also indicate that there are genes at two different loci which have a incompletely dominant effect. Less resistance in the F_1 progeny would serve as evidence that the genes tested were not additive. Resistance screening of the segregating F_2 generation of the crosses would be necessary to establish allelism of genes, but this screening was not completed.

Table 22 gives the results of screening the F_1 progeny of the reciprocal crosses at 10 d pi. Resistance was intermediate when somaclonal lines or GCRI-237 were crossed with susceptible GCRI-26, indicating incomplete dominance of resistance for these genes. It is notable that by classification in S:R or H:M:L ratings there appears to be segregation in the F_1 progeny. This is likely to be due to variable expressivity of these genes, as was also noted in the distribution of ELISA values in Figure 5. It is also possible that at the the time of

Table 22. Results of screening reciprocal crosses made between somaclonal line 12 or 247 and tomatoes bearing the resistance genes *Tm-1/Tm-1* (GCRI-237), *Tm-2/Tm-2* (GCRI-236) or *Tm-2²/Tm-2²* (GCRI-267).

Genotype	Cross	10 d ELISA value (mean)	(S.E.)	S:R rating	H:M:L rating
Sc/+	26 x 247	0.946	0.408	10:2	7:4:1
	247 x 26	0.246	0.306	2:10	1:4:7
	26 x 12	0.414	0.479	5:7	2:4:6
	12 x 26	0.764	0.484	8:4	6:4:2
Tm-1/+	26 x 237	0.671	0.345	9:3	3:6:3
	237 x 26	0.659	0.336	9:3	3:6:3
Sc/*Tm-1*	247 x 237	0.001	0.004	0:12	0:0:12
	237 x 247	0.003	0.005	0:6	0:0:6
	12 x 237	0.062	0.042	0:12	0:0:12
	237 x 12	0.027	0.010	0:12	0:0:12
Sc/*Tm-2*	247 x 236	0.603	0.348	8:4	3:6:3
	236 x 247	0.760	0.452	9:3	4:5:3
Sc/*Tm-2²*	247 x 267	0.012	0.006	0:12	0:0:12
	267 x 247	0.001	0.003	0:12	0:0:12
Controls					
Sc/Sc	247 x 247	0.025	0.044	0:12	0:0:12
	12 x 12	0.003	0.006	0:5	0:0:5
Tm-1/Tm-1	237 x 237	0.149	0.256	1:11	1:0:11
+/+	26 x 26	1.513	0.051	12:0	12:0:0

assay, partial resistance had already been overcome. The results listed here support a genetic model for incompletely dominant resistance in the somaclonal lines, since the *Tm-1* gene has a similar intermediate level of resistance to virus multiplication seen in the somaclone lines.

Significantly, crosses made to GCRI-237 using either somaclonal line produced progeny which were fully resistant to ToMV, indicating additivity of the somaclonal resistance genes with *Tm-1*. Screening of the F_2 plants would allow differentiation between allelism and additivity of resistance genes.

Because the crosses with GCRI-237 using somaclone lines 12 or 247 each resulted in complete resistance, this supported earlier results indicating that there were not differences between the lines of somaclones. Screening of crosses between the somaclonal lines themselves would provide further evidence of allelism of the resistance genes recovered from the somaclones.

When somaclonal line 247 was crossed with GCRI-236 (*Tm-2* gene), the progeny had intermediate levels of virus after 10 d pi. This evidence indicated that somaclonal resistance and the *Tm-2* gene were not additive. In fact, because the *Tm-2* gene did not display dominance in the F_1 progeny, there may have been interference in the resistance normally seen in plants with the genotype *Tm-2/+* by the presence of the somaclonal resistance gene. Control plants of the *Tm-2/+* genotype would be important for comparison, but none were assayed.

When the somaclonal lines were crossed with plants having $Tm\text{-}2^2$, the progeny plants did not allow virus to replicate, thus showing possible additivity of resistance. Alternatively, the $Tm\text{-}2^2$ gene may act almost completely dominant or epistatic for resistance and may

not actually be additive with the somaclonal resistance. Further screening of the F_2 progeny would be necessary to discern between these possibilities. Again, controls with the *Tm-2*^{2}*/+* genotype would have been useful for comparison, but were not screened.

MOLECULAR GENETICS

Because there appeared to be a cytoplasmic factor in the inheritance of TMV resistance in the tomato somaclones, the possibility that there were detectable changes in cytoplasmic nucleic acids was explored. Somaclonal lines were examined for changes in the chloroplast genome and for the presence of unusual nucleic acids (dsRNA) in the cytoplasm which might be associated with the resistance trait. One can imagine that the dsRNA might act as a protective element against viral replication, and could have been activated by the "genome shock" of the tissue culture conditions. Gene activation resulting in the expression of a repressor of TMV replication or excision out of the genome within which it has integrated could confer the resistance seen in the somaclonal lines.

Changes have been detected in mitochondrial DNA of potato (Kemble and Shepard, 1984) and in maize (Gengenbach *et al.*, 1977) after tissue culture operations. Because chloroplasts or mitochondria might be involved in the life cycle of ToMV, changes in the chloroplast genome were studied. Similar attempts to purify and analyze mitochondrial DNA were not successful.

Chloroplast DNA. Restriction fragments of cpDNA from somaclones 12 and 247 were compared to cpDNA of GCRI-26, the parental line. Using four restriction enzymes, no RFLPs could be seen on the gel (Figure 14). This does not mean that there are no changes in either somaclonal

plastid genome, only that if changes occurred, they were not detected by these enzymes. Banding patterns were similar to those published for tomato (Piechulla, et.al., 1985; Palmer and Zamir, 1982). Therefore, if there was a change in the cpDNA which altered virus replication, the change would not be a major one.

Double-stranded RNA. When procedures were used to extract dsRNA from plant material, no dsRNA was detected in GCRI-26 or the somaclones (Figure 15). A band of dsRNA corresponding to the replicative form of ToMV (6.15 kb) was faintly visible after extraction from a mildly infected GCRI-26 plant. As a positive control, dsRNA was extracted from pepper (14 kd). Because no dsRNA was found in the uninfected tomato lines, this provided evidence that dsRNA (expressed constitutively) does not play a role in the resistance seen in the tomato somaclones.

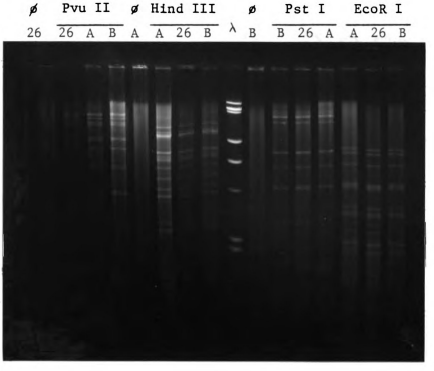


Figure 14. Restriction patterns of cpDNA from parental tomato line GCRI-26 compared to somaclone line 247 (A) and 12 (B). Lanes marked ø are undigested cpDNA. Lambda DNA fragments (restricted by *Hind* III) were used as markers.

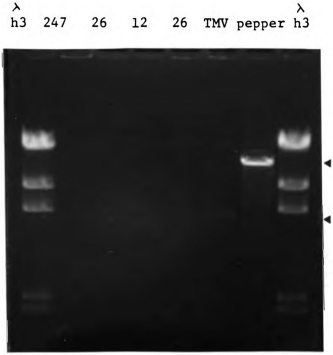


Figure 15. Extraction of dsRNA species from tomato somaclones 12 and 247 and from parental line 26. Controls show bands after extraction from ToMV-infected tomato (faint) at 6.15 kb and from pepper at 14 kb.

GENETIC ANALYSIS

Genetic models. With the completion of resistance screening for the somaclonal lines and crosses, it was possible to test models of inheritance of the resistance. It became clear that there were certain data that eliminated very simple genetic models from consideration. There were three lines of evidence which need to be considered in the proposals for a model of inheritance of the somaclonal resistance.

First, nearly all R_1 plants screened were resistant to ToMV accumulation. Because there was no segregation of self-pollinated progeny into different phenotypes, a single gene for resistance would have had to be homozygous in the original somaclone. If the original somaclone were not homozygous, then multiple genes would be needed in the model.

The second line of evidence involved the maternally biased inheritance. In tomatoes, cytoplasmic inheritance has been reported to be uniparental maternal (Kirk and Tilney-Bassett, 1978), so that a trait inherited from the seed-bearing parent would be passed to all of the progeny, and the trait would not be seen in any progeny if available only from the pollen parent. Yet in the somaclonal resistance, the results of screening progeny of reciprocal crosses indicated intermediate levels of resistance. The maternal bias in the inheritance of resistance nearly disappeared in the F_2 generation and in the backcross progeny. There was no model involving a cytoplasmic gene that would provide an explanation for these results was impossible, so other explanations for the maternal bias in transmission of inheritance were needed.

Lastly, the intermediate levels of virus accumulation seen in F_1 progeny from crosses and backcrosses suggest the possibility that the

somaclonal resistance was due to completely dominant nuclear gene(s) was unlikely. There was more evidence to support models that were based on incompletely dominant or additive genes.

Another more general complication was the high level of variation due to environmental effects that was seen among groups of plants screened at different times. This reduced the ability to resolve differences in resistance among the lines, and in retrospect, more attention was needed in the experimental design of the progeny evaluation. The assay for evaluation of resistance may not have been optimal either, as demonstrated by the large variation seen among plants with the genotype, *Tm-1/+* (Figure 5 and Table 22). To make up for this, care was taken to keep all possible models in mind for evaluation. Two models are presented and evaluated.

Model 1: Two additive genes plus a cytoplasmic effect. In this model, a scheme was proposed for resistance based on two gene pairs plus a cytoplasmic factor. The two genes are additive and unlinked, with alleles for susceptibility (*Ts*) and alleles for resistance (*Tr*) plus the cytoplasmic resistance factor A or the susceptibility cytoplasm a. For this model, the assumption was made that the original somaclone had a heterozygous genotype, but that this heterozygosity was masked in the self-crossed progeny because of the additive effect of two nuclear genes plus a cytoplasmically encoded gene which gave similar phenotypes. The description of this model can be found in Table 23, with the list of assumptions which were made.

The descriptions of phenotypes were dependent on gene dosage, and are listed as susceptible (S) versus resistant (R) or rated on a scale of zero to two (roughly corresponding to the scale of ELISA values). Also listed in Table 23 are the phenotype descriptions of the selfed

Table 23. Model 1: Description of proposed genetic model of inheritance of somaclonally derived resistance to ToMV.

Assumptions:

1. There was a cytoplasmic mutational event that caused a change from **a** to **A** cytoplasm which enhances the effect of nuclear resistance genes.
2. There was a masked heterozygous gene in the susceptible parent line (genotype *TsTsTsTr*).
3. Mutation or deletion occurred at two unlinked nuclear genes which are duplicate and additive.

	GCRI-26		Somaclone
Event:	**a** *TrTsTsTs*	---------→	**A** *TrTrTsTr* (mutation or deletion)

Definitions

A is a cytoplasmically encoded resistance gene
a is the lack of this gene in the cytoplasm
Tr is the additive resistant allele
Ts is the susceptible allele

	Phenotype		Ranking	
Genotype	**a**	**A**	**a**	**A**
TsTsTsTs	S	S	2.0	1.5
TsTsTsTr	S	S	1.5	1.0
TsTsTrTr	S	R	1.0	0.5
TsTrTrTr	R	R	0.5	0.0
TrTrTrTr	R	R	0.0	0.0

Selfed GCRI-26

			Rank	
TrTsTsTs ----------→	1	**a** *TsTsTsTs*	S	2.0
	2	**a** *TrTsTsTs*	S	1.5
	1	**a** *TrTrTsTs*	S	1.0
			all S	1.5 mean

Selfed somaclone

			Rank	
TrTrTsTr ----------→	1	**A** *TrTrTsTs*	R	0.5
	2	**A** *TrTrTrTs*	R	0.0
	1	**A** *TrTrTrTr*	R	0.0
			all R	0.17 mean

GCRI-26 and the selfed somaclonal lines. It was noted that the predicted phenotypes were similar to those observed, and when looking at the distributions of ELISA values for these plant lines (Figure 2) there was close agreement to the predicted distributions.

Table 24 summarizes the predicted results for F_1 progeny of crosses between the somaclonal lines and GCRI-26. The complexity of these predictions increases when all nine genotype combinations are included, but results in only four genotype classes, which are listed. The predicted results are consistent with the observed results for the reciprocal F_1 families, and perhaps might explain some of the variation that was observed between F_1 progeny from different somaclonal R_1 parents. The pooled predicted and observed S:R ratings were the same for the F_1, with 3:1 for the **a** cytoplasm and 1:3 for the **A** cytoplasm. The numerical rankings for predicted and observed crosses were also similar, in that the mean value for the **A** cytoplasm was approximately half of the value for the **a** cytoplasm in both cases. The next step is to examine the results of self-pollination to produce the F_2 progeny, presented in Table 25.

When each of the four F_1 genotypes were selfed to produce F_2 families, different ratios resulted, as shown by both S:R rating and the numerical ranking (with the same scale as ELISA values). In each case, the resistance rating was higher in the presence of **A**, the resistant somaclonal cytoplasm. When all of the resulting F_2 phenotypes were pooled for each of the different cytoplasmic backgrounds, the predicted ratings could be compared to the observed ratings for the pooled somaclonal lines. The predicted S:R ratio of 3:1 for the F_2 progeny with the susceptible cytoplasm (**a**) was the inverse of the observed S:R ratio, which was 1:3. For the resistant cytoplasm (**A**), the

Table 24. Model 1: List of predicted genotypes resulting from all possible crosses between the hypothesized genotypes of somaclonal lines and GCRI-26 of Table 23.

-Cross- Somaclone	GCRI-26	Resulting Genotypes	Class	a S:R	a Rank	A S:R	A Rank
1)*TrTrTrTr* x	*TsTsTsTs*	*TrTsTrTs*	2	S	1.0	R	0.5
2)*TrTrTrTr* x	*TsTrTsTs*	*TrTrTrTs*	4	R	0.5	R	0.0
		TrTsTrTs	2	S	1.0	R	0.5
					1.25		0.25
3)*TrTrTrTr* x	*TrTrTsTs*	*TrTrTrTs*	4	R	0.5	R	0.0
4)*TrTrTsTr* x	*TsTsTsTs*	*TrTsTrts*	2	S	1.0	R	0.5
		TrTsTsTs	1	S	1.5	S	1.0
					1.75		1.25
5)*TrTrTsTr* x	*TsTrTsTs*	*TrTsTsTs*	1	S	1.5	S	1.0
		TrTsTrTs	2	S	1.0	R	0.5
		TrTrTsTs	3	S	1.0	R	0.5
		TrTrTsTr	4	R	0.5	R	0.0
					1.0		0.5
6)*TrTrTsTr* x	*TrTrTsTs*	*TrTrTrTs*	4	R	0.5	R	0.0
		TrTrTsTs	3	S	1.0	R	0.5
					1.25		0.25
7)*TrTrTsTs* x	*TsTsTsTs*	*TrTsTsTs*	1	S	1.5	S	1.0
8)*TrTrTsTs* x	*TsTrTsTs*	*TrTsTsTs*	1	S	1.5	S	1.0
		TrTrTsTs	3	S	1.0	R	0.5
					1.25		0.75
9)*TrTrTsTs* x	*TrTrTsTs*	*TrTrTsTs*	3	S	1.0	R	0.5
			Total	3:1	1.0	1:3	0.54

Table 25. Model 1: Summary of genotypes and phenotypes for resistance to ToMV in tomatoes with an additive resistance plus a cytoplasmic resistance factor.

