# Characterization of Virus Disease Resistance in *Lactuca sativa*

by

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**Suggested short title:**

Characterization of virus disease resistance in lettuce.

**Dedicated**
**to**

*Rahul*
*Krishna*
*Mahipal*

## Abstract

Little is known about the mechanism of virus disease resistance in plants. The aim of the work presented here was to answer whether disease resistance is offered within the cell or at the level of intercellular movement of the virus. The protoplast system was used for this purpose. Conditions were optimized to isolate viable protoplasts from the leaves of *Lactuca sativa* cultivars. Protoplasts and leaves from resistant and susceptible *Lactuca sativa* cultivars were inoculated separately with turnip mosaic virus (TuMV) and lettuce mosaic virus (LMV). Virus multiplication was examined over time using enzyme-linked immunosorbent assay. Resistant cv. Kordaat did not support TuMV multiplication in protoplasts as well as in leaves. The results indicated that resistance to TuMV is available within the cell. The results ruled out the possibility of involvement of cell to cell movement and resistance to TuMV seems to be constitutive. On the other hand, protoplasts and leaves from both resistant and susceptible lettuce cultivars supported LMV multiplication. This suggested that resistance to LMV may not be offered within the cell. The results also indicated that the resistance to LMV was partly due to a hypersensitive response though virus was still able to spread systemically. To contribute towards mapping of the *Tu* resistance gene, the genotype of $F_2$ individuals was determined by screening a $F_3$ population from 71 $F_2$ individuals of a cross between cv. Calmar and cv. Kordaat for TuMV-infection. These data were useful for the production of bulks around the *Tu* locus to facilitate the search for new molecular markers linked to the *Tu* gene.

## Resumé

Peu de choses sont connues concernant les mécanismes de résistance contre les maladies virales chez les plantes. Le but du travail présenté ici était de savoir si la résistance à la maladie s'explique dans la cellule ou au niveau du mouvement intercellulaire du virus. Des protoplastes ont été utilisés à cet effet. Les conditions ont été optimisées pour isoler des protoplastes viables des feuilles des cultivars de *Lactuca sativa*. Les protoplastes et les feuilles des cultivars résistant et sensible de *Lactuca sativa* ont été inoculés séparément avec le virus de la mosaïque du navet (TuMV) et avec le virus de la mosaïque de la laitue (LMV). La multiplication du virus a été examinée à différents moments en utilisant un test ELISA. Le cultivar résistant Kordaat ne supportait pas la multiplication de TuMV dans les protoplastes aussi bien que dans les feuilles. Les résultats ont indiqué que la résistance au TuMV est présente dans la cellule. Les résultats ont permis d'écarter les faits que la cellule soit impliquée dans le mouvement dans la cellule et que la résistance soit présente de façon permanente. D'un autre côté, les protoplastes et les feuilles des deux cultivars de laitue (résistant et sensible) supportent la multiplication de LMV, mais à un niveau moindre dans le cultivar résistant. Ceci suggère qu'au moins une partie de la résistance au LMV provient de la cellule. Les résultats indiquent aussi que la résistance au LMV semble être en partie due à une réponse hypersensible bien que le virus soit toujours capable de se propogager à d'autres parties de la plante. Pour contribuer à la cartographie du gène de résistance *Tu*, le génotype des individus de $F_2$ a été déterminé en testant la population $F_3$ provenant de 71 individus de $F_2$ issus d'un croisement entre Calmar et Kordaat pour l'infection par TuMV. Ces données ont été utiles pour la production de bulks pour le locus *Tu* afin de trouver de nouveaux marqueurs moléculaires liés au gène *Tu*.

## Acknowledgements

I express my sincere gratitude to my supervisor, Dr. M.G. Fortin for his valuable and critical guidance throughout the course of this research. The financial support from his research grant was greatly appreciated. I would like to thank members of my advisory committee, Dr. D. Smith and Dr. T. Paulitz for their cooperation and suggestions. I would like to thank Mrs. H. Cohen-Rimmer for photographic assistance. I wish to thank my labmates Eliane Ubalijoro and Marjorie Robbins for the helpful discussions from time to time (and of course for their great sense of humour). I wish to thank Olivier Leflaëc for translating the abstract. My special thanks are due to friends Rajesh and Laurie, who always encouraged me during the days of my winter depression. Also sincere thanks to Laurie for typing the references. I want to thank my friend Djavad Shateryan for moral support. Last but not the least, heartiest thanks to my fiancee Seema for the constant and inspiring phone calls. I appreciate her patience.

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# I. Introduction

Little is known about how plants offer resistance to viral infections. Viral infections contribute significantly to loss of yield and quality in crop plants (James, 1974). The control measures include crop hygiene and use of virus-free seed (Kwaje and Young, 1979), control of vectors (Zimmerman-Gries, 1979) and virus elimination by heat and chemicals (Walkey, 1980). Chemical treatments are costly and have toxic effects on plants and animals (Cassells, 1983). There is little that can be done once a crop gets infected. The only effective and long-term protection seems to be the availability of resistance in a particular crop species (Fraser, 1986).

An understanding of the mechanism involved in disease resistance would be the first step to control virus diseases of plants. Cultivar resistance can be due to inhibition of either viral replication, translation, post-translational processing of viral polyprotein or cell-to-cell movement of the virus. The aim of the work presented in this thesis was to answer whether resistance to two viruses in *Lactuca sativa* is offered within the cell or at the level of cell to cell movement. The plant-microbe interaction system examined in this thesis consists of one crop (*Lactuca sativa*) and two infecting potyviruses. Resistance to turnip mosaic virus (TuMV) is conferred by a single dominant gene *Tu* (Zink and Duffus, 1970) and to lettuce mosaic virus (LMV) by a single recessive gene *mo* (Ryder, 1970). This offered an opportunity to study a system where the mode of resistance conferred by a dominant and recessive gene can be compared at the same time. In this study, the *L. sativa* cv. Calmar (susceptible) and cv. Kordaat (resistant)

were used for TuMV infection. The lettuce cvs. Vanguard (susceptible) and Vanguard 75 (resistant) were used for LMV infection.

Knowledge of the resistance mechanism alone is not enough. The knowledge of a resistance gene and its gene product is necessary in order to characterize the phenomenon of disease resistance completely. Resistance to virus diseases is mostly conferred by a single dominant or a recessive gene (Fraser, 1987). There has been slow progress towards cloning of viral disease resistance gene(s) in plants. No one has yet cloned any virus disease resistance gene in plants (Wilson, 1993). Map-based cloning is a promising approach to isolate genes of interest. A part of this study was aimed at contributing towards mapping of the resistance gene *Tu*. Map-based cloning requires finding molecular markers closely linked to the resistance gene *Tu*. Molecular markers may be detected using bulk-segregant analysis in which two DNA bulks are prepared from homozygous individuals of resistant and susceptible plants (Michelmore *et al.*, 1991) and then examined for polymorphism which will be linked to the resistance gene.

By combining the understanding of biochemical reactions involved in disease resistance and sequence information from the resistance gene (once it is cloned), it may be possible to understand the phenomenon of virus disease resistance.

## Objectives

**1. Standardization of conditions leading to high viability of protoplasts and their subsequent inoculation with virus.**

To examine the multiplication of virus in isolated cells, the experiment designed required a large number of protoplasts which would remain viable for a long period (one week). To maintain the viability of the protoplasts, conditions were standardized to maintain pH of the culture medium and to eliminate bacterial contamination, which otherwise kill the protoplasts.

**2. Comparison of levels of virus multiplication in resistant and susceptible lettuce lines.**

The aim of this study was to test whether resistance to different potyviruses in lettuce is offered intracellularly (inhibition of viral replication or translation) or intercellularly (inhibition of cell-to-cell viral movement). The protoplasts from respective resistant and susceptible lettuce lines were inoculated with TuMV and LMV. The level of viral coat protein with time was estimated using enzyme linked immunosorbent assay (ELISA). The same experiment was done on intact young leaves and results compared with those from protoplasts.

**3. Examination of viral induction of hypersensitive response i. e. (HR).**

A number of biotic and abiotic stress factors induce defence enzymes and

pathogenesis-related (PR) proteins. These proteins restrict the multiplication and spread of pathogen to neighbouring cells from the site of invasion in resistant plants. This is termed HR. A typical HR consists of formation of necrotic lesions resulting from death of cells at the site of infection. Microscopic examination of inoculated leaves was carried out for this purpose.

**4. Screening of $F_3$ population for resistant and susceptible phenotype (using ELISA) for its subsequent use in bulk-segregant analysis (BSA).**

This screening data was needed to make DNA bulks for subsequent detection of molecular markers linked to the *Tu* resistance gene in *Lactuca sativa*. Eighteen $F_3$ individuals from each of the 71 $F_2$ individuals of a cv. Calmar x cv. Kordaat cross (made by M. G. Fortin in our lab.) were screened for TuMV-infection using ELISA.

# II. Review of Literature

## 1. Plant Viruses

Plant RNA viruses have been classified into two groups (Goldbach, 1987): the "sindbis-like viruses" (eg. tobamo-, bromo-, cucumo-, tobra- and ila viruses) and "picorna-like viruses" (e.g., poty-, como- and nepoviruses). The RNA genome of the first virus group is monocistronic and individual genes are translated separately, while that of the second is continually translated into one large polyprotein which is cleaved into functional proteins by a virus-coded protease (Atabekov and Taliansky, 1990).

### 1.1 The Potyvirus group

The potyvirus group is the largest known group of plant viruses and very high crop losses have been attributed to this group (Ward and Shukla, 1991). These viruses share a common feature of induction of pinwheel inclusion bodies in the infected host cells (Edwardson, 1974).

The potyviruses have a positive single strand RNA genome, approx. 10,000 nucleotides long. A viral protein genome-linked (VPg) is covalently attached at 5' terminus and a polyadenylate region is present at 3' terminus (Dougherty *et al.*, 1985). It contains a single open reading frame that is translated into a polyprotein, which is cleaved into smaller polypeptides by virus coded proteases (reviewed by Riechmann *et al.*, 1992).

### 1.2 Polyprotein processing of potyviruses

By using a cell-free translation system, expression of a number of potyviral genomes has been studied (Dougherty and Hiebert, 1980; De Mejia *et al.*, 1985). Using a cell-free translation system, the phenomenon of polyprotein processing has been analyzed. Two virus-encoded proteases, NIa (Carrington and Dougherty, 1987a; Hellmann *et al.*, 1988; Garcia *et al.*, 1989; Ghabrial *et al.*, 1990) and HC-Pro (Carrington *et al.*, 1988a) process the polyprotein co- and post-translationally. Demangeat *et al.* (1991) reported *in vitro* polyprotein processing in tomato black ring virus and grapevine chrome mosaic virus; both are nepoviruses (a group related to potyviruses). Later, Demangeat *et al.* (1992) confirmed these results *in vivo* using infected *N. tabacum* cv. Xanthi protoplasts and leaf extracts. Polyprotein processing has also been shown in plum pox potyvirus (Garcia *et al.*, 1992).

Polyprotein processing has been studied from the pathogen point of view only. Little information is available on whether plants use inhibition of viral polyprotein processing as a mechanism of virus disease resistance.

## 2. Molecular interactions in host-virus relationship

### 2.1 Molecular determinants

In the case of bacterial and animal viruses, some virus-coded proteins recognize host proteins specifically. The tetramer replicase of bacteriophage Qβ contains three proteins from the host and only one is virus-coded (Blumenthal and Carmichael, 1979). Orsini and Brody (1988) showed that bacteriophage $T_4$-coded proteins bind host RNA

polymerase to enhance viral DNA transcription while inhibiting host transcription. Inhibition of host transcription by poliovirus infection (picornavirus group) has been shown to occur (Rubenstein and Dasgupta, 1989). This group of viruses has been shown to express a specific inhibitor of translation (a protease) which destroys a host component which binds capped messenger RNA.

In contrast, no such specific viral interaction at the cellular level has been described in plants.

## 2.2 Genetics and Virus Disease Resistance Phenotype

In plants, resistance to viral infections is mostly conferred by single resistance genes (Fraser, 1990; Matthews, 1991). According to Fraser (1986), the most common result of the expression of a single dominant resistance gene against a virus is the localization of the virus with the formation of necrotic lesions, i. e. the hypersensitive response (Fraser, 1986). In cases of resistance controlled by recessive or incompletely dominant genes, the host plant may cause complete suppression of virus multiplication or may cause tolerance, reducing symptom severity and virus multiplication (Fraser, 1986). In that case, virus is able to spread systemically. Most of the incompletely dominant or recessive genes do not function to form necrotic lesions (Fraser, 1986). Exceptions to this include the recessive gene *tm* in *Phaseolus vulgaris* cv. Scotia causing local lesions after inoculation with tobacco mosaic virus (TMV) (Thompson *et al.*, 1962). In *Capsicum* species, necrotic localization to TMV infection has been reported being associated with a recessive resistance gene (Bouwkma, 1980).

## 2.3 Models for virus disease resistance

Fraser (1987) proposed models for the action of disease resistance genes. In the positive model, the resistant plant produces a factor which will be inhibitory to virus replication (Fraser, 1987) and will result in complete immunity. Resistance mechanisms causing total immunity operate in protoplasts also (Motoyoshi and Oshima, 1977; Barker and Harrison, 1984). The resistance mechanism in such cases seems to be constitutive. Ponz *et al.* (1988) reported presence of a protease inhibitor in cowpea cv. Arlington inhibiting polyprotein processing of cowpea mosaic virus (CPMV). Resistance in this case is conferred by a single dominant gene which may be expressed constitutively (Bruening, 1987). In the negative model for resistance, Fraser (1987) proposed that the host plant lacks a gene product required for virus replication and this leads to immunity to its pathogen. In the third model, the resistance response is determined by the quantitative interaction between host and viral functions resulting in either resistance or susceptibility (Fraser, 1987) depending on the concentration and nature of the function coded by host and pathogen.

# 3. Mechanism of virus disease resistance in plants

Resistance may be defined as any inhibition of virus multiplication or its pathogenic effects on the host (Fraser, 1986). The terms *resistant* and *susceptible* are used to describe the degree of virus replication and accumulation (Cooper and Jones, 1983). All members of a plant species are immune to a particular viral infection in cases of non-host resistance. No definite mechanism of virus disease resistance has

been established in plants.

Siegel (1979) identified six steps in the virus life cycle where resistance could be conferred by the host plant: (1) entry into the host cell (2) uncoating of viral nucleic acid (3) translation of viral protein (4) replication of viral genome (5) assembly of progeny and (6) cell-to-cell movement of virus (Mansky and Hill, 1993). These are discussed below.

### 3.1. Entry and uncoating of virus particle

The majority of plant viruses are wound pathogens entering plant cells damaged by insects, nematodes or fungal zoospores (Wilson *et al.*, 1990). Most plant viruses have single-stranded RNA as their genome (Zaitlin and Hull, 1987) which is protected by a capsid protein.

Wilson *et al.* (1984 a,b) studied cotranslational disassembly of tobacco mosaic virus (tobamovirus group) using cell-free translation systems. Uncoating of viral RNA proceeds in the 5' to 3' direction by the first ribosome to translate viral RNA. *In vitro* studies with tobamovirus group (Register and Beachy, 1988; Roenhorst *et al.*, 1989), furoviruses (Shirako and Ehara, 1986) and icosahedral RNA viruses (Brisco *et al.*, 1985, 1986; Roenhorst *et al.*, 1989) have shown that mildly alkaline conditions and chelation of divalent metal ions is required to initiate cotranslational uncoating *in vitro*. It is still unclear that how these conditions are met *in vivo*. It has been shown that calmodulin-regulated low level of intracellular $Ca^{+2}$ ions is responsible for disassembly of both isometric and rod-shaped nucleocapsids (Allan and Hepler, 1989; Durham,

1978). Not enough literature is available to show that resistance to viral infection is offered at the level of uncoating of virus particle.

### 3.2 Inhibition at translational/post-translational level

Kiefer *et al.* (1984) showed that CPMV inoculated protoplasts from cowpea cv. Arlington (resistant) yielded less coat protein and viral RNA as compared to the inoculated protoplasts from susceptible cowpea line. This suggested that resistance to CPMV is offered within the cell. Later, Bruening *et al.* (1987) showed three activities in leaf extracts of Arlington cowpea, which might be involved in resistance: (i) proteinase (s) that degrade CPMV proteins (ii) inhibitor(s) of translation of CPMV RNAs and (iii) an inhibitor of proteolytic processing of the polyprotein. The latter activity has been detected by *in vitro* assays of extracts of both leaves and protoplasts (Ponz *et al.*, 1988). Inhibition of polyprotein processing by a protease inhibitor may be a general phenomenon in restricting viral infections. The same has been detected in cultured human cells (Korant *et al.*, 1985).

It is interesting to note that many members of the Solanaceae and Fabaceae families show high levels of expression of proteinase inhibitors in leaves upon injury (Richardson, 1977). These inhibitors are active against proteinases of microbial/animal origin but not against that of plants (Richardson, 1977). Rickauer *et al.* (1989) showed induction of proteinase inhibitors in tobacco cell suspension cultures using elicitors from *Phytophthora parasitica* var *nicotianae*. Recently, Atkinson *et al.* (1993) have reported cloning of a cDNA which encodes a protein inhibitor (a protein) from *Nicotiana alata*.

This clone may be used as the heterologous probe to detect the presence of an inhibitor in response to viral infection.

### 3.3 Inhibition at the replication level

Another mechanism by which plants may confer resistance to viral infection is by inhibiting virus replication. Loebenstein and Gera (1981) showed release of an inhibitor of virus replication (IVR) from TMV-infected protoplasts of resistant tobacco leaves and not in susceptible cultivar. Later, Gera and Loebenstein (1983) reported IVR-inhibitors of TMV replication in tobacco and tomato leaves. They also observed IVR inhibition of potato virus X (PVX) and cucumber mosaic virus (CMV) in different tissues showing that IVR is specific neither to the virus nor its host. Spiegel *et al.* (1989) reported recovery of this IVR protein in intercellular fluid of resistant tobacco leaves infected with TMV. An antiviral factor (AVF) was reported to be produced in virus infected leaves of tobacco (Antignus, 1977).

### 3.4 Cell to cell Movement of Virus

Virus infection spreads in a plant by its movement from the site of infection to the other parts of the plant (Hull,1989). The two forms of virus spread within the plant are short-distance movement from cell-to-cell through plasmodesmata and long-distance movement via the vascular system (Hull, 1989).

### 3.4.1 Short-distance virus movement.

Tomato gene *Tm*-1 confers a symptomless reaction to TMV (Motoyoshi and Oshima, 1979). The *Tm*-1 resistance is also maintained in tomato protoplasts (Watanabe *et al.*, 1987). On the other hand, *Tm*-2 and *Tm*-$2^2$ confer a hypersensitive response (Pelham, 1966, 1972). *Tm*-2 and *Tm*-$2^2$ resistance allowed TMV replication in a small number of initially infected cells but not virus movement from cell to cell (Nishiguishi and Motoyoshi, 1987). Expectedly, protoplasts from *Tm*-2 and *Tm*-$2^2$ plants allowed virus replication. This observation suggested that the resistance to TMV is offered at the level of movement across cell wall. The 30K protein encoded by TMV has been shown to dilate the plasmodesmata and hence potentiate cell to cell movement (Deom *et al.*, 1990).

#### 3.4.1.1 Virus coded functions for viral movement from cell to cell

It is not known how a plant offers resistance to virus movement from cell to cell. Much of the evidence that cell to cell movement is a function of the viral genome came from TMV mutants defective in movement across the cell wall. Three potyviral proteins P1, HC and CP are involved in cell-to-cell virus movement (reviewed by Riechmann *et al.*, 1992). The P1 protein of TVMV showed sequence similarity with the movement protein of TMV (Lain *et al.*, 1989a ; Robaglia *et al.*, 1989). The homologous TMV protein binds to viral RNA (Citovsky *et al.*, 1990). Recently, Citovsky *et al.* (1992) reported electronmicrographs of complexes of TMV ssRNA/cDNA and virus coded movement protein P30. They also reported that these

complexes increase plasmodesmatal permeability which facilitates cell-to-cell movement of TMV. Derrick *et al.* (1992) demonstrated an increase in plasmodesmatal permeability during cell spread of tobacco rattle virus (TRV; tobravirus group) in *Nicotiana cleavelandii* using a microinjection technique (Wolf *et al.*, 1989) devised to inoculate single leaf trichome cells. There are uncertainties over the role of pinwheel proteins in virus movement from cell to cell. They could be involved in virus transport through the plasmodesmata (Calder & Ingerfeld, 1990).

### 3.4.2 Long-distance virus movement

Not much research has been done on resistance to long-range movement of viruses. The research done so far emphasizes how viruses move to different parts within the plant. A procedure of immunogold staining was developed in which cowpea chlorotic mottle virus (CCMV) antigen in cowpea was detected early in phloem parenchyma and bundle sheaths, later spreading to mesophyll cells (van Lentt, 1988). Mutations in the coat-protein (CP) gene made TMV defective in rapid long-range movement (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988) suggesting that it is a virus-encoded function. Recently, in *Arabidopsis*, it has been shown that the conditions influencing the rate of plant development impact the long-range movement of cauliflower mosaic virus (CaMV) (Leisner *et al.*, 1993). The rate of plant development under normal conditions closely matched the kinetics of virus movement. Viral genes required for long-distance movement seem to be different from those required for cell to cell movement. It is not clear whether long-distance spread is simply a passive

movement of virus through vascular system (Hull, 1989). Also it is still unknown how the virus moves back from the phloem to other tissues (Hull, 1989).

### 3.5 Defence related plant proteins

When a viral pathogen attacks, an "induced resistance" is triggered in the plant. This results in a hypersensitive response by the plant leading to necrosis at the site of infection thereby localizing the pathogen. Bol *et al.* (1988) described the cascade of enzymes involved in the production of aromatic compounds, cell wall components and the extracellular pathogenesis-related (PR) proteins. PR protein induction has been observed in more than 20 plant species upon infection with microorganisms including viruses (reviewed by Bol, 1988; Carr and Klessig, 1989). Kauffmann *et al.* (1987) showed that four PR proteins of *Nicotiana tabacum* cv Samsun NN, reacting hypersensitively to TMV, have 1,3-$\beta$-glucanase activity. The same group of researchers reported four other PR proteins which show chitinase activity (Legrand *et al.*, 1987). Glycan hydrolase activities have been shown to be associated with PR proteins in potato (Kombrink *et al.*, 1988), tomato (Fischer *et al.*, 1989; Joosten and De Wit, 1989) and maize (Nasser *et al.*, 1988) showing that these proteins play a general role as anti-microbial defence enzymes. Plants attacked by pathogens accumulate enzymes of the phenylpropanoid pathway (Van Loon, 1982), hydroxyproline rich glycoproteins (HRGPs) (Lawton and Lamb, 1987; Wycoff *et al.*, 1992), proteinase inhibitors (Thornburg *et al.*, 1987), peroxidases (Lagrimini *et al.*, 1987) and superoxide dismutase (Bowles *et al.*, 1989).