Cross	Genotypes	Phenotype: Cytoplasm a		Phenotype: Cytoplasm A	
F_1		26 x Sc		Sc x 26	
26 x Sc		Rank		Rank	
Sc x 26	1. *TrTsTsTs*	S	1.5	S	1.0
	2. *TrTsTrTs*	S	1.0	R	0.5
	3. *TrTrTsTs*	S	1.0	R	0.5
	4. *TrTrTsTr*	R	0.5	R	0.0
		3S:1R	1.0	1S:3R	0.5
Expected F_2: (four types of self-crosses)					
1. *TrTsTsTs* ------>	1 *TrTrTsTs*	S	1.0	R	0.5
	2 *TrTsTsTs*	S	1.5	S	1.0
	1 *TsTsTsTs*	S	2.0	S	1.5
		all S	1.5	3S:1R	1.0
2. *TrTsTrTs* ------>	1 *TrTrTrTr*	R	0.0	R	0.0
	2 *TrTrTsTr*	R	0.5	R	0.0
	1 *TrTrTsTs*	S	1.0	R	0.5
	2 *TrTsTrTr*	R	0.5	R	0.0
	4 *TrTsTrTs*	S	1.0	R	0.5
	2 *TrTsTsTs*	S	1.5	S	1.0
	1 *TsTsTrTr*	S	1.0	R	0.5
	2 *TsTsTrTs*	S	1.5	S	1.0
	1 *TsTsTsTs*	S	2.0	S	1.5
		11S:5R	1.0	5S:11R	0.53
3. *TrTrTsTs* ------>	all *TrTrTsTs*	all S	1.0	all R	0.5
4. *TrTrTsTr* ------>	1 *TrTrTrTr*	R	0.0	R	0.0
	2 *TrTrTsTr*	R	0.5	R	0.0
	1 *TrTrTsTs*	S	1.0	R	0.5
		1S:3R	0.5	all R	0.13
Pooled total for F_2		3S:1R	1.0	1S:3R	0.54

predicted and observed resistance ratios were very close to the same. The predicted numerical rankings between these reciprocal F_2 progeny were similar to those of the F_1 progeny, with the resistant cytoplasm (A) reducing the value by about half. However, the observed ELISA means of the reciprocal F_2 progeny were not significantly different from each other, so that there was a clear discrepancy between the predicted and the observed values. Though this weakened the support for this model, it did not eliminate the model, since it was possible that the observed resistance rating were skewed by inadvertant selection of more resistant F_1 plants for self-pollination to get the next generation. To examine the data for this possibility, it was necessary to predict the possible results from each crossing combination.

From the previous two tables, the predicted values were listed to show the ranking of F_2 progeny from each of the nine F_1 crosses. These are presented in Table 26. From this chart, it was then possible to look at the crossing pedigree of individual somaclones to see if the model would fit. The comparison of predicted and observed ratings was made using dat from somaclonal line 247, which had the most plants sampled (Table 27).

In Table 27, the observed results of resistance screening of line 247 are listed on the left side of the table, and the best fit for the predicted genotypes is listed on the right of side of the table. It seemed that some of the data did fit the predicted model, but with notable exceptions, especially when both S:R ratings and ELISA means were compared. For instance, only the progeny of the first cross of 247 x 26 gave a close fit of observed and predicted values. In the other crosses, the ELISA values were fairly similar between observed values and predicted rankings, but the S:R ratios were very different.

Table 26. Model 1: List of combinations of F_1 and F_2 predicted results from crosses of all possible genotypes of somaclonal lines and GCRI-26.

A) 26 x Sc (cytoplasm a)

F_1 Cross	S:R rating F_1	S:R rating F_2	rank F_1	rank F_2
1)	all S	11:5	1.0	1.0
2)	1:1	11:5	0.75	1.0
		1:3		0.5
3)	all R	1:3	0.5	0.5
4)	all S	all S	1.25	1.5
		11:5		1.0
5)	3:1	all S	1.0	1.5
		11:5		1.0
		all S		1.0
		1:3		0.5
6)	1:1	all S	0.75	1.0
		1:3		0.5
7)	all S	all S	1.5	1.5
8)	all S	all S	1.25	1.5
		all S		1.0
9)	all S	all S	1.0	1.0

B) Sc x 26 (cytoplasm A)

F_1 Cross	S:R rating F_1	S:R rating F_2	rank F_1	rank F_2
1)	all R	5:11	0.5	0.5
2)	all R	5:11	0.25	0.5
		all R		0.13
3)	all R	all R	0.0	0.13
4)	1:1	1:3	0.25	1.0
		5:11		0.53
5)	1:3	1:3	0.5	1.0
		5:11		0.53
		all R		0.5
		all R		0.13
6)	all R	all R	0.25	0.5
		all R		0.13
7)	all S	1:3	1.0	1.0
8)	1:1	1:3	0.75	1.0
		all R		0.5
9)	all R	all R	0.5	0.5

Table 27. Model 1: Comparison of predicted and observed results of an additive duplicate gene model using data from somaclonal line 247.

Cross	F1 S:R	F1 ELISA mean	F2 S:R	F2 ELISA mean	Cross type*	predicted F1 S:R	predicted F1 rank	predicted F2 S:R	predicted F2 rank
26 x 247									
1.	4:5	(0.33)							
	3:5	(0.50)	----11:5	(0.74)	3	all R	(0.5)	-- 1:3	(0.5)
2.	11:1	(0.96)	----4:12	(0.48)	5	1:1	(0.75)	-- 1:3	(0.5)
			6:10	(0.73)				or 11:5	(1.0)
								or all S	(1.0)
								or all S	(1.5)
			----0:13	(0.09)					
			0:14	(0.02)					
3.	10:2	(1.20)	----none		4,8	all S	(1.25)	-	-
247 x 26									
1.	0:12	(0.00)	----0:16	(0.05)	3	all R	(0.0)	--all R	(0.13)
			1:15	(0.24)					
			----0:16	(0.03)					
			----0:14	(0.10)					
2.	0:5	(0.00)	----12:4	(0.93)	4	1:1	(0.25)	-- 1:3	(1.0)
								or 5:11	(0.5)
3.	4:2	(0.80)	----11:5	(0.89)	8	1:1	(0.75)	-- 1:3	(1.0)
			4:9	(0.55)				or all R	(0.5)
4.	2:10	(0.33)	----none		2,6	all R	(0.25)	-	-

*In reference to cross types listed in Table 26.

Thus, this proposed model was not supported by the data from resistance screening of either pooled somaclonal lines nor by comparisons of individual plant lines. The complexity of the analysis of this model was also a serious drawback, especially with the high level of environmental error. Further analysis, including the backcross results, failed to provide support for the model. Therefore, due to its great complexity, the evaluation of backcross results is not presented.

Model 2: Incompletely dominant gene(s) with an additive cytoplasmic interaction. One major problem in formulating models for testing was that the maternal bias in the inheritance of the resistance was not seen consistently through all generations of crosses. It seemed that there was not a feasible model which incorporated an organellar gene for resistance. The maternally biased inheritance that was so strong in the F_1 progeny of the reciprocal crosses was not evident in the F_2 progeny nor the backcrosses.

The possiblity that seed transmission of ToMV was the reason for the greater virus titers in susceptible plants was eliminated in the experiments on seed transmission. If this maternal bias was not due to a resistance gene encoded in an organelle, nor due to transmission of virus through the maternal cytoplasm, nor due to dsRNA presence, few choices remain to explain this aspect of inheritance of the resistance. Perhaps a maternal effect, also called predetermination, or nuclear-cytoplasmic interaction may be involved, though a mechanistic model or explanation for these would be difficult to formulate.

Maternal predetermination describes a transitional phenotypic effect that normally wears off in one or more generations (Levine, 1973). It usually involves the imposition of characteristics on the cytoplasm of the ova by an outside force. Strictly speaking, this is a

developmental process rather than a mode of inheritance, but it can mimic extrachromosomal inheritance. Maternal effect, essentially the same as predetermination, is the determination of the phenotype of the offspring by cytoplasmic elements coded for by the nuclear genotype of the mother (Elseth and Baumgardner, 1984). Maternal effects can be due to complex interactions, as seen in mice in which adult size is influenced by maternal uterine genotype (Cowley, *et al.*, 1989). This is in contrast to nuclear-cytoplasmic gene interactions, such as cytoplasmic male sterility and the nuclear restorer genes of maize, which are due to the interaction of products encoded chromosomally and cytoplasmically (Elseth and Baumgardner, 1984).

This model, described in Table 28A, includes a single nuclear gene and incorporates a maternal effect. It is assumed that the gene was homozygous in the original somaclone, and was expressed in an additive way, so that heterozygous plants would show an intermediate level of resistance. A precedence for an incompletely dominant ToMV resistance allele is the tomato resistance gene, *Tm-1* (Fraser and Loughlin, 1980). Because of the additivity seen between *Tm-1* and the somaclonal resistance, a tentative term used for the somaclonal gene in this model was *Tm-3*. Final designation depends on further genetic analysis to determine allelism to *Tm-1*. Additionally, there was a favorable change in the offspring, determined by the presence of the resistance genotype in the female parent, which was also dependent upon dosage of the resistance gene. The gene dosage ranking for this model is presented in Table 28B. Values are assigned depending on the gene dosage of the plant, and an additional lesser value was assigned that was dependent upon the genotype of the female parent.

Table 28. Model 2: Description of a proposed genetic model for the inheritance of ToMV resistance seen in tomato somaclone lines. The model is based on an incompletely dominant nuclear gene with a maternal effect. A) Definition of terms. B) Description of genotypes and additive rank resulting from gene dosage.

A. Definition of terms.

Tm-3 is an incompletely-dominant nuclear-encoded resistance gene.
+ is the wild-type susceptible allele.

AA is a cytoplasm type determined by the homozygous nuclear resistance gene of the female parent, *Tm-3/Tm-3*.
A is a cytoplasm type determined by the heterozygous female parent, *Tm-3/+*.
a is the normal type of cytoplasm determined by the wild-type female parent, *+/+*.

B. Additive ranking (gene dosage)

Genotype	Assigned gene dosage
+/+	0
Tm-3/+	2
Tm-3/Tm-3	4

Female genotype	Cytoplasm	Assigned gene dosage
Tm-3/Tm-3	**AA**	2
Tm-3/+	**A**	1
+/+	**a**	0

This ranking was composed of an additive nuclear component (incompletely dominant) and an additive maternal effect factor which increased resistance by a factor which was dependent on gene dosage of the female parent. A gene dosage value was assigned to each category, which were then added together for ranking of the genotypes.

Detailed description of genotypes and additive gene dosage ranking are presented in Table 29. The homozygous starting material (**A** *Tm-3/Tm-3*) bred true for resistance through all generations. The F_1 progeny of reciprocal crosses of somaclonal lines with GCRI-26 produced plants with intermediate resistance (*Tm-3/+*). Because of the maternal effect, there was an enhancement of the resistance of *Tm-3*, resulting in a lower level of virus accumulation seen among the progeny. Upon self-pollination of the F_1 to get the segregating F_2 population, there would be three nuclear genotypes with respect to virus resistance: *Tm-3/Tm-3*, *Tm-3/+* and *+/+*. Each of these would be more resistant in the presence of (**A** or **AA**), from the maternal effect. There would be four types of backcrosses of F_1 to the parent lines; those which involved the somaclonal line twice and had the somaclonal cytoplasm (**AA**), those which involved the somaclonal line twice but had the susceptible cytoplasmic background (**a**), those which involved the somaclonal line once and had the somaclonal cytoplasm (**A**), and those which had the somaclonal line involved once but had the susceptible cytoplasmic background (**a**). Table 29 lists the predicted genotypes and additive resistance ranking (gene dosage) derived from these crosses.

To examine the data for evidence of nuclear-cytoplasmic interaction, this resistance-ranking of the cross-pollinated lines and the backcrosses was used for comparison of the model with observed crosses. ELISA values were employed. Since the S:R rating system was

Table 29. Model 2: Additive nuclear-cytoplasmic interaction.
A) Assignment of additive ranking values for different genotypes.
B) Proposed genotypes and additive gene dosage values.

A. Assigned gene dosage values.

Progeny genotype	Value	Female parent genotype	Value
+/+	0	*+/+*	0
Tm-3/+	2	*Tm-3/+*	1
Tm-3/Tm-3	4	*Tm-3/Tm-3*	2

B. Description of genotypes and additive rank resulting from crosses.

Parents	Resulting Genotypes	Additive Rank	Mean
Selfed *Tm-3/Tm-3*	All **AA** *Tm-3/Tm-3*	2+4=6	6
Reciprocal crosses (F_1)			
Tm-3/Tm-3 x *+/+* (Sc x 26)	All **AA** *Tm-3/+*	2+2=4	4
+/+ x *Tm-3/Tm-3* (26 x Sc)	All **a** *Tm-3/+*	0+2=2	2
Reciprocal crosses (F_2)			
Selfed *Tm-3/+* (Sc x 26)	1 **A** *Tm-3/Tm-3*	1+4=5	3
	2 **A** *Tm-3/+*	1+2=3	
	1 **A** *+/+*	1+0=1	
Selfed *Tm-3/+* (26 x Sc)	1 **A** *Tm-3/Tm-3*	1+4=5	3
	2 **A** *Tm-3/+*	1+2=3	
	1 **A** *+/+*	1+0=1	
Backcrosses			
+/+ x *Tm-3/+* (A and B)	1 **a** *Tm-3/+*	0+2=2	1
	1 **a** *+/+*	0+0=0	
Tm-3/+ x *+/+* (C and D)	1 **A** *Tm-3/+*	1+2=3	2
	1 **A** *+/+*	1+0=1	
Tm-3/+ x *Tm-3/Tm-3* (E and F)	1 **A** *Tm-3/Tm-3*	1+4=5	4
	1 **A** *Tm-3/+*	1+2=3	
Tm-3/Tm-3 x *Tm-3/+* (G and H)	1 **AA** *Tm-3/Tm-3*	2+4=6	5
	1 **AA** *Tm-3/+*	2+2=4	

qualitative, based on virus presence or absence, the intermediate levels of virus titer could not be identified using the S:R ratings. As a more direct measure of virus titer, the standardized ELISA values from each plant were used to calculate the mean. To test for significant differences among the ranks, analysis of variance and range tests were done on all of the data, and results of the LSD multiple range test were presented in Table 30. In some cases there were significant differences of the experimental results between crosses with the same rank, possibly due to the relatively small sample number.

When the additive gene dosage values were plotted versus mean ELISA values (Figure 16), there was a linear relationship. Linear regression analysis gave a correlation coefficient (r^2) of 0.844, which indicated there was a fairly good fit. Thus a model using an incompletely dominant nuclear gene which also gives an additive maternal effect fits the experimental results found after resistance screening of the somaclonal lines.

The mechanism of a nuclear gene with a cytoplasmic effect for this resistance could take many forms, involving the interaction of gene products at any of several locations. These locations could include components of the membrane of either the plastids or the mitochondria, or perhaps even the thylakoid membranes. Because the method of evaluation of resistance in these studies involved inoculation of the cotyledons, it was possible that there was still an effect from an element of the maternal cytoplasm. Further speculation on a mechanism for resistance that incorporates nuclear-cytoplasmic interaction, requires more detailed knowledge of the replication and life cycle of ToMV.

Table 30. Model 2: Additive nuclear-cytoplasmic interaction. Comparison of predicted resistance rank with mean ELISA values (from 10 d pi) for pooled somaclonal families.

Parents	additive gene dosage	ELISA mean	SE	LSD
26 x 26	0	1.75	0.015	a
A) 26 x [26xSc]	1	1.38	0.064	b
B) 26 x [Scx26]	1	1.45	0.063	b
C) [26xSc] x 26	2	1.13	0.139	c
D) [Scx26] x 26	2	1.11	0.084	c
26 x Sc (F_1)	2	0.79	0.038	d
[26xSc]x[26xSc] (F2)	3	0.63	0.040	e
[Scx26]x[Scx26]	3	0.46	0.037	f
Sc x 26 (F_1)	4	0.38	0.044	f
E) [26xSc] x Sc	4	0.41	0.071	f
F) [Scx26] x Sc	4	0.76	0.297	de
G) Sc x [26xSc]	5	0.64	0.115	de
H) Sc x [Scx26]	5	0.33	0.074	f
Sc x Sc (R_1)	6	0.03	0.011	g

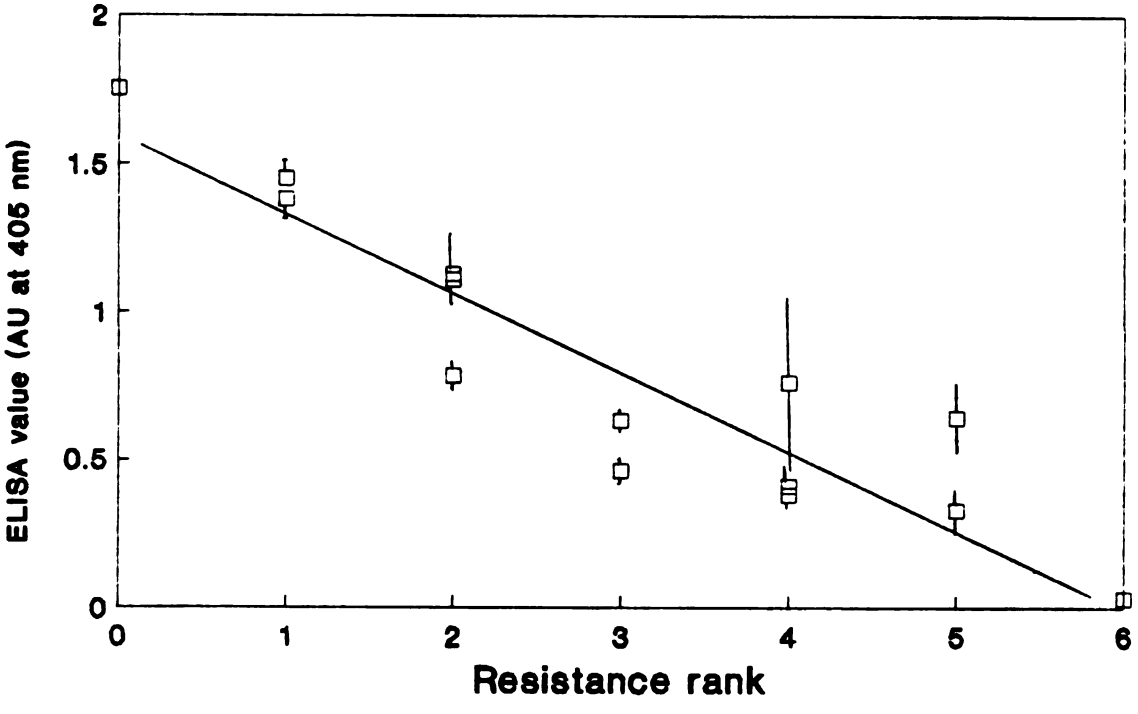


Figure 16. Graph of resistance ranking versus mean ELISA values for each cross and backcross between GCRI-26 and the pooled somaclonal lines. (Line was determined by linear regression analysis.)

SUMMARY

In characterizing the resistance to ToMV in tomato somaclones, it was shown that symptom formation was suppressed and virus multiplication was limited within the plants. The somaclonal lines appeared to be normal otherwise, with the gross morphology, pollen viability and chromosome number being similar to the susceptible parental line.

The resistance to ToMV found in these six tomato somaclonal lines is stably inherited through several generations, with almost complete penetrance of the resistance trait, and very little variation in expressivity in the selfed generations, thus appearing to breed true. After progeny of reciprocal crosses were screened and rated for resistance, it was shown that there was a 37.1% difference in ratings favoring progeny of the crosses in which the somaclonal lines were used as the female parent compared to the reciprocal cross. This maternal bias in the inheritance of resistance was not seen in all generations of crosses and backcrosses, eliminating involement of classical cytoplasmic inheritance.

Although a maternally transmitted cytoplasmic factor was concluded to contribute to the inheritance of TMV resistance, the major portion of the resistance appeared to be determined by an incompletely dominant nuclear gene. This pattern of inheritance was found in varying degrees for all six of the resistant somaclones regenerated (representing approximately 1.6% of the somaclones regenerated in the study). Though careful records were not kept regarding the source of each somaclone, at least two of the TMV resistant somaclones were isolated from separate calli (Barden, 1985). To determine the relationships of the resistance from different somaclonal lines, test crosses between lines would be

necessary. If crosses are made between different somaclonal lines, the resulting progeny can be screened for resistance to ToMV. If the genes are additive, full resistance would result in the progeny, and resistance screening of the segregating F_2 generation would give the information to establish whether the genes are allelic or not. For further characterization and comparison to each other, these somaclonal genes could each be tested for linkage to different markers (including isozymes and/or RFLPs known in tomato).

It was shown that the resistance of these six tomato somaclonal lines derived from tissue culture was a type similar to that of the *Tm-1* gene, an incompletely dominant gene originally found in *L. chilense* (Pelham, 1972). Because the somaclonal resistance gene was phenotypically additive with *Tm-1* in crosses, the somaclonal gene was tentatively called *Tm-3*. In addition to the incompletely dominant nuclear gene(s), the somaclonal plant lines had a maternally transmitted component that enhanced the resistance of *Tm-3*. Because the genetic evidence for maternal cytoplasmic inheritance was atypical, a maternal effect of the nuclear gene was deduced, which could be from a nuclear-cytoplasmic interaction in which a cytoplasmic component limited the multiplication of ToMV when the nuclear resistance gene was present in the female parent. This limitation of virus multiplication was dependent on the nuclear gene dosage itself, and the gene dosage of the female parent.

A similar pattern of inheritance was described in TMV-resistant hybrid tomato plants produced by conventional breeding methods between *L. esculentum* and *L. chilense* (Phillip *et al.*, 1965). An intermediate level of resistance was seen in the reciprocal crosses with a slight maternal bias. Further genetic characterization was not completed,

though it was later speculated that this resistance gene was probably *Tm-1*, with variation of expression due to the genetic background of the plant cultivar.

In the further genetic characterization of the somaclonal resistance genes, several steps would be useful for analysis. First, the completion of tests for allelism and for linkage between *Tm-3* and *Tm-1* should be done. This would include screening the segregating generation of the crosses between GCRI-237 (*Tm-1*) and the somaclonal line 247. If all of the F_2 progeny are resistant, then the genes are allelic (or closely linked). To test for linkage, progeny of the following cross, ([237x26] x [247x26]) x 26, would be screened, however, because of the additive nature of the resistance, the results might not be clear.

It would also be possible to determine which chromosome carries the somaclonal resistance gene. Sets of seeds are available with markers for each chromosome of tomato. By crossing the somaclonal lines with these and then screening the F_2 generation, the resistance gene could be mapped to a specific chromosome. It might not be as simple as stated, since the chromosome which carries the resistance gene *Tm-1* has not yet been identified, though early attempts reported mapping of resistance to chromosome 5 (Clayberg *et al.*, 1960).

To gain further information for testing proposed models of inheritance of the somaclonal resistance genes, it would be useful to try to separate the nuclear gene from the cytoplasmic gene, thus eliminating the added complexity. It might then be possible to determine whether the somaclonal resistance is encoded by a single, incompletely dominant gene or not. Inversely, the strength of the

cytoplasmic factor could be determined (if it could be separated from the nuclear portion of the resistance).

As another step in characterization, screening of the next generation of the crosses and backcrosses would be particularly useful, especially if these progeny were selected from parent plants which were identified as susceptible versus resistant. This would allow for the confirmation of genotypes that were predicted in the genetic models.

In all further screening, the experimental design should follow a randomized block design, with careful attention paid to environmental differences between replications. Though many precautions were taken in the characterization of the somaclonal resistance, additional steps may help in identifying the sources of the variation in the experimental results. The amount of genetic variation could then be determined and separated from the environmental variation. When these are separated, the differences between progeny of each cross would be more meaningful, and generation mean analysis could be done.

Overall, the use of somaclonal variation to generate a beneficial trait was successful. Yet it was suprising that mutations occurred in as many as six somaclones, resulting in a resistance trait which is very similar to one found in wild tomato. This leads to speculation on mechanisms of mutation, which might involve areas of DNA that are more susceptible to mutation, possible involvement of deletions of highly repetitive gene families, or secondary structural changes which change the expression of genes. These can only be tested by gaining more information on the role of the resistance genes in inhibiting virus multiplication. The next part of this work includes characterization of the type of resistance observed in the tomato somaclones.

CHAPTER 2

CHAPTER 2

CHARACTERIZATION OF PLANT-VIRUS INTERACTIONS OF THE ToMV RESISTANT SOMACLONES

INTRODUCTION

In characterizing the type of resistance to ToMV found in tomato somaclones, several approaches were taken. Plants were challenged with different strains and viruses under different conditions, systemic virus movement was studied, and infected protoplasts were studied. This resistance, encoded by both nuclear and cytoplasmic genes, offered a unique system for study. The objectives were to study the mechanisms involved in the resistance and to use that knowledge to gain a better understanding of the plant-pathogen relationship.

To gain an understanding of the mechanisms that may be employed in the resistance of the tomato somaclones, the current knowledge about the cycle of the virus and about other resistance genes was reviewed.

LITERATURE REVIEW

Tomato mosaic virus life cycle. ToMV, a member of the tobamovirus group, is composed of a single strand of RNA and is coated with protein subunits to make a straight rod of about 300 x 18 nm. The virus particles enter the epidermal cells through breaks in the cell membrane caused by injury (Figure 17a). It is believed that 1×10^6 virions per cell are required to initiate infection (Takebe, 1983). However, when plants are inoculated with more than one strain, the infection is established with only one of the strains (Otsuki and Takebe, 1978).

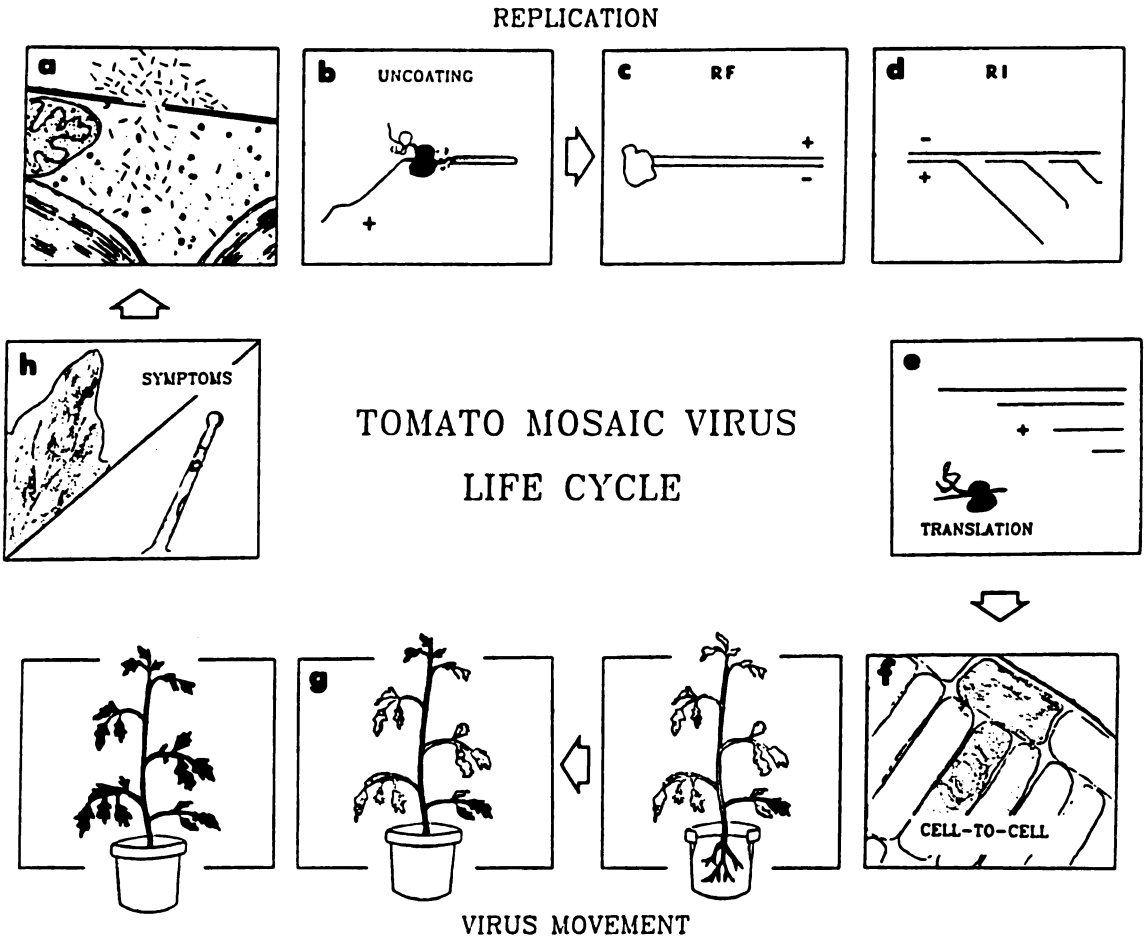


Figure 17. Tomato mosaic virus life cycle. a) virus enters cell through broken membrane and b) is uncoated while being translated from the 5' end c) the RNA is replicated (found in the double-stranded replicative form) and many subgenomic RNA's are made (found as the replicative intermediate with single-stranded RNA tails). e) viral proteins are synthesized from the subgenomic RNA's on the plant ribosomes. Virus is transported from f) cell to cell and then g) throughout the plant. h) typical symptoms are mosaic leaves, and viral inclusions can be detected in the leaf hair cells.

Once in the cell, the virus is uncoated as the 5′ end is translated (Figure 17b). This process, called cotranslational disassembly (Wilson and Watkins, 1986), occurs on the host ribosomes. The first protein product is associated with replication of the viral RNA, probably a component of the viral replicase. Replication of the negative strand of RNA (opposing base pairs of the coding strand) begins early in the infection (Figure 17c). Once there are copies of the negative strand, double-stranded RNA (dsRNA) can be extracted from plant cells (Ralph and Wojcik, 1969). This double-stranded form has been called the replicative form (RF). From the negative strand of RNA, many sub-genomic copies are made of the RNAs which code for viral proteins (Figure 17d). This complex can be isolated as the replicative intermediate (RI), with many single-stranded tails. The subgenomic pieces of RNA are then translated on plant ribosomes (Figure 17e).

Four viral proteins have been well characterized (Goelet *et al.*, 1982; Atebekov and Dorokov, 1984). A 126-kD protein is encoded in the first part of the genome (5′ end) and a 183-kD protein has also been found which is a readthrough product of the 126-kD protein. Both of these proteins are implicated in replicase function, and have been detected in the cytoplasm of infected tobacco cells by immuno-gold labelling (Hills *et al.*, 1987). The viral proteins, including the 126- and 183-kD proteins, are thought to be associated with granular inclusion bodies found in virus-infected cells (Saito *et al.*, 1987; Hills *et al.*, 1987).

A 30-kD protein is made from another reading frame downstream from the first two proteins. This protein is involved in cell-to-cell transport of the virus (Ohno *et al.*, 1983; Deom *et al.*, 1987). This protein has been localized by immuno-gold labelling to the plasmodes-

mata (Tomenius *et al.*, 1987). Virus strains with mutations in this coding region are unable to move from the initially infected cell (Leonard and Zaitlin, 1982).

The fourth gene is the 18-kD coat protein gene, which is found near the 3' end of the genome. Coat protein accumulates in the thylakoid membranes of the chloroplasts (Reinero and Beachy, 1986) as well as throughout the cytoplasm, and assembles with the nascent positive-stranded RNA to form new virions.

Virus transport, reviewed by Atabekov and Dorokov (1984), is active at two steps. Cell to cell transport, probably via plasmodesmata, is active (Figure 17f). The virus then enters the phloem, is rapidly carried to the roots and the top of the plant (Figure 17g), and then over a longer period (15 to 20 d) spreads to all parts of the plant. It has been suggested that movement both into the phloem and back out to the mesophyll cells are active steps in viral movement.

After movement of the virus through the plant, symptoms of virus infection are apparent, including the typical mosaic mottling of the leaves (Figure 17h) and the appearance of viral inclusions in the leaf hair cells. The inclusions are made from millions of virus particles which form hexagonal crystals that can be detected with a light microscope (Figure 1).

The life cycle of the virus is complex and not completely understood at this time, but what is known can be used as a basis for speculating about the mechanisms which plants use to interfere with the life cycle to cause resistance.

TMV resistance genes in tomato. There are three well-characterized ToMV resistance genes in tomato, which have each been incorporated into cultivated tomato from wild tomato species. There is also pathogen-derived resistance in transgenic plants expressing TMV coat protein (CP) (Nelson *et al.*, 1988). Table 31 lists the resistance genes and several characteristics of the resistance traits.

Characteristics of resistance genes. There are three well-studied genes for resistance to ToMV in tomato. The gene *Tm-1* is co-dominant for limitation of virus replication and dominant for suppression of symptom formation (Fraser and Loughlin, 1980). At a different locus, the allelic genes, *Tm-2* (Hall, 1980) and $Tm\text{-}2^2$ (Alexander, 1963) effectively prevent infection of ToMV by a hypersensitive response, usually evident as local lesions. At higher temperature, or in heterozygous plants, the hypersensitive resistance response may cause systemic necrosis.

These three genes have been bred into many commercial cultivars as *Tm-1/Tm-1*, $Tm\text{-}2/Tm\text{-}2^2$ to give complete resistance to date. Also, each of these alleles has been incorporated into isogenic lines of tomato, and have been tested with different virus strains (see Table 31). This work done by Pelham (1972) has been very useful in aiding studies of resistance in tomato, and serves as an ideal system for study of resistance mechanisms. Because the resistant somaclones in this study were derived from GCRI-26, one of Pelham's near-isogenic plant lines, direct comparison of resistance can be made between the somaclones and the other resistance alleles.