No causal relationship has been shown between any of the defence proteins and the resistance phenotype.

## 4. Use of plant protoplasts in understanding virus disease resistance

Over the years, protoplasts have proved to be an important tool for understanding the mechanism(s) of disease resistance. As described earlier, protoplasts from *Tm*-1 containing tomato, Arlington cowpea and *Rx* and *Ry* potatoes do not support viral multiplication. Resistance in these cases seems to be offered within the cell. The more common observation is that protoplasts from resistant as well as susceptible plants support virus multiplication. This suggests a resistance mechanism involving inhibition of cell to cell movement of the virus through plasmodesmata. The number of examples available suggests that resistance to viral infections, in general, is operating at the intercellular level more than within the cell. The use of protoplasts in cell biology has facilitated understanding of viral disease resistance mechanisms which may eventually be helpful in manipulating disease resistance for crop protection.

## 5. Resistance mechanisms with special reference to potyviruses

As mentioned earlier, potyviruses constitute the largest and economically most important group of plant viruses. Therefore understanding the mechanism of resistance to potyviruses will play an important role in crop protection (Mansky and Hill, 1993). At present, resistance to only four members of the potyvirus group, soybean mosaic virus (SMV), tobacco vein mottling virus (TVMV), potato virus Y (PVY) and maize

dwarf mosaic virus (MDMV) has been studied (reviewed by Mansky and Hill, 1993).

### 5.1 SMV

In soybean, a single dominant allele (*Rsv*) confers resistance to most SMV strains (Buss *et al.*, 1987, 1988). Mansky *et al.* (1992) examined protein extracts of the resistant cultivar Davis for protease inhibitor activity (as reported in cowpea cv. Arlington; Bruening *et al.*, 1987). They could not detect such an activity.

### 5.2 TVMV

A comparison of TVMV multiplication in resistant and susceptible cultivars of tobacco showed that TVMV spread within the inoculated leaf but could not spread to uninoculated leaves (Gibb *et al.*, 1989). Protoplasts from both cultivars supported virus multiplication although with lesser yield of TVMV in resistant protoplasts. This observation suggests inhibition of long-range movement of TVMV in tobacco.

### 5.3 PVY

In potato, resistance to PVY is conferred by a single dominant allele *Ry* (Barker and Harrison, 1984). Protoplasts from a large number of resistant potato cultivars containing *Ry* supported PVY replication except two cultivars namely Corine and Pirola, where protoplasts showed no accumulation of PVY. This suggests that resistance in these two cultivars may involve inhibition of virus replication within the cell rather than inhibition of cell-to-cell movement of PVY (Mansky and Hill, 1993).

### 5.4 MDMV

Resistance to MDMV in maize is conferred by a single dominant allele *Mdm*-1 (McMullen and Louie, 1989). MDMV replicates locally in the resistant maize cultivar and does not spread systemically, indicating that resistance is offered at the level of cell to cell movement of the virus.

From the above mentioned examples, it is clear that there are more than one mechanisms of resistance operating against potyvirus infections. Also it may be concluded that resistance to potyviruses can be offered by the host plant either at intra-, intercellular level or long range movement.

### 5.5 Resistance to TuMV in *L. sativa*

The mechanism of resistance to TuMV is not known. TuMV is a member of the potyvirus group and its genome has been fully sequenced and some of its cistrons have been cloned into expression vectors. (Nicolas and Laliberte, 1992; Laliberte *et al.*, 1992). The coat protein (CP) clone was used to express the protein in *E. coli* and antibodies have been raised against the CP (Robbins *et al.*, 1994). TuMV causes systemic mosaic, stunting and circular chlorotic areas in susceptible *Lactuca sativa* CV. Calmar (Zink & Duffus, 1969). They observed that cultivars of *L. sativa* were either completely resistant or susceptible to TuMV infection. A single dominant host gene *Tu* confers resistance to TuMV (Zink & Duffus, 1970). No HR has been reported in this system.

### 5.6 Resistance to LMV in *L. sativa*

The mechanism of resistance to LMV is also not known. LMV is an aphid and seed transmitted potyvirus which causes severe damage to lettuce crops (Dinant and Lot, 1992). The symptoms include dwarfing, mottling, leaf distortion, yellowing necrotic spots or vein necrosis. Resistant lettuce cultivars may be symptomless or show faint mottling depending upon the genetic background of the cultivar (Marrou, 1969; Tomlinson, 1962, 1970). Recently, Dinant *et al.* (1991) determined the nucleotide sequence of the 3' terminal region of LMV which showed homology with the coat protein genes of other potyviruses.

Resistance in a few crisphead commercial cultivars like Salinas 88 and Vanguard 75 was introduced from Egyptian cultivars (Dinant and Lot, 1992). Resistance to LMV (Dinant and Lot, 1992) was first reported as tolerance in lettuce cultivar Gallega de Invierno (von der Pahlen and Crnko, 1965) which was inherited as a single recessive gene *g* (Bannerot *et al.*, 1969). Ryder (1968) reported similar resistance in three Egyptian wild *L. sativa* lines and named the recessive gene *mo* (Ryder, 1970). These genes allow symptomless systemic infection of LMV (Dinant and Lot, 1992). The genes *g* and *mo* are in fact different alleles or very closely linked genes (Lot and Deogratias, 1991). *mo* conferred a quasi-immunity to LMV infection whereas the *g* gene didn't.

## 6. Map-based cloning of virus disease resistance gene

The resistant host plant should be able to restrict the spread of the virus. The genetics of such resistance has been reviewed by Fraser (1990). In some plant species,

resistance is conferred by a single dominant (Shattock and Stobbs, 1987) or recessive (Fraser, 1987) gene. Progress in molecular genetic analysis has facilitated the mapping and cloning of the gene(s) of interest. However, to date no one has cloned any host gene involved in virus disease resistance (Wilson, 1993).

In the past few years, human disease genes have been isolated on the basis of their position on the chromosome (Rommens *et al.*, 1989). In tomato, a disease resistance gene active against bacteria has been cloned using the same strategy (Martin *et al.*, 1993). This approach is called positional cloning or map-based cloning. It is possible to detect the variation between individuals at the DNA sequence level in the form of restriction fragment length polymorphism (RFLP) (Kan and Dozy, 1978). Probes of known chromosomal location that detect such variation can be used to study cosegregation with the disease phenotype (Wicking and Williamson, 1991). The RFLPs serve as molecular markers. Nearest flanking markers are identified by segregation analysis. The segregation data allows construction of a linkage map of the region in which the target gene can be located (Lathrop *et al.*, 1984).

In a few studies on plants, molecular markers which are linked and flanking the target gene have been identified (Gebhart *et al.*, 1993; Martin *et al.*, 1993; Timmerman *et al.*, 1993). The next step is to estimate the physical distance between markers using pulsed-field gel electrophoresis (PFGE). The tight linkage may mean that the physical distance is small enough to allow chromosome walking, a technique to reach the gene of interest. This is not always true. Young *et al.* (1988) found markers closely linked to the *Tm-2a* gene conferring resistance to TMV in tomato. But the physical distance

between *Tm-2* and the marker (1cM = 4Mb) was greater than the average of 500 Kb per centiMorgan in tomato (Ganal *et al.*, 1989). This gene is close to the centromere and recombination frequency in the region is low (Tanksley *et al.*, 1992). It was suggested that a larger population should be screened to identify recombinants to get a higher resolution of the map.

Michelmore *et al.* (1992) reviewed the map-based cloning approach to clone the disease resistance gene. No knowledge of the gene product is required in this strategy. As mentioned earlier, no virus disease resistance has been cloned yet. The first step towards cloning a target gene is to identify molecular markers which are closely linked and flanking the target gene.

## 6.1 Types of molecular markers

### 6.1.1 Restriction fragment length polymorphisms (RFLPs)

Mapping using RFLPs began in early eighties with the human genetic linkage map (Botstein *et al.*, 1980). Restriction endonucleases are used to fragment DNA from the resistant and susceptible plants. Single copy DNA is cloned from a species of interest used as a probe to follow the segregation of homologous regions of the genome in individuals from a segregating population (Tanksley *et al.*, 1989). Polymorphism is detected using Southern blots (Zabeau and Roberts, 1979). Economically important plant genomes such as corn and tomato have been mapped using RFLPs (Coe *et al.*, 1990; Tanksley and Mutscher, 1990).

### 6.1.2 Random amplified polymorphic DNA.

An alternative to RFLPs is a polymerase chain reaction (PCR)-based technique where oligonucleotide primers are used to generate polymorphisms (Williams *et al.*, 1990). This technique does not require knowledge of DNA sequence from the target genome. The PCR technique uses a heat-stable DNA polymerase, nucleotide triphosphates (NTPs), oligonucleotide primers and target DNA.

## 6.2 Populations used to identify molecular markers linked to resistance gene

To increase the chances of a tight linkage between the target region and polymorphic loci, the approaches used in mapping plant genomes are near-isogenic lines and bulk-segregant analysis.

### 6.2.1 Near-isogenic lines (NILs)

NILs produced for a resistance locus differ in resistance gene(s) and are used to find markers at the resistance gene locus. These markers are likely to be linked to the resistance gene locus (Michelmore *et al.*, 1991). To produce NILs, at least six backcrosses are made. By the eighth backcross generation, less than 0.2 % of unlinked donor genome remains in the progeny (Young and Tanksley, 1989). Producing NILs is a time consuming process. The generation time of a particular species will determine the time required for NILs production. In *Arabidopsis thaliana*, it takes two years to produce NILs. NILs have also been used to identify markers linked to resistance genes like *Tm 2A* and *Pto* in tomato (Young *et al.*, 1988; Martin *et al.*, 1991) and *mlo* in

barley (Hinze *et al.*, 1991). In lettuce, NILs have been used to identify RFLP and RAPD markers linked to the *Dm* resistance gene conferring resistance to downy mildew (Paran *et al.*, 1991). The backcrossing is difficult in *Lactuca sativa* as it is a self pollinating species. Special care has to be taken to prevent self pollination while making crosses.

### 6.2.2 Bulk-segregant analysis.

This method is rapid and doesn't require NILs. A single segregating population is used for the identification of molecular markers linked to the target gene (Michelmore *et al.*, 1991). This method involves screening for polymorphism between two bulked DNA samples from a segregating population. The two bulks differ at the target locus. Each bulk contains individuals that have identical genotypes at the target locus. The two DNA bulks (resistant and susceptible, for example) are screened for polymorphism with 10-mer oligonucleotide primers. Bulk-segregant analysis has advantages over NILs. Bulks can be made instantaneously from any segregating population and for any region of the genome for which there is a marker (Michelmore *et al.*, 1991). From $F_2$ data, heterozygotes cannot be distinguished from dominant homozygotes and only RAPD markers in *cis* with the dominant allele will be identified. Availability of $F_3$ data increases the probability of selecting homozygous individuals for making the bulks (Michelmore *et al.*, 1991). Heterozygotes are not included in the analysis allowing RAPD markers to be identified that are both in *cis* and *trans* to the dominant allele.