The transgenic tomato lines that express TMV-CP provide resistance to all strains of ToMV tested, but are not resistant to TMV-RNA (no coat protein) (Nelson *et al.*, 1988). This mechanism of resistance, which

Table 31. List of TMV resistance genes in tomato and some of their characteristics.

Gene	Gene Source	Mode of Inheritance	Type of Resistance	Overcoming Strains	Response to Temperature	Resistance Level
Tm-1[a]	Lycopersicon chilense	nuclear (dominant/ co-dominant)	limited multiplication, symptom suppression	ToMV-1 ToMV-1.2 ToMV-1.2[2]	sensitive/ resistant	protoplast
PI# 235673[b] (Tm-1)	L. chilense	nuclear plus maternal	limited multiplication	n.d.	n.d.	protoplast
Tm-2[c]	L. peruvianum	chromosome 9 co-dominant	hypersensitive (local lesion)	ToMV-2 ToMV-1.2	sensitive	callus
Tm-2[2d]	L. peruvianum	chromosome 9 co-dominant	hypersensitive (local lesion)	ToMV-2[2] ToMV-1.2[2]	sensitive	callus
+CP trans-genic[e]	pathogen-derived (TMV)	nuclear	cross-protection	TMV-RNA	n.d.	protoplast
Sc[f]	tissue cultured L.esculentum	nuclear, maternal effect	limited multiplication	ToMV-1	resistant	n.d.

[a]Holmes, 1954.
[b]Phillip et al., 1965.
[c]Hall, 1980.
[d]Alexander, 1963.
[e]Nelson et al., 1988.
[f]Barden et al., 1986.

resembles cross-protection, is thought to be due to inhibition of uncoating and initial translation of viral protein (Register and Beachy, 1988).

Temperature sensitivity. When resistant tomato plants are inoculated with virus and incubated at high temperature (28-30° C), there is sometimes a breakdown in the resistance. With *Tm-1*, the results have been ambivalent with reports of temperature sensitivity, especially when heterozygous (Pelham, 1972) and stability (Fraser and Loughlin, 1980). The genes *Tm-2* and *Tm-2*^{2} both lose resistance at higher temperature (Pelham, 1972; Stobbs and MacNeill, 1980; Cirulli and Alexander, 1969), allowing systemic virus multiplication and spread. When such plants are moved to normal temperatures, the plants become systemically necrotic. Protoplast infection. When isolated protoplasts were inoculated with ToMV, the cells with *Tm-1* inhibited virus multiplication (Motoyoshi and Oshima, 1977), thus the resistance was expressed at the cellular level. However, protoplasts from lines with *Tm-2* or *Tm-2*^{2} were as susceptible to ToMV inoculation as were protoplasts from the fully susceptible line, GCRI-26 (Motoyoshi and Oshima, 1975; Stobbs and MacNeill, 1980). The resistance was not expressed at the cellular level, but when callus cultures were inoculated with ToMV, the *Tm-2* and *Tm-2*^{2} lines showed resistance (Toyoda *et al.*, 1983). There was no resistance seen in GCRI-26 callus cultures.

Protoplasts from transgenic tobacco plants (similar to the tomato transgenic resistance) were resistant to infection by TMV, but not by TMV-RNA or TMV which had been treated at pH 8.0 (Register and Beachy, 1988). This result provides evidence for the hypothesis that protection is due to prevention of TMV uncoating.

RNA polymerase activity. In ToMV-infected susceptible tomatoes, activity of RNA-dependent RNA polymerase increases two times the normal levels observed in healthy plants (Evans *et al.*, 1984). Infection with most RNA viruses leads to increased enzyme activity (May *et al.*, 1970; Romaine and Zaitlin, 1978), and some or all of this activity may be responsible for virus reproduction. In tomato plants with the *Tm-1* resistance gene, there is no increase in this RNA polymerase activity (Evans *et al.*, 1984). Furthermore, when RNA-dependent RNA polymerase was purified and ToMV-RNA added as a template, the replicative form (RF) and replicative intermediate (RI) of ToMV were detected only when enzyme of ToMV-infected susceptible plants, but not the enzyme from healthy susceptible plants or inoculated plants containing the *Tm-1* gene was used. Strains of TMV that overcome *Tm-1* resistance did stimulate RNA polymerase activity (Evans *et al.*, 1985). These results suggest that *Tm-1* resistance operates by preventing synthesis or modifying RNA-dependent RNA polymerase activity needed to form a functional viral replicase, though other explanations are possible (Fraser, 1985).

Grafting experiments. Study of virus movement in reciprocally grafted tomato has been done for each resistance gene. Selman and Yahampath (1973) made reciprocal grafts between a cultivar with the *Tm-1* gene and a susceptible cultivar with a different genetic background. The scion was inoculated with ToMV and virus was assayed by inoculation on *N. glutinosa*. When the rootstock was susceptible, the resistant scion was less resistant, allowing the multiplication of virus. When the rootstock was resistant, the susceptible scion became infected with virus, indicating that there was no viral inhibitor being transported upward. In either case, the virus moved freely across the graft and

replicated throughout the plant, indicating that the *Tm-1* gene did not inhibit transport of virus or cause systemic resistance.

Pilowsky (1971) studied reciprocal grafts between plants with *Tm-2*^{2} and infected susceptible plants, and found that in either position (rootstock or scion) the *Tm-2*^{2} plants became systemically necrotic, even when the graft was severed after seven days. Stobbs and MacNeill (1980) studied virus movement when grafts were made between plants expressing *Tm-2* and susceptible plants. Stem inserts of one genotype were made between rootstock and scion of the other genotype, and ToMV was inoculated on the rootstocks, after the graft unions were established. The resistant insert did not alter virus movement; when a leaf of the susceptible rootstock was inoculated, the virus moved into the scion, but when a leaf of the resistant rootstock was inoculated, the virus remained in the inoculated leaf. When grafted as rootstock or scion, the resistant plants developed systemic necrosis when the grafted partner was TMV-infected. In the same study, virus movement was found to occur through the phloem when examined using electron microscopy. It was suggested that the virus by-passes the normal genetic resistance mechanisms by entering the resistant mesophyll from the phloem. The virus was localized if the only inoculum was on a resistant leaf.

Preliminary reports of grafting experiments with transgenic tobacco plant material are intriguing. When inserted stem sections of a transgenic plant with TMV-CP were grafted between the top and bottom of a systemic host of TMV (*N. tabacum* cv. Xanthi) followed by inoculation of a lower leaf with TMV, virus movement through the graft was not inhibited (Wisniewski and Beachy, 1988). However, when the stem section included a leaf, the virus did not move through the graft to infect the top of the plant. One explanation is that resistance might be due to

the export of viral coat protein from the transgenic leaf into other parts of the plant. It was suggested by Beachy (1988) that this resistance may be caused by a physiological boost from nutrients produced in the healthy transgenic leaf which caused greater expression of the CP in the phloem cells, thus stopping transport.

Resistance mechanisms. Hypothetically, there are three kinds of general resistance mechanisms (Fraser, 1985). Positive resistance is that in which the host produces a factor which stops the virus. The hypersensitive response to virus might exemplify this kind of resistance mechanism. Negative resistance is due to a factor which the host lacks that is essential to viral replication, as might be the case with a protein product such as RNA-dependent RNA polymerase to which a specific virus may be highly adapted. The other category includes physical or chemical barriers, to either prevent access of the virus or to cause destabilization of the virus with changes in pH or ionic strength.

There are also a variety of resistant phenotypes (Fraser, 1985), including plants which are entirely symptomless, plants which localize the virus into necrotic or chlorotic lesions, and plants which allow systemic spread of the virus but are not affected with symptoms. In any of these phenotypes, the increase of virus in time and space is limited, as shown by reduced levels of virus multiplication at the cellular, plant or crop level. The possibility that host genetic background may influence resistance expression emphasizes the importance of using near-isogenic lines when comparing resistance genes. It becomes more critical to test the resistance in different ways in order to establish a mechanism. The following section of experiments does this.

MATERIALS AND METHODS

RESISTANCE RESPONSE TO DIFFERENT CHALLENGES

Response of somaclones to different virus strains. Tomato seeds were planted and cultivated as described previously. Groups of tomatoes representing the R_1 generation of all six somaclonal lines and the susceptible line, GCRI-26, were divided and inoculated with ToMV-0, TMV-Fl or a strain that overcomes the resistance of the *Tm-1* gene, TMV-1 (described by Pelham, 1972 and kindly provided by F. Motoyoshi, University of Tokyo, Japan). Assay of the inoculated plants was done at 10 d pi, and continued for 20, 30, 50, and 70 d for ToMV-0 and TMV-Fl. Plants that became infected with TMV-1 were discarded immediately after assaying to prevent unintentional spread to other tomatoes. Assay of virus presence was done using ELISA as described except for TMV-1, which was detected by observation of symptoms and inclusion bodies in leaf hair cells. Comparisons of ToMV-0 and TMV-Fl were repeated three times with six to 16 plants of each line. Comparison of TMV-1 to ToMV-0 was done once with twelve plants of each line.

Response of somaclones to inoculation of different viruses. Groups of tomatoes representing each of the R_1 generation somaclonal lines (except 12) and GCRI-26 were divided and inoculated with the pepper strain of cucumber mosaic virus (CMV, CMI/AAB Description of Plant Viruses, 1979) or with the wild tobacco strain of tobacco etch virus (TEV, CMI/AAB Description of Plant Viruses, 1980), which were both provided by T. Zitter, Cornell University, New York. The inoculum was prepared from frozen, infected tobacco tissue which was ground at 1 g:20 ml of 0.01 M NaK-phosphate buffer, pH 7.0. Detection of the virus was done using

ELISA, with gamma-globulin specific for each virus. Each gamma-globulin was purified from rabbit antiserum (from American Type Culture Collection, Rockville, MD) as described previously. Plants were assayed at 10 and 20 d pi. Twelve plants of each line were tested with each virus.

Response of somaclones to inoculation and incubation at different temperatures. Groups of tomatoes representing R_1 and R_2 generations of somaclones, GCRI-26 (+/+), and the resistant tomato line GCRI-237 (*Tm-1/Tm-1*) were inoculated with ToMV-O and divided into sets which were incubated at 25° C or 30° C, with otherwise similar conditions. Plants were assayed for the presence of virus at 10, 20, and 30 d pi (and one group was assayed at 50, 70 and 150 d pi as well) using ELISA. The first experiment included six plants of each line for each temperature and was replicated using six to nine plants of each line for each temperature.

VIRUS MOVEMENT

Virus movement in tomato. Plants were transplanted into 4" pots at 12 d. The groups represented R_2 plants from somaclone lines 12, 215 and 247, and the susceptible line, GCRI-26. One group was inoculated with ToMV-O when the plants were 32 d old and the second group was inoculated at 25 d old. The outermost leaflet of the first true leaf was marked by punching a hole with a wide-bore needle, and after dusting with carborundum, it was inoculated with virus. The inoculum was not rinsed off. Ten to 12 plants of GCRI-26 and somaclone 247 were tested in each replication. At each time point (4, 7, 10 and 20 days pi), leaflets were removed from each leaf of the plant as shown in Figure 18. Two or three plants of each line were sampled each day. Once plants had been

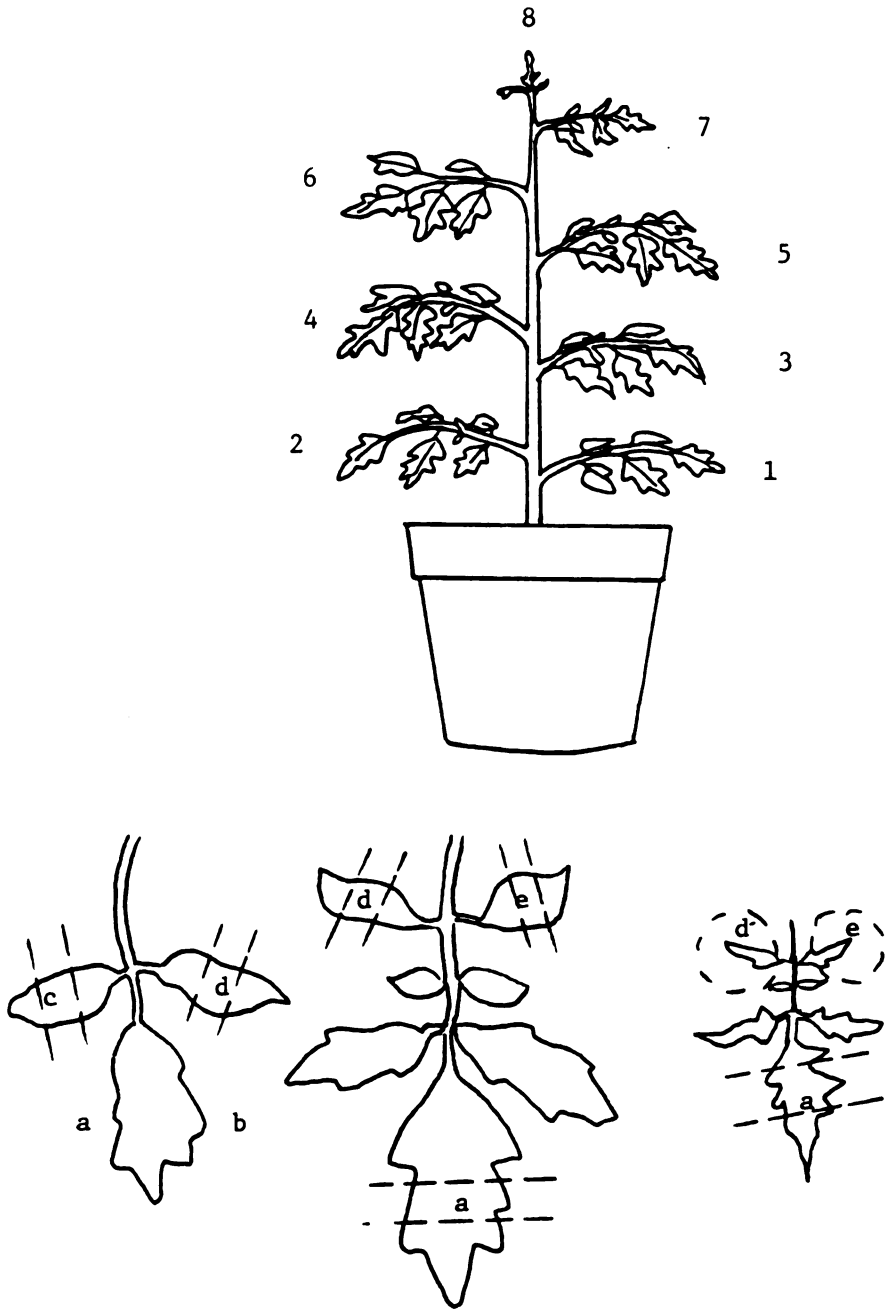


Figure 18. Diagram of sampling of inoculated tomato plants for detection of virus spread over time and space. The triangle indicates the leaflet which was inoculated. Numbering and lettering identified each leaflet for later reference.

sampled, they were not used for further assay, to eliminate the liklihood that plants were inadvertantly inoculated during sampling. Leaf samples were assayed using ELISA, and the absorbance values for leaflets from each corresponding leaf were averaged. Plants from the second repetition were used to isolate protoplasts for detection of infection at the cellular level.

As controls, susceptible and resistant plants were mock-inoculated and assayed. For an added control in the second group, the inoculated leaflet was detached at 48 h to determine the time required for the virus to leave the inoculated leaf.

Virus movement in grafted tomatoes. To study the movement of virus between reciprocal grafts of resistant and susceptible plant material, several types of grafts were established. Whip grafts (see Figure 19A) were done using young seedlings by wetting a clean razor blade and removing the top of 18-d-old plants with a V-shaped cut. Tops were removed from two plants and exchanged, resulting in a rootstock with cotyledons only, and a scion with three young leaves. The graft was held in place using 1/2" adhesive tape. Plants were sealed in polyethyene bags to increase the humidity, and were allowed to bond for several weeks. A cotyledon of the rootstock of the grafted plant was then inoculated. Of the ten whip grafts made, three grafted plants survived for the assays.

The second set of plants was grafted, in this case using older plants (20 to 30 d). Again, V-shaped cuts were made near the tops of two plants and the tops were exchanged. After the whip graft was established (25-30 d), there were two true leaves remaining below the graft plus secondary shoots, and two to three young leaves on the scion. The outermost leaflet of the first true leaf of the rootstock was

Figure 19. Diagram of whip graft (A) and whip and tongue graft (B).

inoculated. Of the ten grafts made, again only three were successful and used for the assays.

The third type of graft was established using an approach graft which incorporated a tongue (whip and tongue graft, Figure 19B). This method was used successfully on older plants (33-39 d) and was less stressful to the plants. The graft was made on the stem between the third and fourth true leaves in most cases, and was bound with adhesive tape. After two weeks, one stem was severed below the graft and the opposing top was removed. The outermost leaflet of the first true leaf was inoculated a week after that. Of the ten whip and tongue grafts made, five were used for assay. Two others were contaminated with virus before the date of inoculation and were eliminated.

Assay of the grafted plants was done using ELISA at 0, 5, 7 and 10 d pi. Some plants were assayed further at 21, 34, 39 and 44 d pi. The first group of grafted plants were assayed from every leaf of both rootstock and scion at each time point. Later groups were assayed in one or two leaves from both parts.

Three pairs of reciprocal grafts were established between somaclone 247 (R_2) and GCRI-26, and one pair between between somaclone 12 (R_3) and GCRI-26. Two control grafts were made between two plants of GCRI-26 to eliminate the possibility that delayed virus spread was due to the graft itself.

PROTOPLAST ISOLATION AND SCREENING FOR INFECTION

Protoplast isolation. Tomatoes were grown in the laboratory (22-25° C) under cool white fluorescent lights supplying 60 $uEm^{-2}s^{-1}$ on a 16 hour light cycle. The plants were fertilized daily when they were watered with one tablespoon of Peters 20-20-20 per gallon. At about four weeks

plants were used for isolation of protoplasts. The condition of the plants was optimal for about six weeks when new leaves were used, but the yield of protoplasts per gram of leaf declined as the plants aged. Light green, tender leaves were picked, weighed and used to isolate protoplasts.

Protoplast isolation procedures were adapted from Kassanis and White (1974). Leaves were surface sterilized by washing for ten min in 15% bleach solution containing two drops of Tween-20 per 250 ml. In a laminar flow hood, the leaves were then rinsed three times with sterile distilled water. All following steps were performed using aseptic techniques. From leaves which were infected with TMV, leaves were stacked and sliced transversely in 1 mm thin sections. Leaf strips were placed in a petri dish containing 20 ml of an enzyme solution consisting of 1% cellulysin, 0.5% macerase, (Calbiochem, Behring Diagnostics, San Diego, CA), 0.4 M sorbitol in cell protoplast wash (CPW) salts (0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4 \cdot 7\ H_2O$, 1 uM KI, 0.1 uM $CuSO_4 \cdot 5\ H_2O$, and 10 mM $CaCl_2 \cdot 2\ H_2O$) at pH 7.0. This was incubated on a slow shaker in the dark at room temperature, until the solution was visibly green and many protoplasts could be seen when viewed using the inverted microscope (12 to 18 h).

The enzyme solution containing protoplasts and leaf debris was filtered through a 60 um sieve into a petri plate, and remaining leaf debris was gently rinsed with CPW salts with 0.4 M sorbitol at pH 6.5. The protoplasts were transferred carefully to a 30 ml round-bottomed centrifuge tube to pellet the protoplasts. The protoplasts were centrifuged at 35 x g or 400 rpm in an HNS-II IEC No. 2355 tabletop centrifuge for 10 min. The supernatant was removed by suction, the

pellet was gently resuspended by rolling in a small amount of washing buffer, and the protoplasts were moved to a 15 ml tube. The protoplasts were washed in 15 ml of sorbitol-CPW buffer, centrifuged again at 35 x g and the supernatant was removed by suction. The protoplasts were rinsed a total of three times in this manner. The final step in purification of the protoplasts was to float the intact protoplasts on a solution made of Lymphoprep (Accurate Chemical and Scientific Corp., Westbury, NY) and 0.5 M sucrose-CPW salts at 1:2 for healthy protoplasts or at a 2:1 ratio for protoplasts which were isolated from virus-infected plants. To determine whether the exposure to Lymphoprep (9.6% w/v sodium metrizoate and 5.6% w/v Ficoll; 1.077 g/ml density and 300 mOsm osmolarity) inhibits virus infection or detection, some isolations of protoplasts were done omitting the flotation step.

Yield of protoplasts was determined by counting them with a hemocytometer, and calculating the number of protoplasts released per gram of starting material. Concentration of protoplasts was then adjusted to the proper amount for each experiment.

Protoplast viability. The viability of protoplasts after isolation was determined in two ways. Protoplasts were examined using an inverted light microscope and rated for their general appearance. Living cells remained perfectly round with an intact membrane. Dead cells were misshapen or shriveled. The second method to determine viability was to observe cells after the addition of drops of Evan's blue dye (0.05% w/v Evan's blue in 0.7 M mannitol, pH 7). Live cells excluded the dye, but dead cells took on a blue color after several min.

Preparation and staining of infected protoplasts. Following the procedure by Otsuki and Takebe (1969), the infected protoplasts were harvested and concentrated by centrifugation. A small drop of solution

containing protoplasts was placed onto a glass slide which was coated with Mayer's albumin (egg white + phenol), swirled to spread the cells evenly and allowed to dry. The dried protoplasts were fixed by immersion in 95% ethanol for 10 min at room temperature, followed by a rinse for 10 min in PBS. To stain the protoplasts, 20 ul of FITC-conjugate (antibody against viral coat protein labelled with fluorescein isothiocyanate) was placed on the slide, covering the cells. The slide was incubated for one h in a moist chamber at 37° C in a water bath. The chamber was lined with wet filter paper so as to maintain a high level of humidity. Excess FITC-conjugate was removed by a 10 min rinse in PBS, then the sample was prepared for viewing by carefully blotting the excess PBS away, adding a drop of 10% glycerol (in PBS) and placing a coverslip over the cells.

Detection of virus infection by fluorescence microscopy. FITC-labelled cells were viewed with a Zeiss GFL epiluminescent microscope equipped with exciter filters KP-490 and LP-445, dichroic reflector 510 and barrier filter 520. Infected cells contained specks which were a bright yellow-green in color. Uninfected cells exhibited slight light green fluorescence, and dead cells appeared dull orange-brown in color. When chlorophyll was present, cells fluoresced red.

Photography. Photographs of the fluorescing cells were taken with ASA 400 daylight film at 12 volts. Exposures were between 12 min (for heavily infected cells) and 28 min (for uninfected cells).

Preparation of FITC-conjugate. The procedure used was adapted from that of Spendlove, 1967. Serum was collected from rabbits following three virus injections at 2-week intervals. The gamma globulin fraction was precipitated by the dropwise addition of 25 ml $(NH_4)_2SO_4$ (saturated

solution) to 50 ml of serum with stirring. This was mixed for 3 h at 0° C. The precipitate was collected by centrifugation at 5000 rpm in a SS-34 rotor for 15 min. The pellet was then dissolved in 40 ml of PBS, and was precipitated a second time by the dropwise addition of 20 ml saturated $(NH_4)_2SO_4$ while stirring for one h at 0° C. After centrifugation at 6000 rpm for 15 min, the pellet was dissolved in 6 ml PBS. The gamma globulin was then dialyzed against five changes of PBS over a period of 24 h. The solution was then clarified by centrifugation at 14,000 x g for 30 min and the supernatant containing the gamma globulin was collected. The protein concentration was determined by measuring the absorption at 280 nm, using the extinction coefficient of 1.8 for rabbit gamma globulin (McGuigan and Eisen, 1968).

To label the gamma globulin with fluorescein isothiocyanate (FITC) (ICN Immunobiologicals, Lisle, IL) 2 ml of the purified gamma globulin was stirred while adding 1.2 ml of FITC solution dropwise. For the FITC solution, 15 mg FITC was mixed with 1 g protein, made in freshly prepared 0.1 M Na_2HPO_4. An additional 0.4 ml of freshly prepared Na_2PO_4 was added, followed by the addition of 0.4 ml 0.04 M NaOH (pH 9.4-9.6). The solution was incubated for 30 min at 25° C without stirring. The solution was loaded onto a Sephadex G-25 column (2.5 x 15 cm) which was previosly equilibrated with PBS. The conjugate was then eluted with PBS and the first visible (greenish-yellow) band was collected in fractions. The eluate was centrifuged at 15,000 rpm for 30 min to clarify. The supernatant containing the conjugate was adjusted to 0.1% NaN_3 and stored at 4° C.

Cross absorption of FITC-conjugate with acetone powder of healthy tobacco leaves. Acetone powder of tobacco leaves was prepared according to the procedure of Otsuki (1976). Ten g of healthy leaves were picked,

placed in 100 ml cold acetone (0° C) and homogenized at maximum speed in a Waring blender for 5 min. More acetone was added to make a volume of 300 ml. The homogenate was washed on a Buchner funnel lined with Whatman No.1 filter paper with cold acetone until the filtrate was nearly colorless. The homogenate was further pulverized by grinding in a dry mortar, resulting in an almost powder-like substance. This was dried under vacuum overnight and stored in a dessicator at room temperature.

The acetone powder was prepared for cross-absorption by several washes with PBS, pH 7.0. To 40 ml PBS, 0.2 g of powder was added and stirred for 10 min. The suspension of powder was centrifuged at 3000 rpm in an SS-34 rotor for 7 min, and the pellet was resuspended in 40 ml PBS, and centrifuged again as before. The pellet containing the acetone powder was saved, resuspended in 35 ml PBS and divided into two tubes for cross-absorption of the different FITC-conjugate bands collected from the Sephadex column. To determine the optimum amount of cross-absorption necessary, one tube contained approximately twice the amount of the pellet as the second tube. Each fraction was centrifuged again and 2 ml of FITC-conjugate solution was added to each pellet. This was stirred with the aid of a glass rod and the mixture was incubated for 30 min at 37° C. After unwanted antibodies were absorbed to the tobacco acetone powder, a centrifugation at 3000 rpm for 7 min removed the solids, and the supernatant fraction containing the FITC-conjugate was saved. Tests for the optimum amount of acetone powder needed for cross-absorption were made by trial and error method using identical sets of infected protoplasts. The various fractions were labelled and stored at 4° C.

RESULTS AND DISCUSSION

RESPONSE TO VIRUS CHALLENGE

Response to different strains of ToMV. The somaclonal lines displayed similar resistance to ToMV-0 and TMV-Fl, but were not resistant to TMV-1 (Table 32). This response was similar to that reported for plants with the *Tm-1* gene for resistance (Pelham, 1972).

Table 32. Response* of somaclonal lines to inoculation by three strains of TMV.

Line	ToMV-0	TMV-Flavum	TMV-1
12	R	R	S
215	R	R	S
219	R	R	S
247	R	R	S
322	R	R	S
330	R	R	S
GCRI-26	S	S	S

*Ratings were based on the presence of virus, detected by ELISA, symptoms or inclusion bodies. R = resistant, S = susceptible.

Response of somaclonal lines to other viruses. After inoculation with ToMV-0, the somaclones were virus-free for more than 30 d (Figure 20). There were several plants among somaclone lines 215, 219, 247 and 330 which showed a delay in infection after inoculation with CMV, but all plants were infected by 20 d pi. When inoculated with TEV, all plants had high levels of virus at 10 d pi. It was concluded that the somaclones which had been selected for TMV resistance were not resistant to other viruses. This was in contrast to a type of resistance found in a

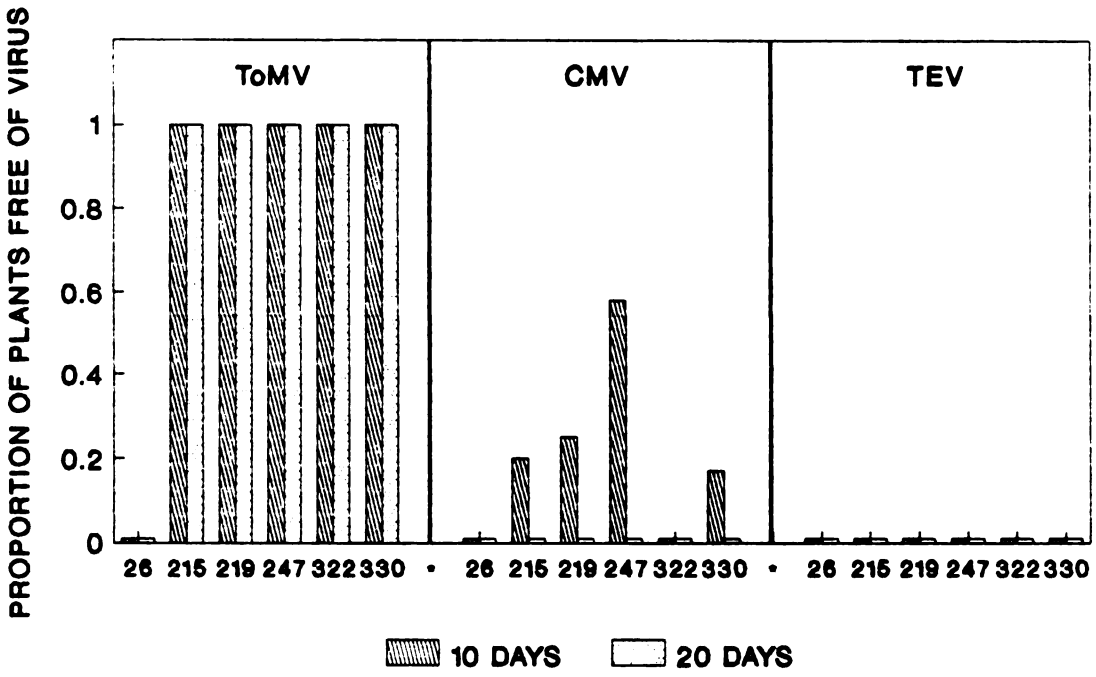


Figure 20. Response of resistant tomato somaclonal lines to virus challenge with ToMV, CMV or TEV.

tobacco line, T.I. 245, which gave a non-specific resistance to virus infection (Thomas and Fulton, 1968) and which was correlated with a reduced number of ectodesmata.

Response of somaclonal resistance to high temperature. The somaclonal resistance to ToMV was not changed when inoculated plants were grown at high temperature (Table 33). The resistance ratings of the somaclone lines at 25 and 30° C are similar, but plants did not thrive in the warmer conditions and were stunted. Severe stunting

Table 33. Ratings (S:I:R) of plants inoculated with ToMV-O and incubated at either 25 or 30° C for 30 d. ( ) indicates number of plants tested.

Plant line	25° C	30° C
Somaclone 12	0:0:6 (6)	0:0:6 (6)
Somaclone 215	0:0:15 (15)	0:0:15*(15)
Somaclone 219	0:0:15 (15)	0:0:15 (15)
Somaclone 247	0:0:15 (15)	0:1:14*(15)
Somaclone 322	1:0:11 (12)	0:2:10 (12)
Somaclone 330	0:2:10 (12)	1:0:11 (12)
GCRI-26	12:0:0 (12)	12:0:0 (12)
GCRI-237 (*Tm-1/Tm-1*)	0:0:9 (9)	2:0:7 (9)

*group with plants which recovered from infection.

was seen in infected GCRI-26 plants at the higher temperature. There were several cases of 'recovery' in the somaclones (marked with asterisk), when a plant showed high virus titer at one sample time and did not show the presence of virus at the next sampling. Two plants of

line 237 (*Tm-1/Tm-1*) which had a medium virus titer at 10 d died after necrotic collapse before they could be sampled again at 20 d. It is possible that line 237 was less resistant at the higher temperature, however, due to the small sample size no conclusive statement can be made. The somaclonal lines were not affected by the higher temperature.

MOVEMENT OF VIRUS

Systemic movement of virus in susceptible and resistant plants. The movement of ToMV-O was fairly rapid in susceptible plants (Figure 21). At four days, virus was detected in the inoculated leaflet and in neighboring leaflets. By the seventh day after inoculation, there was a high titer of virus in the upper leaves of the plant, which were expanding with rapid growth. The virus spread through the rest of the plant. By 20 d, the whole plant contained a high virus titer. This result substantiated the classical work done on virus movement in tomato by Samuel (1934) and the more recent report of movement of potato spindle tuber viroid in potato (Palukaitis, 1987). The long-distance, intraplant transport was consistent with the pattern of movement of photosynthetic products, moving in the phloem from fully expanded leaves, to developing upper leaves.

When this experiment was repeated using somaclonal line 247, high levels of virus did not develop anywhere in the plant. The inoculated leaf had a detectable level of virus, and over time, a trace level of virus was measured in the upper parts of the plants, but at the lower limits of detection by ELISA (values in the range of 0.02 to 0.09 AU).