## III. Materials and Methods

### 1. Virus propagation

*Brassica perviridis* was grown under greenhouse conditions to propagate the TuMV isolate described by Tremblay *et al.* (1990). Crude extracts of infected leaves (0.25 g/ml in 0.1M $KPO_4$, pH 7.5) were used for inoculation. The carborundum was dusted on the leaves using a cotton swab. The cotton swab was soaked in infected leaf extract and gently rubbed on the young leaves. *Chenopodium quinoa*, *C. amaranticolor*, *B. perviridis*, *L. sativa* cultivars, Cobbham Green and Kordaat were used as indicator plants.

The same conditions were used for LMV except that the host was *L. sativa* cv. Climax. *Chenopodium amaranthicolor* was used as an indicator plant.

### 2. Virus Purification

### 2.1 TuMV

TuMV was purified from infected *Brassica perviridis* leaves using the protocol of Choi *et al.* (1977). The TuMV-infected leaf material was stored at -70°C. The frozen tissue (100 grams) was homogenized in 0.5 M $KPO_4$ buffer pH 7.5 containing sodium EDTA (0.01 M) and mercaptoethanol (0.1 %). The homogenate was filtered through Miracloth (Calbiochem). The crude filtrate was centrifuged at 3,200 x g for 10 min and the pellet was discarded. Triton X-100 (1 %), NaCl (0.1 M) and polyethylene glycol (PEG, mol. wt. 6000) (4 %) were added to the supernatant and was stirred for three

hours at room temperature. The mixture was centrifuged at 8,500 x g for 10 min. The supernatant was discarded at this step and the pellet resuspended in 0.5 M $KPO_4$, pH 7.5 containing 0.01 $MgCl_2$. The suspension was centrifuged at 8,500 x g for 10 min. The supernatant was spun at 65,900 x g for 90 min. The pellet from previous spin was resuspended in 0.5 M $KPO_4$ pH 7.5, containing 0.01 M $MgCl_2$ and spun at 65,900 x g for 90 min. The supernatant from both of these spins was layered on a sucrose gradient (10, 20, 30 and 40 %) and was centrifuged at 61,000 x g for 2 hours. The grey band in 10 % sucrose was pipetted out and diluted in 0.01 M $KPO_4$, pH 7.0 and centrifuged again for 90 min. at 69,000 x g. The pellet was resuspended in 0.01 M $KPO_4$, pH 7.0 and centrifuged at 8,500 x g for 10 min. The supernatant containing purified TUMV was stored at -70°C.

## 2.2 LMV

LMV from *L. sativa* cv Climax was purified as described by Tomlinson (1964). All the operations were carried out at 3°C. The 100 grams of frozen infected leaf material was homogenized in 150 ml 0.5 M borate buffer, pH 7.5, containing sodium EDTA (0.001 M) and thioglycollic acid (0.1 %). The homogenate was filtered by passing through a double layer of cheese cloth. The residue was discarded. To the filterate was added 8.5 % n-butanol and the mixture stirred for 45 min. The mixture was centrifuged at 8,000 x g for 30 min. The pellet was discarded and the supernatant was again centrifuged as in the previous step. This pellet was also discarded and the supernatant was spun at 30,000 x g for 60 min. The pellet formed was resuspended in

0.05 M borate buffer and kept on ice for two hours, then centrifuged at 10,000 x g for 10 min. The pellet was discarded. The supernatant containing partially purified LMV was stored at -70°C.

### 3. Virus multiplication in resistant and susceptible lines.

#### 3.1 Preparation of protoplasts

The protocol described by Berry *et al.* (1982) was followed with slight modifications. Lettuce leaves (5-6 weeks old) from plants grown in growth chambers were first weighed and then washed under tap water; then surface sterilized for 2 min in 10 % w/v bleach solution containing 200 μg/ml cefotaxime, washed six times in sterile water, and cut into thin strips on a sterile glass plate using a sterile blade. The stripped leaves were plasmolysed in CPW solution (Frearson *et al.*, 1973) containing 13 % w/v mannitol for 1 hour (Berry *et al.*, 1982). Plasmolysed leaves were then incubated with gentle shaking (40 r.p.m.) at room temperature (in the dark) in CPW solution containing 13 % mannitol, 1 % cellulase w/v (Onozuka, Japan), 0.3 % w/v Macerase, 20 mM 2-(N-morphaline) ethane sulphonic acid (MES) and 200 μg/ml cefotaxime. After 8-9 hours of incubation, protoplasts were filtered through a 62 μm sieve and washed in CPW solution containing 13 % mannitol by centrifugation at 100 g for 6 min. They were further purified in two changes of CPW solution containing 21 % w/v sucrose (Berry *et al.*, 1982).

### 3.2 Inoculation of protoplasts with virus

The procedure of Otsuki *et al.* (1972) was followed with slight modifications. The purified protoplasts (as in section 3.1) were again resuspended in CPW medium containing 13 % mannitol and were counted using a haemocytometer. Purified virus (TuMV or LMV) (1 $\mu$g/ml) was mixed with poly-L-ornithine (1.5 $\mu$g/ml) in 0.025 M potassium phosphate buffer pH 6.0 containing 13 % mannitol for 30 min. Protoplasts were suspended in this solution ($1x10^6$ cells/ml) and incubated for 1 hour at room temperature to allow infection with virus. The percentage of infected protoplasts was estimated using a fluorescent antibody staining method (as described in the next section). Protoplasts were washed three times with CPW (13 % mannitol solution) as before and finally resuspended at the concentration of 4 x $10^5$ cell/ml in $K_p$ culture medium (Kao, 1977) pH 5.6, containing 20 mM MES and 200 $\mu$g/ml cefotaxime. The pH of the culture medium was adjusted after addition of the antibiotic cefotaxime. All these operations were performed under sterile conditions in the laminar flow hood. All the media and solutions mentioned above were filter-sterilized using 0.22 um pore size filters. The protoplasts in petri dishes were handled with sterile Pasteur pipettes and were incubated at 28°C under continuous illumination of 3000 lux (37.8 $\mu$moles $m^{-2}s^{-1}$. Samples in triplicates were harvested at definite time intervals (as mentioned in results section) in 1.5 ml eppendorf tubes and frozen at -20°C.

### 3.3 Fluorescent antibody staining

This technique was used to estimate the percentage of infected protoplasts in the begining of the experiment. The procedure described by Otsuki and Takebe (1969) was followed. One drop of protoplast suspension in mannitol solution was air (warm) dried rapidly on a glass slide previously coated with Mayer's albumen (spread using the finger). The protoplasts were fixed in 90 % ethanol for 10 min. and incubated with anti-TuMV coat protein serum (Robbins *et al.*, 1994) diluted 1:1000 in phosphate-buffered saline (PBS), for 1 hour at 37°C. After washing with PBS, protoplasts were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit protein A (ICN Biochemicals) for 1 hour at 37°C, washed with PBS and mounted in 40 % glycerol for microscopic examination under uv-illuminated (365 nm) field of a Zeiss fluorescence microscope.

### 3.4 Fluorescein diacetate (FDA) staining

This method was used to estimate viability of protoplasts. Procedure described by Huang *et al.* (1986) was followed. A stock solution of FDA (5 mg FDA /ml acetone) was prepared and then diluted 50 times in CPW solution containing 13 % mannitol. For staining, equal volumes of protoplast suspension and dye were mixed on a glass slide. After 3-5 min., a drop of this mixture was transferred onto a haemocytometer and protoplasts were counted under fluorescence microscopy using UV light (365 nm) (Larkin, 1976).

### 3.5 Enzyme linked immunosorbent assay (ELISA)

#### 3.5.1 Detection of TuMV

With slight modifications, the protocol of indirect ELISA as described by Clarke *et al.* (1986) was followed. The washing of wells of microtitre plates after each step was done six times using phosphate-buffered saline (PBS; 0.137 M NaCl, 27 mM KCl, 43 mM $Na_2PO_4$, 14 mM $KH_2PO_4$, pH 7.0) containing 0.05 % Tween- 20 (PBST). All the steps were performed at room temperature. The protoplast sample (100 $\mu$l) was mixed in 1:1 ratio with extraction buffer (0.15 M $Na_2CO_3$, 35 mM $NaHCO_3$ and 2 % polyvinylpyrolidone 44,000) and 200 ul was loaded in triplicates in the wells of a microtitre plate (Falcon). Leaf samples were ground in the same extraction buffer (100 mg/ml extraction buffer) and spun at 14,000 x g for one minute in a microcentrifuge. Twenty $\mu$l in 180 $\mu$l of extraction buffer was loaded in microtitre plate wells. Free sites were blocked with 2 % bovine serum albumin (BSA) in PBST. Antibody to coat protein (anti-TuMV CP, Robbins *et al.*, 1994) (1:1000 dilution in PBST containing 0.2 % BSA and 2.0 % PVP) was incubated to allow it to bind viral antigen. Further detection was done with goat-anti rabbit alkaline phosphatase conjugate (Bio-rad) diluted 1:5000 in the same buffer. The substrate p-nitrophenyl phosphate (Sigma) was added (1 mg/ml) in substrate buffer (9.7 % diethanolamine, pH 9.8) and the resulting color was read at 405 nm using Biorad's microtiter plate reader.

#### 3.5.2 Detection of LMV

The LMV antigen was detected using the double antibody sandwich ELISA

procedure described by the antibody supplier (Agdia Inc, Indiana). The anti-LMV serum and alkaline phosphatase conjugated anti-LMV were obtained from Agdia Inc. The microtitre plate wells were coated with anti-LMV serum (1:1000 dilution in coating buffer, pH 9.6, containing 0.159 % $Na_2CO_3$, and 0.293 % $NaHCO_3$) for 18 hrs at 4°C. After four washings with PBST, free sites were blocked with PBST containing 3 % BSA. The leaf extract was prepared in extraction buffer at a dilution of 1:10 (sample weight/buffer volume). For protoplasts, the sample was mixed in a 1:1 ratio with extraction buffer. After overnight incubation at 4°C and four washings with PBST, the wells were coated with alkaline phosphatase-conjugated anti-LMV solution at a dilution of 1:1000 dilution in conjugate buffer (PBST containing 0.2 % BSA and 2.0 % PVP) and incubated for 6 hours. After four washings with PBST, the substrate solution was added and plates were incubated in the dark for color development. Fifty $\mu$l of 3 M NaOH was added to the wells to stop the reaction. The optical density was measured at 405 nm using Bio-Rad's microtitre plate reader.

### 3.6 Whole leaf blot assay

The technique described by Polston *et al.* (1991) was used to detect virus coat proteins on whole leaf blots. The resistant (Kordaat) and susceptible (Calmar) lettuce cultivars were grown in growth chamber. Infected *B. perviridis* was used as positive control. The seedlings were inoculated with crude extract from TuMV infected *B. perviridis* leaves (as described in section 1) at the three-leaf stage and whole leaves were removed and washed with water in triplicates at each sampling time i. e. 0, 1, 2,

4, 6, 8, 14 and 26 days after infection. At each sampling, proteins were transferred from leaves onto a nitrocellulose membrane using a hydraulic laboratory press (10,000 psi, 5 min.). Viral coat protein was detected by incubating the membrane in anti-TuMV serum (1:800 dilution in PBS containing 1 % BSA). This antigen-antibody complex was detected (Gallagher *et al.*, 1989) using goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) (1:3000 dilution in PBS) and a nitroblue tetrazolium assay (Bio-Rad).

### 3.6.1 India ink staining of proteins

Leaf and viral proteins were detected using the protocol described by Hughes *et al.* (1988). The nitrocellulose membrane blots from whole leaf blot assay were stained in a solution of 50 $\mu$l per ml of India ink in a buffer containing 0.15 M NaCl, 10 mM $Na_2HPO_4$, and 0.5 % Triton X-100, pH 7.2. The staining was done for two hours with agitation at room temperature. The blot was rinsed in deionized water to remove excess stain.

## 4. Induction of the hypersensitive response

The growth chamber-grown four week-old lettuce leaves of resistant and susceptible cultivars were inoculated with their respective pathogens (TuMV/LMV) using carborundum and cotton swabs (see section 1). Microscopic examination of inoculated and mock inoculated leaves was performed every day in order to detect the induction of the hypersensitive response (necrotic spots or dead cells) in which phenolic compounds are produced by infected and surrounding cells (which are fluorescent under

UV light).

**5. Screening of an $F_3$ population for the production of bulks for the *Tu* locus**

A cross between Calmar (male parent) and Kordaat (female parent) was used to screen 71 $F_2$ plants for their response to TuMV infection. The seeds were collected from these plants and 18 $F_3$ plants from each of 71 $F_2$ individuals were planted in the greenhouse and inoculated with TuMV-infected *B. perviridis* (Robbins, M.Sc. thesis, 1993). The testing for TuMV infection was done using ELISA (section 3.5.1) twice, at three and six weeks after inoculation. The genotype of the $F_3$ families was determined based on the phenotypic ratio as determined from the ELISA data.

## IV. Results and discussion

### 1. Optimization of conditions leading to high viability of protoplasts for their subsequent inoculation with virus.

To understand the mechanism of virus disease resistance, lettuce protoplasts were used as a system to study virus multiplication in isolated cells. For this purpose, protoplasts (Figure 1) were isolated and purified from the leaves of *Lactuca sativa* cv. Vanguard (susceptible to LMV) and cv. Vanguard 75 (resistant to LMV). Their viability was estimated using FDA staining (Figure 2). A significant bacterial contamination was observed in the culture medium. Cefotaxime (200 $\mu$g/ml) as an antibiotic was used in the bleach solution for surface sterilization of leaves before protoplast isolation and also in the enzyme solution for protoplast isolation. The pH of the medium decreased to 3.0 after 48 hrs of incubation (Figure 3a) and percent viability decreased also (Figure 3b). The viability was 0 % (cv. Vanguard) and 25 % (cv. Vanguard 75) after 4 days of incubation. There was still some bacterial contamination observed after one day of incubation in culture medium. It was decided to also use cefotaxime (200 $\mu$g/ml) in the protoplast culture medium. Also, lettuce cells are known to secrete acidic substances into the culture medium (Engler and Grogan, 1983). Different concentrations of MES buffer (5mM, 25mM, 50mM and 100mM) were used in the culture medium and pH (Figure 4) and percent viability (Figure 4) were examined over time. One sample was drawn for each observation because each treatment had one petri dish containing the protoplasts and much variation was not expected within one petri dish. The initial pH of the medium

was adjusted to 6.1 (Engler and Grogan, 1983). In both lettuce cultivars, pH of the medium was maintained when higher than 5 mM concentrations of MES were used. Using cv. Vanguard, after 6 days of incubation of protoplasts, pH of the culture medium (Figure 4a) was 5.6, 5.8 and 6.0 when MES concentrations used in the culture medium were 25 mM, 50 mM and 100 mM respectively. Similarly in cv. Vanguard 75, the corresponding pH values (Figure 4b) were 5.6, 5.7 and 6.1. When a lower concentration (5 mM) was used, the pH of the medium decreased to 4.6 (Vanguard) and 5.1 (Vanguard 75) after 6 days of incubation of protoplasts.

The proportions of viable protoplasts after 0, 1, 2, 3 and 4 days of incubation are shown in Figure 5. The viability was more than 60 % up to 2 days in all the concentrations of MES. For 5mM MES, the percent viability decreased from 90 % to 46 % (cv. Vanguard) and from 82 % to 45 % (cv. Vanguard 75) after 4 days of incubation of protoplasts. This was attributed to low pH of the culture medium (Figure 3a) due to the weak buffering capacity of 5 mM MES. When 25 mM MES was used in culture medium, the pH of the medium was maintained at 5.6 in both cultivars (Figure 4) for up to 6 days of incubation. The viability of protoplasts was relatively higher with 25 mM MES (Figure 5a,b). As is obvious from Figure 4, the pH of the culture medium was also maintained by higher concentrations of MES (50 mM and 100 mM) but the percent viability of the protoplasts was severely affected (Figure 5). The 25 mM MES concentration appeared to be more effective as far as balance between pH of the medium and percent viability of the protoplasts was concerned. To further reduce the chances of damage to protoplasts, 20 mM MES concentration was used in culture medium for

Figure 1. Photomicrograph of lettuce protoplasts isolated from leaf mesophyll tissue. The protoplasts were viewed with bright light and picture taken after 24 hrs of incubation in Kp culture medium (3200 x magnification). The protoplasts were at the concentration of 1 x $10^6$/ml.

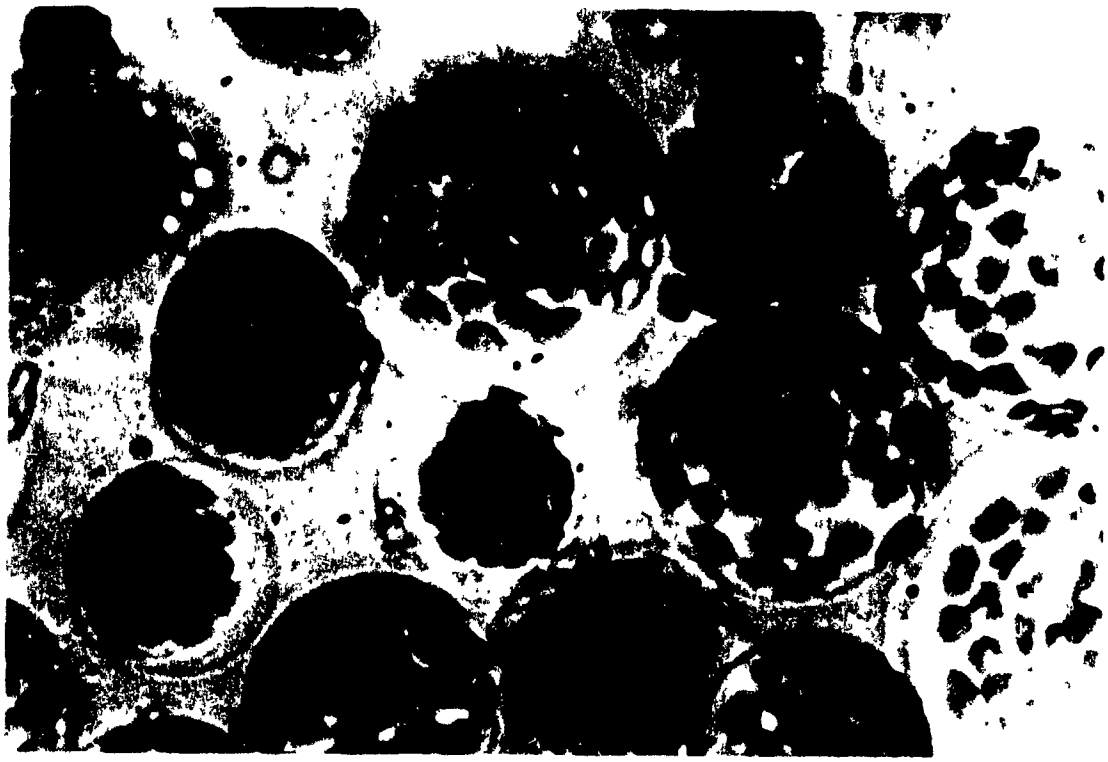
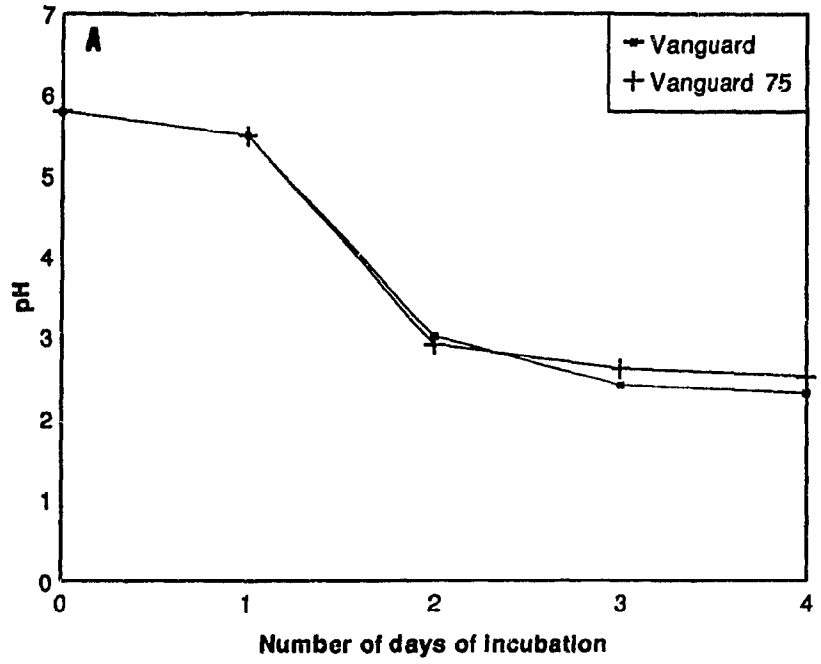


Figure 2. Lettuce protoplasts isolated from leaf mesophyll tissue. A. Fluorescent protoplasts, viewed with uv light after staining with FDA. B. Protoplasts viewed with bright field illumination (2720 x magnification).

A
B

Figure 3. The pH of the Kp culture medium (containing 5 mM MES buffer) in which lettuce protoplasts were incubated (A) and percent viability of the protoplasts incubated in culture medium with 5 mM MES (B). The percent viability was estimated by FDA staining and the number of viable protoplasts was calculated per ml of the culture medium. Each point in the line graph represents one sample from petri dish containing protplasts.