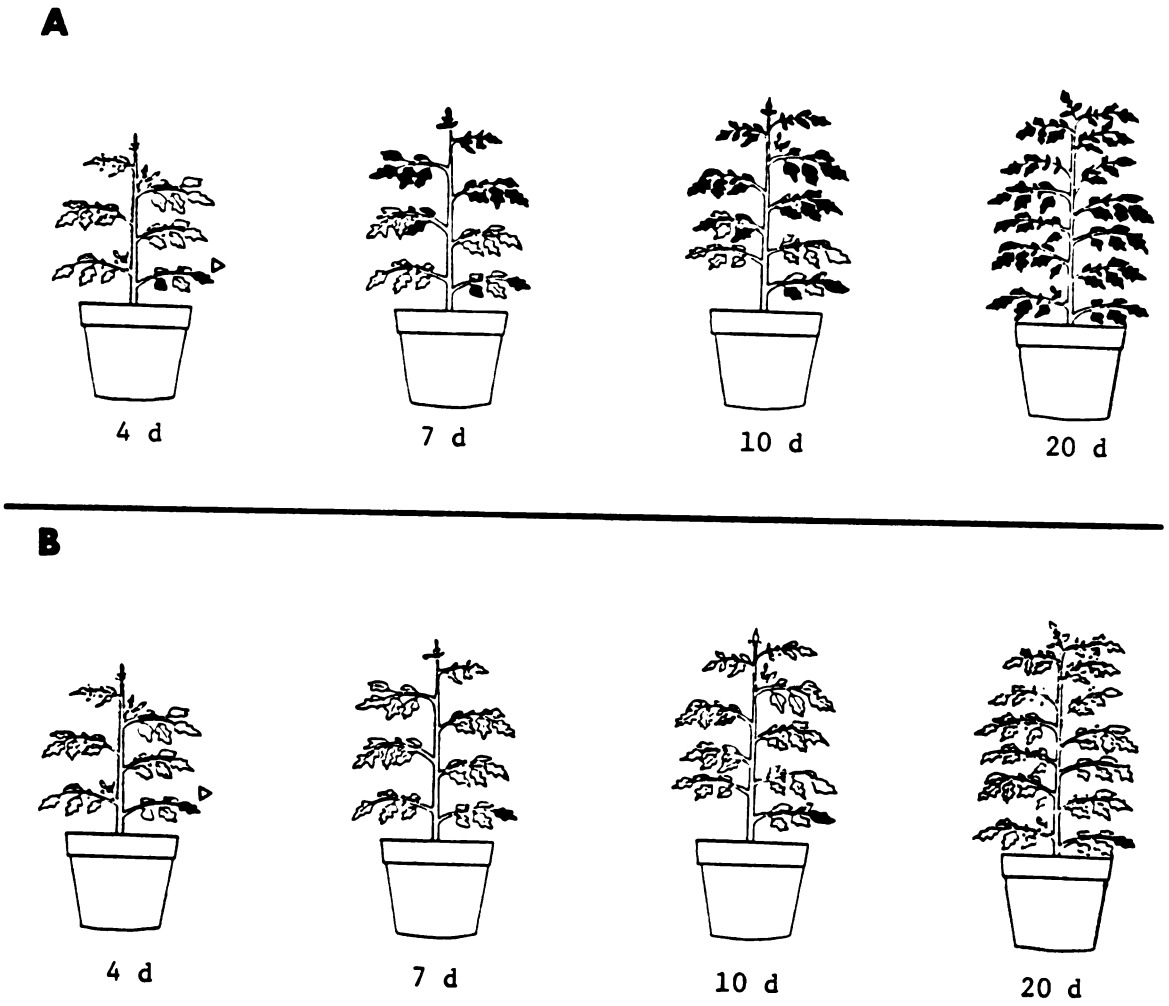


Figure 21. The movement of ToMV-O in tomatoes. Susceptible GCRI-26 tomatoes (A) allow the movement of virus as illustrated by the shaded leaves. At four d, the virus was spreading in the inoculated leaf, then moved quickly to the upper part of the plant. After 20 d the entire plant contained virus. In the resistant somaclonal line 247 (B), the virus remained in the inoculated leaf. An open triangle marks the inoculated leaflet.

Intermediate levels of virus (ELISA values in the range of 0.05 to 0.32 AU) were detected in plants of line 12 at 15-20 d after inoculation. It was evident that the virus was allowed to multiply and move systemically in these plants, but the multiplication was limited, since the virus titer was much lower than expected for fully susceptible plants. Differences in the resistance gene from each line may explain the consistently greater virus titer in somaclone line 12 compared to line 247 . It would be of interest to test for complementation between these two lines. Perhaps both somaclonal lines limit replication, but line 247 may limit systemic transport of the virus as well, but at this point it is difficult to separate the two effects.

Movement of virus in grafted plants. In the control grafts that were made with GCRI-26 on GCRI-26, there was no delay in virus movement (Figure 22A). Virus appeared in the rootstock leaves and the scion leaves in equal amounts at five d (ELISA values from 0.50 to 0.89 AU) and at ten d (values from 1.11 to 1.20 AU). Because the virus multiplied and spread to all parts of the grafted plant quickly, the possibility that limitation of virus movement due to the graft union was eliminated.

When the susceptible line GCRI-26 was used for the inoculated rootstock and the resistant line 247 as the scion, the virus replicated and moved throughout the leaves of the susceptible rootstock to attain a high titer (ELISA values from 1.70 to 1.75 AU) by 7 to 10 d (Figure 22B). In each case, accumulation of virus was delayed in both rootstock and scion compared to the control grafts. In one graft (Figure 22, B1) the virus was delayed in multiplying to a high titer in the resistant scion, even with the presence of the large virus concentration in the

MEAN ELISA VALUES (AU at 405 nm)

Scion / Rootstock	0 d	5 d	7 d	11 d	21 d	39 d
A) Control						
1. 26	0.01	0.59	1.14	1.68	-*	-
26	0.00	0.50	1.11	1.68	-	-
2. 26	0.00	0.86	1.20	1.68	-	-
26	0.00	0.89	1.18	1.68	-	-
B) 247 scion						
1. 247	-	0.16	0.04	0.02	1.35	1.03
26	-	-	1.75	1.75	1.77	0.80
2. 247	-	0.02	0.00	0.00	1.36	1.00
26	-	-	0.14	0.20	1.75	0.88
3. 247	0.00	0.14	1.55	-	-	-
26	0.00	0.26	1.64	-	-	-
C) 247 rootstock						
1. 26	-	0.08	0.04	0.09	0.01	0.33
247	-	-	0.20	0.24	0.11	0.69
2. 26	0.00	0.00	0.00	0.00	0.00	0.02
247	0.00	-	0.00	0.00	-	-
3. 26	0.00	0.02	0.00	0.09	-	-
247	0.00	0.00	0.00	0.07	-	-

*not determined

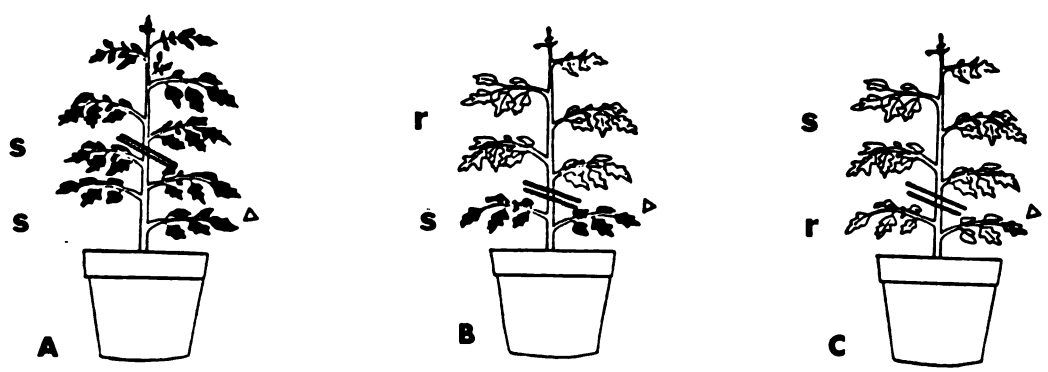


Figure 22. Results of experiments in which grafted plants were inoculated in the outermost lower leaf of the rootstock, and the movement of the virus was followed, using ELISA to measure virus titer in young leaves of the rootstock and scion. Grafts were made between A) two susceptible plants as a control. B) and C) reciprocal grafts between resistant (somaclonal line 247) and susceptible (GCRI-26) plants. Illustrations represent grafts at 10 d.

rootstock. In two other grafts of this type, there was a lower titer of virus present in the rootstock, and virus moved into the resistant scion and reached high titer in 7 to 21 d. The accumulation of virus in the somaclonal material could have been due to the selection of a virus strain which was able to overcome the somaclonal resistance. In each grafted plant, the delay seen in virus multiplication in the resistant scion was significant when compared to the control graft of susceptible plants.

In the reciprocal graft of GCRI-26 (scion) and somaclone line 247 (rootstock), the inoculated resistant rootstock did not show the presence of virus (except for the virus in the inoculated leaf, with ELISA values from 0.00 to 0.14 AU) (Figure 22 C). The virus did not appear in the susceptible scion for more than 21 days pi, and when it did appear (with ELISA values of over 1.00 AU), the rootstock became infected as well. This later appearance of virus could be due to selection of a more virulent virus strain or by unintentional inoculation of the susceptible scion during sampling. In one grafted plant, the virus did not multiply in the susceptible scion for more than 40 d, which indicated that the resistant rootstock was restricting the movement of the small amount of virus that was present in the rootstock. If infective virus had moved into the susceptible scion, there would have been a high virus titer in five d. Because of the long delay in accumulation of virus in the scion, it was concluded that the virus was not allowed to spread systemically to the upper part from the resistant rootstock. Perhaps the active step of virus movement into the phloem was stopped, or there was simply not enough virus to allow for movement. Other explanations are possible as well. Resistance due to a systemically active molecule could not be eliminated.

Somaclone 12	MEAN ELISA VALUES (AU)					
Scion / Rootstock	0 d	5 d	7 d	11 d	21 d	39 d
A. 12	-*	0.01	0.45	0.44	0.24	1.00
26	-	-	1.70	1.76	1.03	0.93
B. 26	0.00	0.00	0.00	0.05	-	-
12	-	0.68	-	0.04	-	-

*not determined

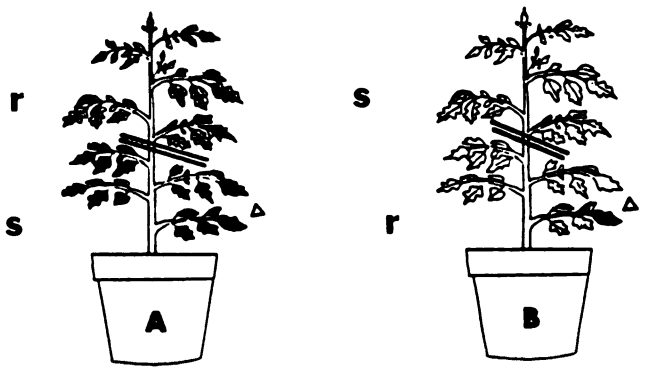


Figure 23. Results of grafting experiments using resistant (somaclonal line 12) and susceptible (GCRI-26) plant material after inoculation with ToMV-0 and screening young leaves from rootstock and scion using ELISA. Illustrations represent the grafts at 10 d.

The reciprocal grafts between GCRI-26 and somaclonal line 12 were similar to the above grafts in their response (Figure 23), but the resistance did not seem as strong in general, which was consistant with the results from the study of virus movement in plants of line 12. When GCRI-26 was the rootstock, there were high levels of virus present in the lower leaves, but only intermediate levels of virus accumulated in the leaves of the resistant scion (ELISA values from 0.45 to 0.24 AU) for longer than 20 d (Figure 23A). This indicated that there was an active limitation of virus multiplication in the resistant scion, but not complete inhibition.

When somaclonal line 12 served as the rootstock, a fairly high level of virus was detected in the inoculated leaf at 5 d (0.68 AU), but did not multiply in the susceptible scion for more than 10 d (Figure 23B). This delay was significant when compared to the control susceptible grafts, and gave support to the hypothesis that the somaclonal resistance includes restriction of systemic movement in the plant. Another explanation could be that the virus only moves out of the leaf when it has reached a high enough titer, but there was no evidence to discern between these two causes of limited virus spread.

PROTOPLASTS

Isolation of protoplasts from inoculated plants. Infected protoplasts seemed very fragile, and protoplast yields from plants with high virus titer (ELISA values of more than 1.00) were lower than yields normally obtained from healthy plants. Protoplasts isolated from infected GCRI-26 (ELISA value of 1.85 AU) were smaller than protoplasts from healthy plants, and were associated with more cellular debris (Figure 24A). After staining with FITC-anti-coat protein antibody conjugate, 51.4% of the protoplasts prepared from infected GCRI-26 were highly fluorescent,

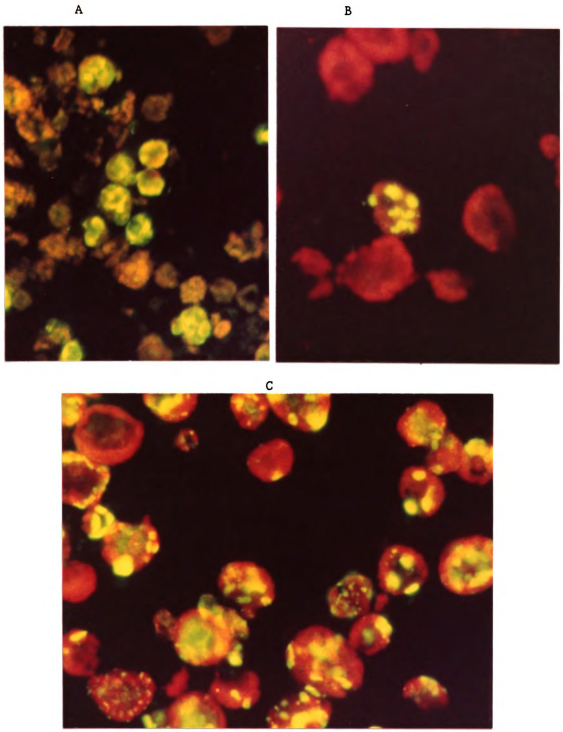


Figure 24. A) Infected protoplasts isolated from GCRI-26. B) One infected protoplast was present among thousands of healthy protoplasts in plant 12-1. C) Protoplasts from resistant plant 247-4 show low levels of virus in most protoplasts. (x600)

where the entire protoplast fluoresced with bright yellow-green, indicating the presence of virus. Uninfected protoplasts appeared darker. Cells which had been in contact with Lymphoprep during the isolation appeared red. Presumably less chlorophyll was extracted during fixation because the Ficoll left on the protoplast interfered with the extraction.

Protoplasts which were isolated from inoculated somaclones were variable in yield and in proportion of infected to healthy protoplasts. The mean ELISA value of the plants (averaged from leaves throughout the plant) did not correlate very well with the appearance of infected protoplasts after protoplast isolation, even though samples for each were collected the same day (Table 34). The breaking and loss of infected protoplasts during the purification may account for this dicrepancy. For several of the protoplast preparations, the sorbitol-CPW buffers from rinsing the protoplasts were saved for testing by ELISA. These ELISA values give an estimation of the amount of virus released during the protoplast isolation procedure.

As shown in Table 34, one plant (12-1) which had an ELISA value of 0.79 AU, yielded only a few infected protoplasts out of thousands of healthy protoplasts (Figure 24B). This was repeated with several plants. Another suprising exception was with protoplasts isolated from plants with very low ELISA values (0.05 AU) that appeared to contain virus in many of the protoplasts at a low level (Figure 24C). In this example, protoplasts were isolated from a plant (247-4) which was symptomless and had an ELISA value of 0.05 AU at 15 d pi. Of these protoplasts, 65.6% were labelled with the FITC-antibody. However, the fluorescent labelling was not seen completely throughout each

Table 34. Correlation of ELISA values to protoplast yield and to percentage of infected protoplasts isolated from susceptible and resistant tomato lines. Also included are ELISA values of protoplast samples and samples of CPW buffer used in rinsing the protoplasts during preparation.

plant	mean plant ELISA	protoplast yield/gm ($\times 10^6$)	mean protoplast ELISA	% infected protoplasts	ELISA of CPW buffer
GCRI-26					
Healthy					
26-17	0.00	4.53	-	-	-
26-18	0.00	5.29	-	-	-
26-19	0.00	8.76	-	-	-
26-21	0.00	6.54	-	-	-
		6.27 (mean)			
Infected					
26-22	1.58	3.55	0.96	-	-
26-24[a]	1.83	2.96	1.61	51.4	-
26-26	1.14	0.77	0.96	56.9	1.53
		2.43 (mean)			
Inoculated somaclone lines					
247-4[c]	0.06	3.17	0.00	65.6*	-
247-8	0.13	3.26	0.00	<0.01	-
247-5	0.04	2.16	0.01	<0.01	0.00
247-6	0.05	1.71	-	29.1*	0.10
12-1[b]	0.13	4.62	0.02	<0.01	-
12-1	0.15	3.15	0.00	<0.01*	0.02
215-1	0.16	5.10	0.00	<0.01	-

*indicates plants from which partially infected protoplasts were detected.
[a]see Figure 16A.
[b]see Figure 16B.
[c]see Figure 16C.

protoplast, unlike protoplasts from infected GCRI-26 plants. Instead, the virus seemed to be localized within compartments in the cytoplasm. The same phenomenon was found in several plants sampled.