A
Vanguard
Vanguard 75
pH
0
1
2
3
4
5
6
7
0
1
2
3
4
Number of days of incubation

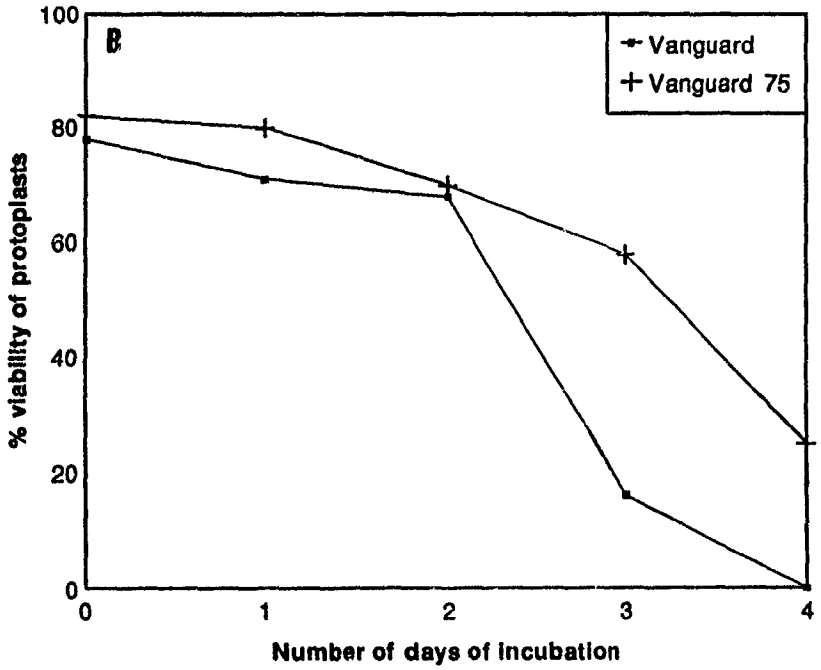
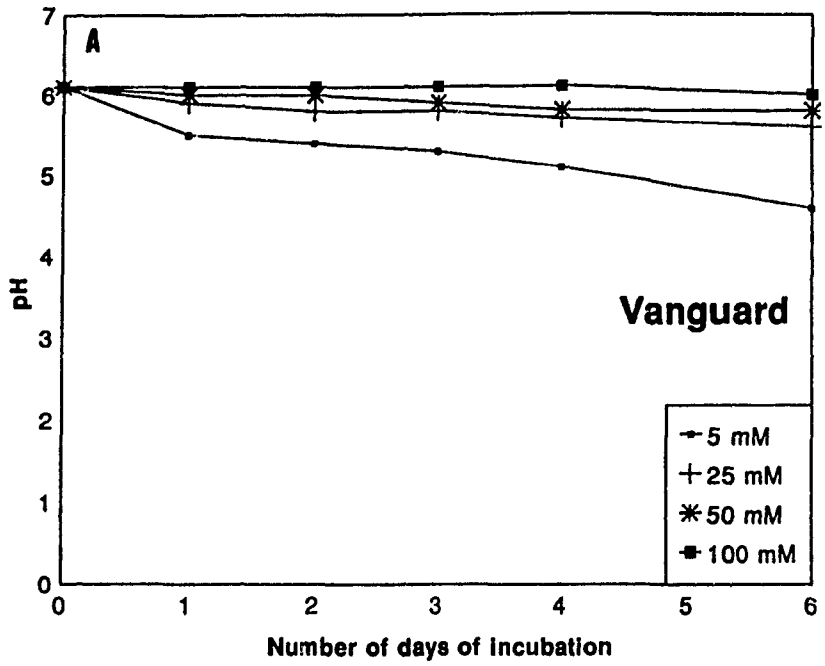

B
Vanguard
Vanguard 75
% viability of protoplasts
0
20
40
60
80
100
0
1
2
3
4
Number of days of incubation

Figure 4. Effect of adding different MES concentrations (5mM, 25 mM, 50 mM and 100mM) in the culture medium on pH of the culture medium in which lettuce protoplasts were incubated. (A) cv. Vanguard (B) cv. Vanguard 75. Each point in the line graph represents one sample.


A
Vanguard
5 mM
25 mM
50 mM
100 mM
pH
Number of days of incubation

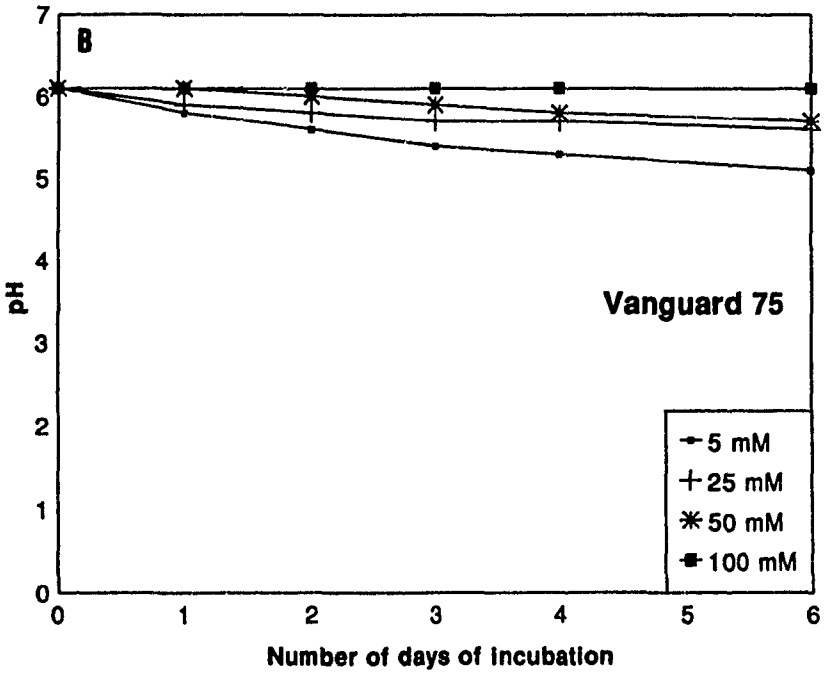
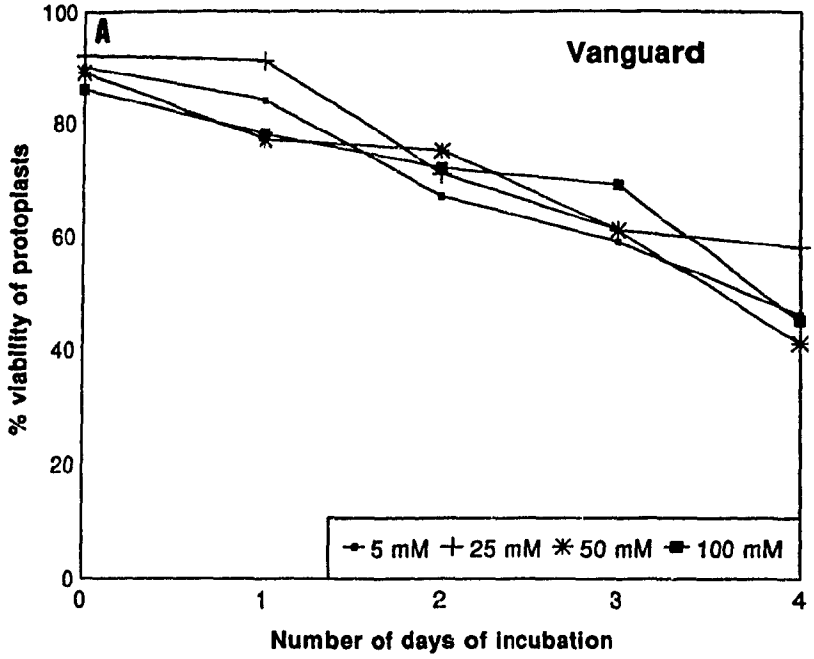
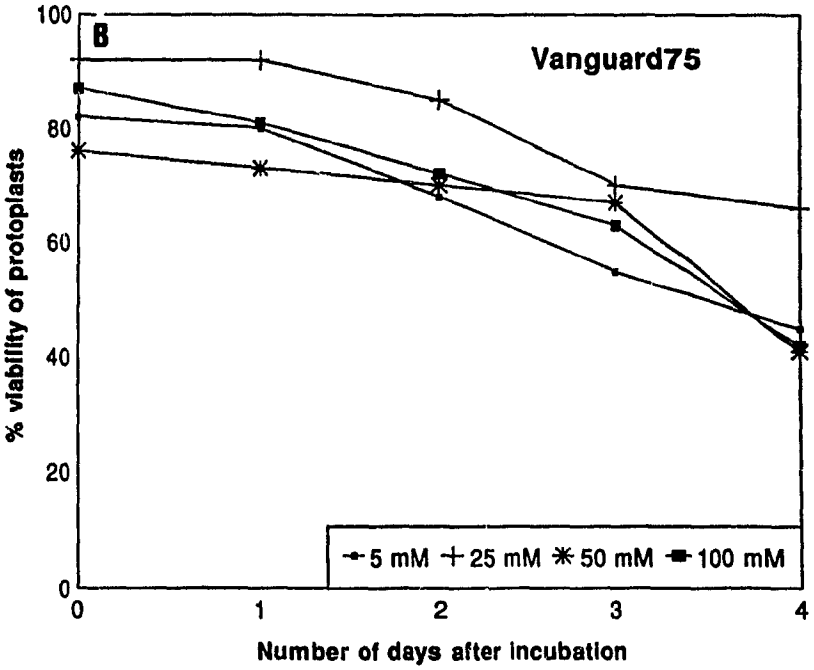

B
Vanguard 75
5 mM
25 mM
50 mM
100 mM
pH
Number of days of incubation

Figure 5. Effect of adding different MES concentrations (5 mM, 25 mM, 50 mM and 100 mM) in the culture medium on the viability of the lettuce protoplasts incubated in Kp culture medium. (A) cv. Vanguard (B) cv. Vanguard 75. One sample of protoplasts from petri dish was drawn at each sampling.


A
Vanguard
% viability of protoplasts
100
80
60
40
20
0
0
1
2
3
4
5 mM
25 mM
50 mM
100 mM
Number of days of incubation


B
Vanguard75
% viability of protoplasts
100
80
60
40
20
0
0
1
2
3
4
5 mM
25 mM
50 mM
100 mM
Number of days after incubation

further experiments.

To summarize the outcome of the above mentioned optimization, pH of the culture medium was maintained at 5.6 by adding 20 mM MES. The bacterial contamination was controlled using cefotaxime (200 $\mu$g/ml culture medium). Cefotaxime was mixed in bleach solution used for surface sterilization of leaves before protoplast isolation and also in the enzyme solution used for protoplast isolation. The fungus contamination was controlled by aseptic conditions during protoplast isolation, purification and inoculation with virus.

## 2. Comparison of virus multiplication in resistant and susceptible lettuce lines

### 2.1 Virus multiplication in protoplasts

A technique of infecting tobacco mesophyll protoplasts with a plant virus was developed for the first time by Otsuki and Takebe (1969) and has been widely used to study virus multiplication in plants since then. To study disease resistance mechanisms, the protoplast system was used to facilitate viral infection due to absence of a cell wall, which acts as a mechanical barrier to virus entry into the plant cell. The protoplast experiment was designed to determine whether resistance to virus in lettuce is offered within the cell or at the intercellular level.

The hypothesis was that if protoplasts of the resistant line show no increase in virus content and the protoplasts from the susceptible line show virus accumulation with time, it may be concluded that the host plant offers resistance within the cell. The resistance could be at the level of inhibition of either virus genome replication or

translation/post-translational modification of virus polyprotein. On the other hand, if the protoplasts from both resistant and susceptible lines show an increase in virus content, this would imply that the resistance is not offered within the cell but rather it is offered at the level of either cell to cell movement of virus through the plasmodesmata or long range movement of virus through the vascular tissue.

At present, resistance to only four potyviruses (SMV, TVMV, PVY and MDMV) has been studied *in vivo* (see literature review, section 5). The work presented in this thesis involves one crop *Lactuca sativa* and two infecting viruses, TuMV and LMV. This provided an opportunity to compare the resistance mechanisms involved in two different types of resistance in lettuce.