The presence of virus coat protein has been detected in inclusion bodies in the cytoplasm (Hills *et al.*, 1987) and has also been shown to accumulate in thylakoid membranes (Reinero and Beachy, 1986). It would be interesting to determine the location of the viral coat protein, especially in consideration of the cytoplasmic inheritance displayed by the somaclones.

The results seen in Figure 24C were puzzling, since it was felt that the ELISA was a very sensitive measure of virus (1 ng/ml was the lower limit of detection). Because the ELISA values did not correlate with the number of protoplasts which were stained for virus, an explanation was sought. Perhaps the immunofluorescence microscopy was more sensitive than the ELISA method for detecting low levels of virus. There has been a report of time-resolved fluoroimmunoassay, which uses double antibody sandwich technique with a fluorescent label, capable of detecting virus (PVX) as low as 100 pg/ml (Siitari and Kurppa, 1987). This was found to give a five- to 100-fold increase in sensitivity over conventional ELISA. With this precedent, it may be possible to explain the relationship between ELISA values and the degree of fluorescent labelling.

In the case of a high plant ELISA value with no fluorescence-labelled protoplasts, most of the infected protoplasts could have been lost during protoplast purification, making it impossible to compare these measures of infection. Loss of virus during protoplast rinsing was not evident in the case of one plant examined (12-1) which had ELISA

values of 0.15 AU for the plant which was relatively low, and 0.02 AU for the CPW buffer, which was very low. More of these assays would be necessary for a conclusive statement to be made.

That a low level (0.01-0.06 AU) of virus was present in protoplasts of the highly resistant somaclonal plant lines was somewhat suprising. However, this has also been noted in resistant potato plant lines after inoculation with PVX (Tavantzis, 1988). Further examination of this phenomenon using molecular biological techniques could verify virus presence. Detection of viral RNA by hybridization to labelled cDNA probes would give more sensitivity. One of several types of *in situ* hybridization could be used to localize the virus at the ultrastructural level, possibly giving more insight into the mechanism of resistance seen in the tomato somaclonal lines. It would be important to determine whether the ToMV coat protein is associated with the chloroplast membranes versus inclusion bodies.

SUMMARY AND GENERAL CONCLUSIONS

In characterizing the resistance to ToMV in tomato somaclones, it was shown that symptom formation was suppressed and virus multiplication was limited within the plants. After genetic analysis, this resistance appeared to be encoded by an incompletely dominant nuclear gene which interacted with the cytoplasm to cause a maternal effect. The resistance of the six somaclonal lines seemed to have a similar pattern of inheritance, with the possible exception of somaclonal line 12. Two somaclonal lines, 12 and 247, used in test crosses, were each found to be additive with the ToMV resistance gene, *Tm-1* but not to *Tm-2*. Test crosses with the other known resistance gene, $Tm\text{-}2^2$, produced resistant F_1 progeny, though is have been due to the dominance of $Tm\text{-}2^2$.

The ToMV-resistant tomato somaclonal lines in this study did not respond differently from one another after inoculation with different virus strains and other viruses. Their resistance was limited to the common strains of tobamoviruses. The somaclonal lines were also similar in response to high temperature incubation after inoculation, remaining resistant at 30° C for more than 30 d. The somaclonal resistance was similar to the resistance given by the *Tm-1* gene; each line limited viral multiplication to varying degrees and suppressed symptoms, but was overcome by the tomato mosaic virus strain, TMV-1, and was not affected by high temperature incubation.

Of the six somaclonal lines, the degree of resistance found in somaclonal line 247 was the highest and most consistent. For compar-

ison, somaclonal lines 12 and 247 were chosen for studies of virus movement. In these studies, after inoculation with ToMV, very low virus titers were present in inoculated leaves of somaclonal line 247, but the virus did not move into the plant systemically. This restriction of virus movement could have been due to deficient levels of virus, or to a deficiency in the transport function. However, somaclone 12 allowed a medium virus titer to accumulate throughout the plant, but did not allow a high virus titer. In contrast, susceptible control tomatoes allowed the virus to spread throughout the plant in high titer in 5 d.

In grafts of resistant and susceptible tomato, inoculated susceptible rootstock allowed multiplication of virus, but the upper, resistant scion delayed multiplication of virus. Somaclonal line 247 completely delayed multiplication for more than 10 d, but somaclonal line 12 allowed medium virus titer to accumulate, which was maintained for more than 20 d. In the reciprocal grafts, inoculated resistant rootstock restricted the virus to the inoculated leaf. In susceptible control grafts, virus levels were high throughout the scion and rootstock at 5 d.

When plants infected with ToMV were used as a source of protoplasts, it was found that the protoplast yield was lowered when high levels of virus were present, probably due to protoplast breakage. In a few of the resistant plants from somaclonal lines, there was a low level of virus found in many protoplasts which was detected by fluoresence microscopy. The virus (coat protein) appeared to be limited to discrete areas in the cytoplasm, which might possibly be chloroplasts. One might speculate that this restriction of virus or

virus component to low levels could be related to the mechanism of resistance, and might be due to the genetic element which displays the maternal effect in the inheritance of resistance.

The resistant plants seemed to limit the multiplication of the virus in more than one way. After looking at protoplasts released from inoculated, resistant plants, lower levels of virus were present in each cell, indicating that there was an inhibition of replication at the cellular level. There was also a restriction of virus movement, especially evident in the grafting experiments. How these two effects are encoded and expressed to give resistance is still not clear.

The resistance trait in each somaclonal line may be inherited by a single, incompletely dominant nuclear gene, *Tm-3*, with a maternally transmitted factor as well. The role of each of these components can only be ascertained by further virological and genetical research. Completion of characterization and comparison of each somaclonal line will also provide the information necessary to examine mechanisms of resistance to determine whether they differ among somaclonal lines.

Further study of virus movement in resistant plants is essential. It would be useful to compare susceptible and resistant plants for the cell-to-cell spread of virus in inoculated leaves, and to compare the systemic spread though the plant with *in situ* virus detection methods. Other variations of grafting experiments would also provide more information on the mechanism of virus transport and its inhibition in the resistant plants.

To determine the ability of the somaclonal lines to resist ToMV multiplication at the cellular level, further attempts at protoplast

infection should be made, preferably using electroporation, which can give up to 90% infection levels. The infection of cells from the resistant somaclonal lines should be contrasted to susceptible cells (*+/+*) and cells with the genotype *Tm-1/Tm-1*. First, the infectivity of the common strain of ToMV should be determined, and then the infectivity of TMV-1, the strain which overcomes the resistance of the *Tm-1* gene, should be determined. These results will help characterize the level of resistance and help to clarify the relationship of the somaclonal resistance, *Tm-3* to the resistance of *Tm-1*.

Since RNA-dependent RNA polymerase activity has been implicated in the resistance of the *Tm-1* gene, it would be useful to examine this enzyme activity in the somaclonal lines. Using somaclonal lines versus the control lines GCRI-26 (*+/+*) and GCRI-237 (*Tm-1/Tm-1*), the level of RNA-dependent RNA polymerase activity should be measured in unchallenged plants versus plants inoculated with ToMV-0 or TMV-1. If differences are present which are similar to those reported for *Tm-1*, further studies of this enzyme activity would be useful in understanding the mechanism of resistance to ToMV replication measured at the cellular level.

More information is needed about the life cycle of ToMV and about resistance to virus multiplication in plants to formulate resistance mechanisms. Current rapid gains in knowledge about the order and site of replicative events of ToMV, about the viral genes and gene products, and about the role of plant gene products in the multiplication and transport of ToMV will facilitate testing of hypothesized resistance mechanisms in the next decade. Perhaps the somaclonal lines will provide a source of variation of resistance for testing.

Possible resistance mechanisms might involve ribosomal components, RNA-dependent RNA polymerase activities, components of the membranes (endoplasmic reticulum, mitochondrial or chloroplast) which bind the active RNA polymerase, components of the viral inclusion bodies which seem to play a role in replication and assembly of the virus particle, chloroplast thylakoid membranes which accumulate viral coat protein, or plants cell products which are needed to facilitate transport of the infective virus through the plant. These are only a few of the possible areas in which resistance to virus multiplication could occur, as either positive or negative resistance mechanisms.

Another area of research which deserves further study is the mechanism of mutation in somaclonal variation. Among possible mechanisms to examine are DNA amplification or deletion, somatic rearrangement of genes (particularly those in multigene families), unequal somatic crossing over, altered nucleotide methylation patterns, perturbation of DNA replication by altered nucleotide pools, silencing or activation of genes by mutations in associated non-coding regions and transposable elements (Scowcroft, 1985). The increasing availability and use of molecular probes will provide some of the experimental tools necessary to understand the contribution of such mechanisms to somaclonal variation.

It is of interest that the viral resistances generated through somaclonal variation resemble that of a viral resistance gene found in a wild tomato species, both genetically and in their general response to virus challenge. It is worthy of note as an example of genetic flux, and when better characterized, may give insight into the evolution of disease resistance traits.

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APPENDICES

APPENDIX A

APPENDIX A

Regeneration and screening of tomato somaclones for resistance to tobacco mosaic virus

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REGENERATION AND SCREENING OF TOMATO SOMACLONES FOR RESISTANCE TO TOBACCO MOSAIC VIRUS

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Leaf discs of tomato (*Lycopersicon esculentum*, Mill.) from a fully tobacco mosaic virus (TMV)-susceptible (+/+) isogenic line, GCRI-26 (Glasshouse Crops Research Institute, Littlehampton, U.K.), were used to regenerate somaclones (Sc). Out of 370 somaclones inoculated with TMV-Flavum, six were eventually selected as virus-free and putatively resistant. R, progeny from self-pollination of the six somaclones showed TMV-resistance for varying periods ranging from 28 to 55 days (extent of test) while seedlings of the source plant usually became infected in 10—20 days. In resistant plants, no symptoms were visible and TMV could not be detected either by enzyme-linked immunosorbent assay (ELISA) or by back inoculations to *Nicotiana glutinosa*.

Key words: tomato; *Lycopersicon esculentum*; somaclonal variation; mosaic virus resistance

Introduction

Somaclonal variation is increasingly being investigated as an alternative source of genetic variation for crop improvement [1] and for disease resistance [2]. Tissue culture-derived plants have provided novel sources of resistance to Fiji virus disease of sugar cane [3], early [4] and late [5] blight of potato, southern leaf blight of maize [6], tobacco mosaic virus of tobacco [7], *Verticillium* wilt of lucerne [8], toxins of *Pseudomonas syringae* and *Alternaria alternata* in tobacco [9] and to the toxin, victorin, in oats [10].

In our preliminary work [11], we found 18 of 370 somaclones of a fully susceptible tomato, GCRI-26 [12] were free of virus 1 month after inoculation with TMV-Flavum. We present here results of further studies on the resistance of the somaclones which were derived from GCRI-26.

Materials and methods

Regeneration of tomato plants

Tomato (*L. esculentum*, Mill.) seed of a fully susceptible (+/+) isogenic line, GCRI-26, based on the variety Craigella, were kindly furnished by Dr. T.J. Hall of the Glasshouse Crops Research Institute. Leaf explants were taken from over 50 different 4-week-old plants grown in a soilless peat mixture (Sunshine mix 1, Fisons Western Corp. Vancouver, BC, Canada) at 25 ± 1°C with a 16-h photoperiod (cool white fluorescent tubes supplying 60 $\mu E\ m^{-2}\ s^{-1}$). Fully expanded leaves were surface sterilized for 15 min in a solution containing 10% (v/v) Big Chief Bleach (Patterson Laboratories, Detroit, MI) plus 0.1% polythylene sorbitan monolaurate (Tween 20) (Sigma Chemical, St. Louis, MO 63178) followed by three rinses in sterile water. Leaf discs, 6 mm in diameter, were cut out with the aid of a cork borer and 5—8 were placed on 25 ml of shoot-

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Abbreviations: BA, 6-benzyladenine; ELISA, enzyme-linked immunosorbent assay; IAA, indoleacetic acid; MS, Murashige-Skoog; Sc, somaclone; TMV, tobacco mosaic virus; Tween 20, polyethylene sorbitan monolaurate.

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inducing medium which consisted of Murashige-Skoog (MS) medium [13] supplemented with 0.3 mg/l indoleacetic acid (IAA) (Sigma) and 3.0 mg/l 6-benzyladenine (BA) (Sigma) and solidified with 0.7% (w/v) agar. The explants were cultured in 100 × 15 mm plastic petri plates and maintained at 23 ± 1°C with a 16-h photoperiod (cool-white fluorescent tubes supplying 30 $\mu E\ m^{-2}\ S^{-1}$). Various concentrations of BA and IAA were tested in the shoot-inducing medium to determine the optimum combination for shoot induction. Young shoots formed after 4 weeks; these were subcultured in 150 × 25 mm culture tubes containing 10 ml of root-inducing medium consisting of MS medium supplemented with Gamborg [14] B-5 vitamins. The pH of the medium was adjusted to 5.8 with dilute HCl or NaOH and then solidified with 0.7% agar. The root system was well developed within 2—3 weeks at which time the plants were removed from the tubes and the agar was gently washed off the roots. The rooted shoots were then transplanted in soilless peat mixture in 7.5-cm styrofoam pots. Plants were placed in a plastic box and covered with a polyethylene film for 3—4 days. The film was removed and the plants placed at 23 ± 1°C with a 16-h photoperiod (cool-white fluorescent tubes supplying 65 $\mu E\ m^{-2}\ s^{-1}$) for an additional 4—5 days.

Inoculation of Sc

A yellowing strain of TMV-Flavum was kindly provided by Dr. H. Jockusch, Max Planck Institut, Tubingen, West Germany. The virus was increased in GCRI-26 tomato plants and the sap extracted by grinding leaves in a mortar in 0.01 M potassium phosphate buffer (pH 7.0) (20 ml of buffer/g of leaves). Sc were inoculated by rubbing Carborundum-dusted leaves with a cotton swab previously dipped in infective sap. TMV-Flavum was used because infected plants could be readily distinguished by the yellow-mosaic symptoms. After inoculation, plants were transferred to a growth chamber at 25 ± 1°C with a 16-h photoperiod (cool-white fluorescent tubes supplying 65 $\mu E\ m^{-2}\ s^{-1}$), Symptoms usually appeared in 7—10 days in susceptible control GCRI-26 seedlings.

ELISA

The double antibody sandwich microplate ELISA procedure of Clark and Adams [15] was used to determine the presence or absence of virus. Antiserum to TMV-Flavum was produced in a rabbit by a series of three intramuscular injections of 1 mg each of purified virus. The initial injection was made with virus emulsified in Freund's complete adjuvant and the second and third with Freund's incomplete adjuvant (Difco Co., Detroit, MI 48201). The gamma globulin fraction was obtained by ammonium sulfate precipitation of the antiserum followed by passage through a 1 × 10 cm column of DE-22 (Whatman Ltd.) and was conjugated with alkaline phosphatase (Type VII S, Sigma) [15]. Optimum dilution of gamma globulin for coating plates was 1:1000 (v/v) and for the conjugate was 1:800 (v/v). Leaf tissue samples were ground with a mortar and pestle in PBS-Tween buffer containing 0.2 g KH_2PO_4, 2.3 g Na_2HPO_4, 8.0 g NaCl, 0.2 g KCl and 0.5 ml Tween 20/l; 5 ml of buffer was used per g of tissue. All ELISA buffers contained 0.02% sodium azide as a preservative. Absorbance values at 405 mm were recorded 30 or 60 min following addition of *P*-nitrophenyl phosphate, the substrate for alkaline phosphatase.

Back inoculations were done with the same samples used in the ELISA by applying the extract with a cotton swab to Carborundum-dusted leaves of *N. glutinosa*. The leaves were immediately rinsed with water after inoculation. Four half leaves were inoculated for each sample. Local lesions were counted 4—6 days later.

Selection of virus-free somaclones and progeny

After the initial inoculation with TMV-Flavum and subsequent ELISA test survivors

were transplanted to 10-cm pots of peat mixture and maintained in a greenhouse at 22—28°C with a 16-h photoperiod (cool-white fluorescent lighting supplying 60—65 $\mu E\ m^{-2}\ s^{-1}$). After a second inoculation, survivors were transplanted to 20-cm pots and grown to the flowering stage. The plants were self-pollinated and seeds were collected from each plant in order to evaluate the resistance of the next generation, designated as R_1 [16]. R_1 plants were inoculated with TMV-Flavum and evaluated under the same conditions as described above.

Results

Plant regeneration

The largest number of shoots in this test, 0.7/leaf disc, was obtained with 13.3 μM BA, and 1.7 μM IAA. A total of 370 Sc were regenerated and transplanted to soilless peat mixture.

Virus screening of somaclones and progeny

After the 370 Sc were inoculated with TMV-Flavum, 18 were judged to be free of virus based on ELISA. If ELISA values exceeded 0.50, plants were judged to be diseased and values for 57 symptomatic, infected plants ranged from 0.36 to 2.55. Values for 52 uninoculated healthy plants ranged from 0.00 to 0.03 (Table I). Plants with values less than 0.5 were saved and rescreened. After the second inoculation and screening, surviving plants were considered resistant if ELISA values did not exceed two times the mean of the uninoculated control. Following reinoculation of the 18 survivors, six remained free of TMV-Flavum (Table II). R_1 progeny of Sc 12 and Sc 247 were inoculated with TMV-Flavum. The plants were tested by ELISA over a 28—55-day period. Results shown in Table III indicate that all of the 15 R_1

Table I. Range and mean of ELISA values for healthy and TMV-Flavum infected GCRI-26 tomato plants.

	ELISA values mean ± S.D.	Sample No.[a]	Range of ELISA values
Healthy GCRI-26	0.005 ± 0.01	52	0.00—0.03
TMV-Flavum infected GCRI-26	1.27 ± 0.77	57	0.36—2.55

[a]Samples taken from groups of plants planted at 2—3-week intervals over a period of 8 months and assayed in duplicate.

Table II. Resistance of original somaclones (R) to TMV-Flavum. Back inoculations of extracts of somaclones to *N. glutinosa* were also negative.

	ELISA[a] values	Duration of experiment
Sc 12	0.00	51 days
	0.00	
Sc 215	0.03	161 days
	0.04	
Sc 219	0.03	173 days
	0.02	
Sc 247	0.03	101 days
	0.04	
Sc 322	0.04	137 days
	0.04	
Sc 330	0.04	95 days
	0.04	

[a]ELISA values for GCRI-26 plants inoculated with TMV-Flavum were >0.50 by 20 days; healthy control plants had values ranging from 0.00 to 0.07. Readings are for duplicate wells.

Table III. Resistance of R_1 progeny of somaclones to TMV-Flavum. ( ) = Number of plants in assay.

	ELISA values at 30 days[a]	ELISA values at 55 days[a]
Sc 12	0.00 ± 0.00 (15)	0.03 ± 0.01 (15)[b]
Sc 247	0.01 ± 0.03 (13)	0.05 ± 0.02 (4)[b]
	0.56 (1)	0.74 ± 0.16 (10)
GCRI-26	1.15 ± 0.45 (27)	—
Uninoculated GCRI-26	0.00 ± 0.00 (2)	0.006 ± 0.009 (6)

[a]Mean ± S.D.
[b]Back inoculations of extracts of resistant somaclones at 55 days to *N. glutinosa* were also negative.

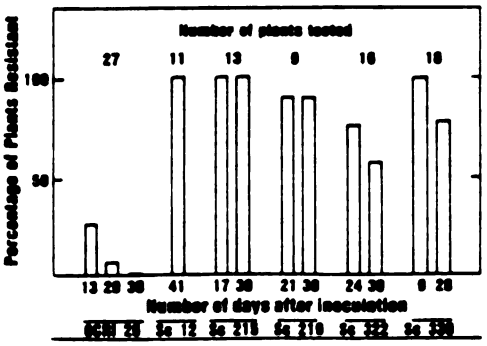

Fig. 1. Resistance of R_1 progeny of somaclones to TMV-Flavum. The mean ± S.D. of 99 ELISA values of putatively resistant somaclones was 0.03 ± 0.07 and the mean ± S.D. of 16 ELISA values of control plants of inoculated GCRI-26 was 0.68 ± 0.19. Back inoculations using extract from surviving somaclones at the end of tests, to *N. glutinosa* were also negative.

progeny of Sc 12 were free of TMV-Flavum. Four of 14 R_1 progeny of Sc 247 were free of TMV-Flavum. Back inoculations to *N. glutinosa* were also negative.

R_1 progeny of Sc 215, Sc 219, Sc 322, Sc 330 and additional Sc 12 were tested later with a second screening. Figure 1 shows that R_1 progeny of Sc 12 and those of Sc 215 were free of TMV-Flavum for the duration of the test but Sc 219, Sc 322 and Sc 330 showed some loss of resistance. Progeny of Sc 219 appeared more resistant than that of Sc 322 while Sc 330 was intermediate. The GCRI-26 control plants became infected at a faster rate and all had symptoms or high ELISA values by 30 days.

Discussion

The results indicate that the type of resistance shown by these somaclones is a delay in symptom expression and virus multiplication lasting for 41—55 days which was the extent of the tests. The resistant somaclones were obtained from a recognized fully susceptible source cultivar using ordinary tissue culture methods without the addition of a known mutagen. A sample of 57 plants from this source showed complete susceptibility, strongly suggesting that the resistant somaclones were displaying a new form of resistance. A similar type of TMV resistance has previously been described in tobacco somaclones following gamma radiation [7]. Different genes for TMV-resistance in tomato have been incorporated into the cultivar Craigella by Pelham [17]. These include the Tm-1 gene which prevents symptom development and multiplication of the common strain of tomato mosaic virus [17,18]. Tm-2 and Tm-2^2 genes elicit a hypersensitive response to the common strain of virus [17]. Tm-2^2 confers a high level of resistance to most strains of TMV [19]. The delay in symptom expression and inhibition of virus multiplication shown by our somaclones seem similar to that expressed by the Tm-1 gene. However, further virological and genetic studies are needed to determine the exact nature of the resistance and its inheritance shown by the somaclones.

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APPENDIX B

APPENDIX B

ELISA Virus Detection Procedures

CONTENTS

1) ELISA Protocol. (See Section 2 for composition of buffers)

Reference: Clark, M. F. and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbant assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Note: All glassware is siliconized to prevent protein absorption.

1. Add 200 ul purified gamma-globulin which has been appropriately diluted in coating buffer per well of the microtiter plate. For each incubation step cover the plate with an airtight cover such as Saran Wrap. Incubate 4 hours at 37° C. Can be stored longer times at 5° or -20° C. (Some coating globulins can be reused: check your system.)

2. Wash by flooding wells with PBS-Tween. Leave at least 3 minutes. Repeat wash three times. Empty plate. Take care to aspirate out bubbles in plate.

3. Add 200 ul aliquots of the test samples appropriately diluted in virus grinding buffer to duplicate wells. Leave at 5° C overnight or at 37° C for 4-6 hours. Include a buffer check, a healthy check and a known virus check in each plate.

4. Wash plate three times as in step 2.

5. Add 200 ul aliquots of enzyme-labelled gamma-globulin (conjugate) of appropriate dilution to each well in PBS-Tween PVP egg albumin buffer. Incubate at 37° for 3-6 hours. (Some conjugates can be rescued: check your system.)

6. Wash plate three times as in step 2.

7. Add 300 ul aliquots of freshly prepared p-nitrophenol phosphate (1 mg/ml) in substrate buffer to each well. Reserve a beaker just for this purpose. Incubate at room temperature for 1 hour of less, as necessary to observe reaction. Save extra substrate for spectrophotometer reference - it should be water-clear.

8. Slow down reaction by adding 50 ul 3 M NaOH to each well. Read final results within 1 hour. Best at OD (A_{405}) near 1; color is not stable.

9. Assess results by a) visual observation, b) measurement of absorbance at 405 nm in a spectrophotometer or ELISA reader when quantitative data are desired. We consider the mean A_{405} of the healthy wells of the plates +/- 2 standard deviations to be the threshold value for positive virus identification in that plate.

2) Chemicals.

1. All glassware used with ELISA antibodies should be pretreated with a silicon coating solution to prevent loss by absorption.

2. Coating buffer (0.5 M sodium carbonate, pH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g (0.02% w/v)

In 1 liter H_2O adjusted to pH 9.6 with HCl.
(Can be stored at 5° C, but check pH. Stock solution okay.)

3. PBS-Tween washing solution (0.1 M sodium-potassium phosphate, 0.85% NaCl, 0.05% Tween-20, pH 7.4)

	1 liter	3 liters	
NaCl	8.0 g	24.0 g	
KH_2PO_4	0.2 g	0.6 g	
Na_2HPO_4 7 H_2O	1.65 g	3.45 g	
KCl	0.2 g	0.6 g	
NaN_3	0.2 g	0.6 g	(0.02% w/v)
Tween-20	0.5 ml	1.5 ml	

In 1 liter H_2O. Should have pH = 7.4.
(Can be stored at 5° C. Stock solution okay.)

4. Virus grinding buffer (also used as conjugate buffer)

PBS-Tween (see 3 above)	1 liter
Polyvinylpyrrolidone (MW 10,000)	20 g (2% w/v)
Ovalbumin (crude egg albumin)	2 g (0.2% w/v)

Store at 5° C and use within one month.
Healthy sap can be added to conjugate buffer to cross-absorb undesirable antibodies in conjugated gamma-globulin. Incubate 1 hour at 25° C before filling wells.

5. Substrate buffer (10% diethanolamine, pH 9.8)

diethanolamine	97 ml
NaN_3	0.2 g

Make up to 1 liter with H_2O. Adjust to pH 9.8 with conc. HCl.
Recheck pH before use and readjust to pH 9.8.
Use liquid diethanolamine.

3) Coating and conjugate globulin preparation.

1. Purification of coating gamma-globulin. (Other recognised procedures may be used instead.) Choose an antiserum with a high gel double diffusion (Ouchterlony) titer against the strain of virus being studied and a low anti-healthy titer.

1. To 1.0 ml antiserum add 9 ml distilled water.
2. Slowly and dropwise add 10 ml saturated ammonium sulphate solution while stirring.
3. Leave 30-60 min at room temperature.
4. Centrifuge at 6000 rpm for 5 min to collect precipitate.
5. Dissolve precipitate in 2 ml 1/2-strength PBS (1:1 PBS:H_2O without Tween-20).
6. Dialyze 3 times against 500 ml 1/2-PBS (including once overnight).
7. Filter through 3-5 cm DE 23 cellulose (diethylaminoethyl cellulose) pre-equilibrated in 1/2-PBS. Use a short chromatographic column or a broken 10 ml pipet plugged with glass wool. See DE 23 manufacturer's directions and follow them carefully. Bio-Rad Affigel Blue has also been suggested for trial as a more rapid one-step globulin purification method.
8. Wash gamma-globulin through DE 23 column with 1/2-PBS.
9. Monitor effluent at 280 nm and collect first protein fraction to elute. Collect in 2 ml fractions. Best should be about 3 OD.
10. Measure A_{280} and adjust strength of gamma-globulin to read aproximately 1.4 OD (about 1 mg/ml).

2. Conjugation of enzyme with gamma-globulin

1. Centrifuge 1 ml (=2 mg) Sigma No. P-4502 alkaline phosphatase at 6000 rpm for 5 min. Discard supernatent liquid. (This step is omitted when using Sigma No. P-5521.)
2. Dissolve precipitate directly in 1 ml (=1 mg) purified gamma-globulin. Be careful of alkaline phosphatase pellet, which is soft. Add globulin to centrifuge tube.

(Continued)

3. Dialyze 3 times against 500 ml PBS (1 X conc, no Tween-20) (twice 1 hour apart + overnight at 5° C).

4. Add fresh glutaraldehyde solution to make a final glutaraldehyde concentration of 0.05% (v/v). Mix well.

5. Leave 4 hours at room temperature. A yellow-brown color should develop. (Color is faint and "sandy" but may stay water-clear and still work.)

6. Dialyze 3 times against 500 ml PBS + 0.01% sodium azide as preservative to remove glutaraldehyde.

7. Add bovine serun albumin, 5 mg/ml and store at 4° C. (Sigma No. A-7638.)

APPENDIX C

APPENDIX C

Pedigrees and ratings of reciprocal crosses.

Table C1. Pedigrees and ratings of reciprocal crosses. Listed on the left is genotype (female parent x pollen parent) followed by the S:I:R rating of the plants. Results of screening of F_2 are presented on the same line as the F_1 (or the next line if there is more than one set). The letters in parentheses denote the groups of plants that were screened at the same time. The last column represents the designation of the F_1 plant which produced the seed for the F_2 screening. *indicates a plant which was screened by TMV-Flavum rather than ToMV-0. "un" indicates that F_2 seed for screening were collected from uninoculated and unrated plants.

Somaclone 12	F_1	F_2	no.
A12-9 x 26	3:2:1(I)	2:2:8(T)	1
A12-12* x 26	6:6:2(I)	2:3:9(T)	6
		2:4:5(n)	7
A12-9-9 x 26	8:4:0(BB)		
26 x A12-8	10:2:0(I)	5:4:7(R)	1un
	7:4:1(Z)	6:5:3(z)	2
26 x A12-9*	10:1:1(N)	3:2:2(z)	1un
	9:3:0(I)		
	3:4:1(Q)	4:1:7(W)	1un
26 x A12-10*	5:7:0(H)	8:3:2(R)	1un
26 x A12-9-9	5:4:3(BB)		

Somaclone 215	F_1	F_2	no.
D215-8* x 26	0:3:9(I)	2:1:13(R)	1un
G215-7 x 26	3:3:0(Q)		
	2:4:0(Q)		
G215-10 x 26	4:4:4(Z)	5:6:5(i)	5
		2:1:9(n)	5
26 x B215-1*	5:7:0(H)	9:3:4(R)	1un
	9:3:0(I)	3:3:6(n)	10
26 x F215-2	1:5:0(Q)		
	3:3:0(Q)		

Table C1. (continued)

Somaclone 219	F_1	F_2	no.
D219-1 x 26	3:3:0(N)	8:3:5(W)	1un
F219-4 x 26	2:4:0(Q)		
G219-5 x 26	1:3:2(Q)	1:2:3(d1)	1un
		3:1:6(d2)	1un
	1:2:5(d1)		
	3:3:1(d2)		
26 x D219-1(O)	8:4:0(N)	4:2:7(W)	un
		3:0:2(d1)	un
		8:2:2(d2)	un
	0:0:2(d1)		

Somaclone 247	F_1	F_2	no.
G247-1 x 26	0:3:9(Q)	0:2:14(c)	1un
		0:0:16(s)	11
		1:1:14(u)	1un
		0:0:14(x)	2un
F247-1 x 26	0:1:4(T)	12:2:2(i)	3
F247-2 x 26	4:1:1(Z)	11:4:1(i)	2un
		4:1:8(s)	2un
G247-4-5-4 x 26	2:9:1(BB)		
26 x A247-15*	4:5:0(H)		
	3:4:1(I)	11:0:5(R)	1un
26 x A247-7	11:1:0(N)	4:5:7(u)	1un
		6:2:8(x)	1un
		0:2:11(s)	2un
		0:2:12(u)	2un
26 x G247-4-5-4	10:2:0(BB)		

Table C1. (continued)

Somaclone 322	F_1	F_2	no.
D322-3 x 26	0:3:0(Q)		
D322-10 x 26	0:3:4(Q)	11:4:1(c)	9
		2:2:12(x)	6
26 x D322-8	5:1:0(N)		
	4:7:1(Z)	10:3:3(i)	12
26 x D322-3	6:0:0(N)	4:5:3(W)	1un
		3:4:9(x)	1un

Somaclone 330	F_1	F_2	no.
D330-5 x 26	0:6:3(S)	11:3:2(c)	9
	2:3:1(Z)	4:5:7(y)	3
D330-7 x 26	0:2:1(S)	2:3:11(x)	11
26 x D330-3	11:1:0(N)	5:3:5(W)	1un
		10:2:4(y)	1un
26 x D330-4	4:5:3(Z)	5:1:10(x)	11
		6:4:3(i)	12