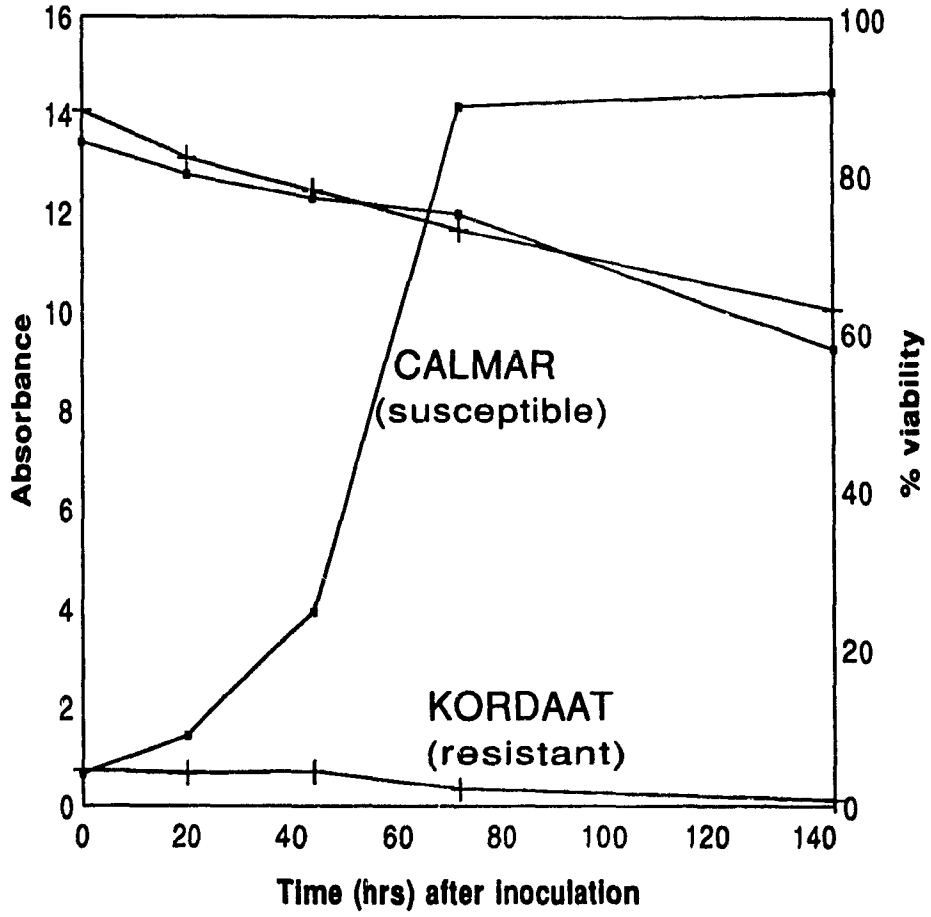
### 2.1.1 Resistance to TuMV

To understand the mechanism of resistance to TuMV, protoplasts from *L. sativa* cv. Calmar (susceptible) and cv. Kordaat (resistant) were infected with purified TuMV. Ninety percent of the protoplasts were found infected. The percent viability was estimated at every sampling by FDA staining (see materials and methods, section 3.4). The TuMV coat protein was detected using coat protein antibody (Robbins *et al.*, 1994) using ELISA. The absorbance thus obtained was calculated per ml (i. e. per $4x10^5$ protoplasts). The percentage of viable cells were calculated in each sample and absorbance values were expressed as per $10^6$ viable protoplasts (Figure 6). Three replicates were used for each sample.

The protoplasts from resistant cv. Kordaat showed no increase in TuMV content

after inoculation whereas susceptible cv. Calmar supported virus multiplication (Figure 6). After 72 hrs, the coat protein content in cv. Calmar was 15 times more than what it was at the time of inoculation. The standard deviation (s.d.) of absorbance for cv. Kordaat ranged from 0.05099 to 0.1219, and for cv. Calmar from 0.0244 to 0.4735. The statistical analysis of the data showed that the TuMV multiplication in cv. Calmar was significantly higher than that in cv. Kordaat ($Pr > F = 0.0001$). Within the cv. Calmar which supported TuMV multiplication, the virus levels were significantly different from each other in all the observations. Since cv. Kordaat is resistant to TuMV, its ability to prevent TuMV multiplication in protoplasts suggests that resistance here is offered within the cell and not at the level of inhibition of cell to cell movement of virus. Within the cell, resistance can be offered at the level of inhibition of either virus genome replication or translation/post-translation processing of viral polyprotein. An inhibitor of virus replication (IVR) was recovered from intercellular fluids of resistant tobacco leaves infected with TMV (Spiegel *et al.*, 1989). TMV does not belong to the potyvirus group. The first report suggesting an inhibitor of virus replication of a potyvirus (PVY) came from screening of resistant potato cultivars. Resistance to PVY in potato is conferred by a single resistance gene *Ry*. Protoplasts from two of the resistant cultivars showed no accumulation of PVY after inoculation (Barker and Harrison, 1984). This suggested a mechanism of resistance to PVY operating within the cell. Potyviruses are known to express their genome through the translation into a polyprotein which is proteolytically processed by three viral proteases (Carrington *et al.*, 1989b; Verchot *et al.*, 1991; Ghabriel *et al.*, 1990). The inhibition of viral polyprotein

Figure 6. TuMV multiplication in protoplasts. The protoplasts from lettuce cvs. Calmar (susceptible) and Kordaat (resistant) were inoculated with purified TuMV in the presence of poly-L-ornithine. The absorbance was calculated per million viable protoplasts. The culture medium contained 20 mM MES and 200 $\mu$g/ml cefotaxime. (Pr > F, 0.0001).


16
14
12
10
8
6
4
2
0
Absorbance
100
80
60
40
20
0
% viability
CALMAR
(susceptible)
KORDAAT
(resistant)
0
20
40
60
80
100
120
140
Time (hrs) after inoculation

processing as a resistance mechanism was first reported in cowpea (against cowpea mosaic virus, CPMV) by Bruening *et al.* (1987). CPMV belongs to the comovirus group which is related to the potyvirus group. Cultured human cells have also been shown to inhibit polyprotein processing of viruses (Korant *et al.*, 1985), suggesting this mechanism to be a general phenomenon in restricting viral infections. The phenomenon of potyviral polyprotein processing has been studied in detail using *in vitro* transcription and translation systems (Garcia *et al.*, 1992; Riechmann *et al.*, 1992). But sufficient literature is not available reporting the inhibition of polyprotein processing, *in vivo*, as mechanism of disease resistance to potyviruses.

As mentioned before, in lettuce, resistance to TuMV is conferred by a single dominant resistance gene. According to Fraser's positive model of virus disease resistance, resistance is dominant where the resistant plant produces a factor inhibitory for virus replication (Fraser, 1987). In such cases, resistance seems to be constitutive. The results from protoplast experiment (Figure 6) appear to fit Fraser's positive model of resistance and it also rules out the possibility that cell to cell movement is involved.

### 2.1.2 Resistance to LMV

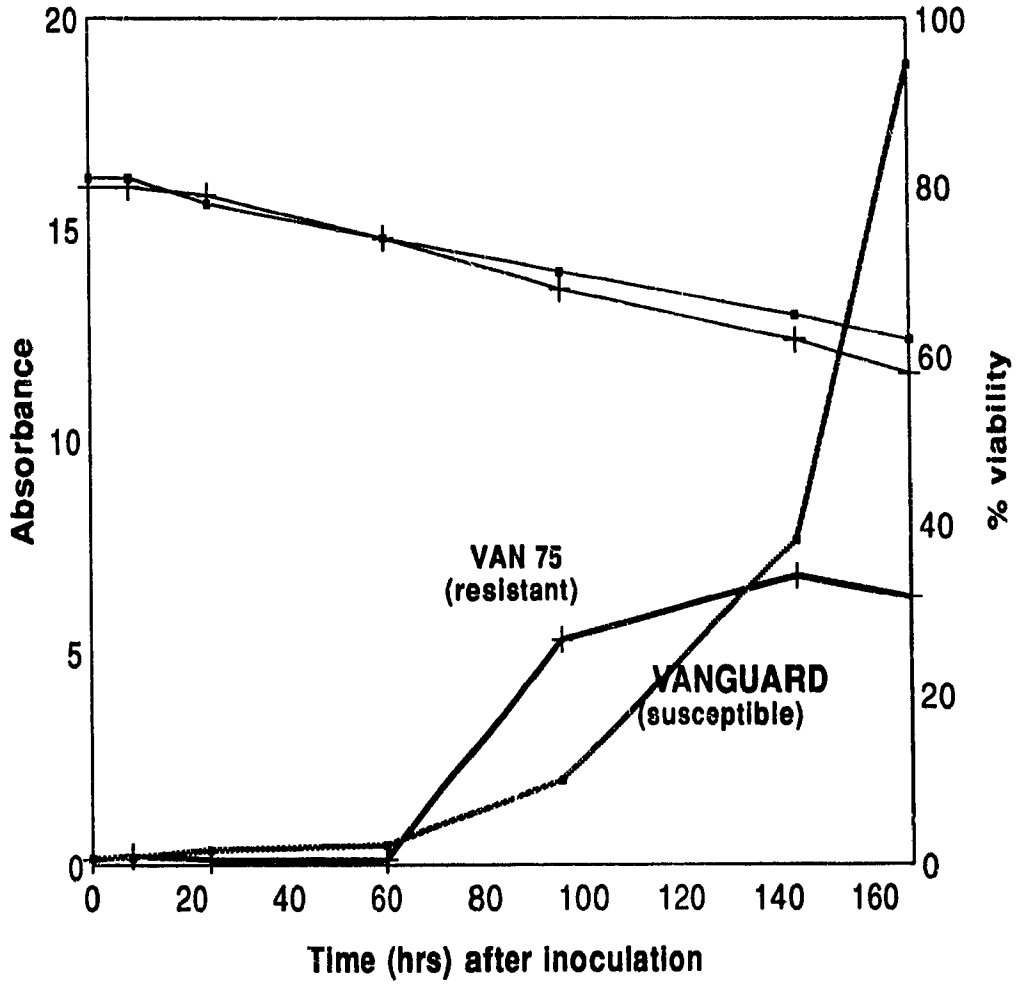
LMV was the second potyvirus used to understand the mechanism of disease resistance in lettuce. The protoplasts from lettuce cv. Vanguard (susceptible) and cv. Vanguard 75 (resistant) were isolated and inoculated as described in the protocol in section 2.1.1. ELISA results from LMV inoculation of protoplasts from resistant and susceptible lettuce lines are shown in Figure 7. Each point represents mean of triplicate

values. The graph shows a lag phase of 60 hrs in resistant cv Vanguard 75. The values at 0, 8, 24 and 60 hrs are not significantly different in two cultivars. LMV started accumulating in susceptible cv Vanguard from 0 hr but did not show a significant increase up to 60 hrs after inoculation. After 60 hrs of inoculation, protoplasts from both cultivars accumulate virus though to a lesser extent in the resistant cultivar. The statistical analysis showed that virus content in cv. Vanguard 75 was significantly lower than that in susceptible cv. Vanguard after 168 hrs of inoculation. This suggests that resistance to LMV is partly offered within the cell. But the possibility that no resistance is offered within the cell cannot be ruled out because only one time point was quantified at 168 hrs and the apparent plateau may thus be an artefactual (Figure 7). Since LMV did multiply in both resistant and susceptible protoplasts, resistance could be offered at the level of inhibition of either cell to cell movement of virus through plasmodesmata or long range movement through vascular tissue. This type of resistance mechanism has been reported in tobacco and maize against potyvirus infections. In tobacco, protoplasts from both resistant and susceptible cultivars supported TVMV (a potyvirus) multiplication although to a lesser extent in resistant protoplasts (Gibb *et al.*, 1989).

## 2.2 Virus multiplication in leaf

To complement the findings from the protoplasts experiments, virus multiplication was examined *in vivo* in leaf tissue. For this, seedlings at the 3-leaf stage were inoculated with crude extracts from virus infected leaves. Leaves were removed in triplicates up to 6 days after inoculation and washed with water after harvesting to

Figure 7. LMV multiplication in protoplasts. The protoplasts from lettuce cvs. Vanguard (susceptible) and Vanguard 75 (resistant) were inoculated with partially purified LMV in the presence of poly-L-ornithine. The absorbance was calculated per million viable protoplasts. The culture medium contained 20 mM MES and 200 $\mu$g/ml cefotaxime. The samples were drawn in triplicates.


20
15
10
5
0
Absorbance
100
80
60
40
20
0
% viability
VAN 75
(resistant)
VANGUARD
(susceptible)
0
20
40
60
80
100
120
140
160
Time (hrs) after inoculation

remove residual antigen on the leaf surface. Each leaf sample was extracted in extraction buffer. In order to standardize the amount of leaf extract to be used for ELISA, different volumes (ranging from 0.1 $\mu$l to 20 $\mu$l) of crude extract of leaves of TuMV-infected *B. perviridis* were tested for virus antigen using ELISA (Figure 8). It was found that 5 $\mu$l of extract gave maximum absorbance reading indicating saturation of the wells of the microtitre plate with TuMV antigen. This data was from using highly infected leaf material for ELISA. But the actual experiment designed to study virus multiplication involved leaf samples inoculated just once and leaves were removed after 0, 1, 2, 4 and 6 days. In order to make sure that virus antigen would be detected in early stages of its multiplication, 20 $\mu$l volume of the leaf extract was used for ELISA instead of 5 $\mu$l.

Figure 9a shows TuMV multiplication in leaves of resistant and susceptible lettuce cultivars. The virus content in cv. Calmar was significantly higher than that in cv. Kordaat ($Pr > F = 0.0001$). The results are similar to those of protoplast experiment in a sense that resistant cv. Kordaat did not support TuMV multiplication. The absorbance values are low in leaf experiment perhaps because of the low number of the initially inoculated cells in leaves compared to 90 % infected cells in protoplast experiment. This logic needs to be confirmed though. As shown in Figure 9a, the TuMV antigen content in resistant cv. Kordaat did not show any accumulation over time and rather decreased and was not detected after four days of inoculation. This may be due to degradation of the inoculated antigen on the plant cell. The results from both leaf and protoplast experiments confirm that resistance appears to be constitutive against TuMV and that resistance is offered within the cell. Cultivar Kordaat (resistant to

TuMV) showed no signs of virus in uninoculated leaf, again confirming that virus is not able to multiply or move to different leaves.

In a similar experiment LMV multiplication was examined in cv. Vanguard (susceptible) and Vanguard 75 (resistant) (Figure 9b). Both cultivars supported LMV multiplication. The statistical analysis showed that the LMV content in two cultivars was not significantly different ($Pr > F = 1.0000$). The data in Figure 9b suggests similarity with the results of protoplast experiment (Figure 7) in a way that protoplasts from both resistant and susceptible cultivars supported virus multiplication. Lettuce cultivar Vanguard 75 (resistant) showed presence of LMV in uninoculated leaf after 5 days of inoculation. This implied that there is no inhibition of long range movement of LMV. Gibb *et al.* (1989) reported that TVMV (a potyvirus) spread within the inoculated leaf in resistant tobacco line but not to the uninoculated leaf, suggesting that virus moved from cell-to-cell through plasmodesmata but its long-range movement to other leaves through vascular tissue was inhibited. In another plant-potyvirus interaction examined, MDMV replicated locally in resistant maize and didn't spread even within the inoculated leaf suggesting a resistance mechanism operating at the intercellular movement of the virus (McMullen and Louie, 1989).

In order to examine the movement of virus from the site of inoculation to the rest of the leaf, whole leaf blot assay was used to detect viral coat protein on leaf blots (Polston *et al.*, 1991). Half of the leaf was inoculated and leaves were removed after definite time intervals and washed with water to remove excess antigen on leaf surface. The proteins from the whole leaf were transferred onto a nitrocellulose membrane using

Figure 8. The effect of dilution of leaf extract on O.D. The extract from TuMV-infected leaves of *B. perviridis* was diluted and TuMV CP was detected by using ELISA. The absorbance was calculated as O.D. per gm fresh leaf weight.

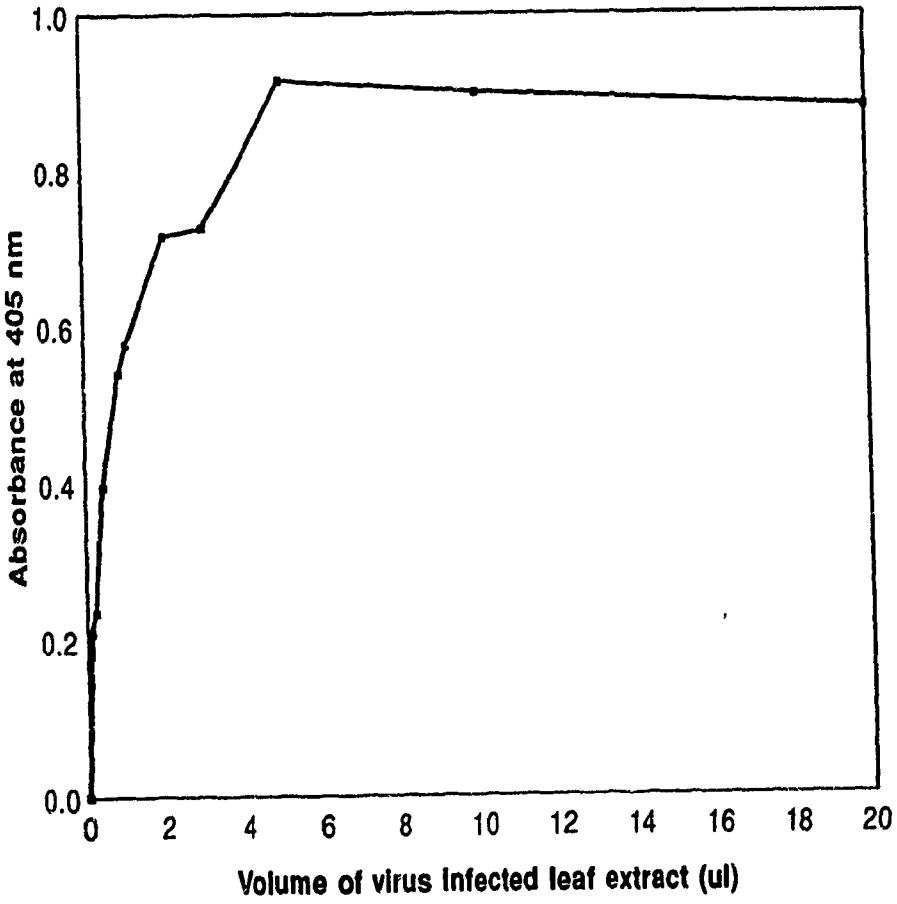
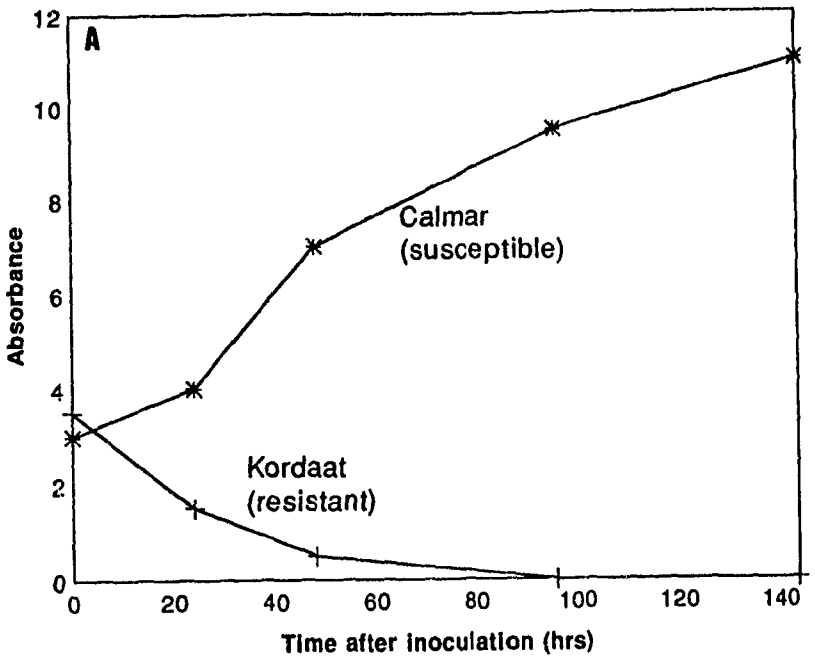
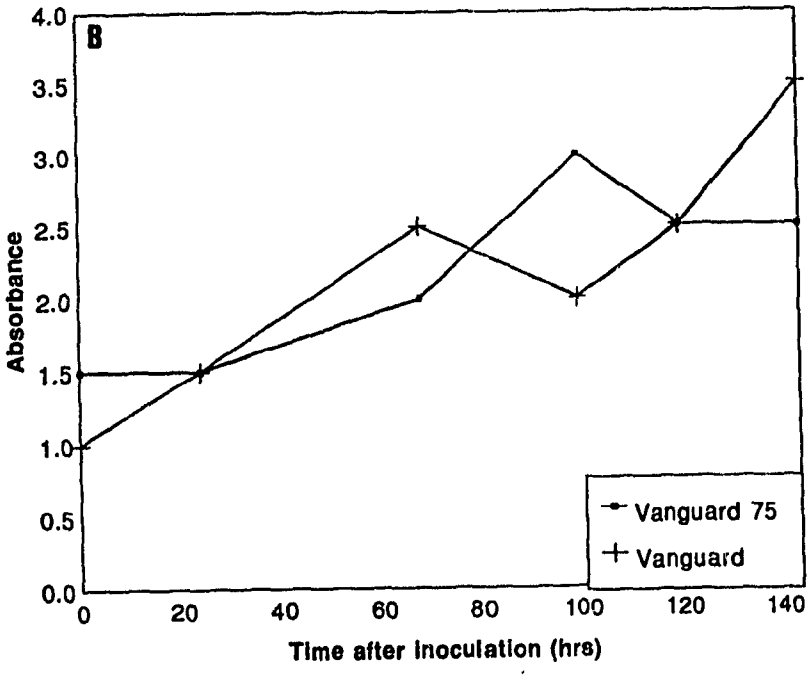

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 405 nm
0
2
4
6
8
10
12
14
16
18
20
Volume of virus infected leaf extract (ul)

Figure 9. Virus multiplication in lettuce leaves. A. TuMV multiplication in resistant (cv. Kordaat) and susceptible (cv. Calmar) lettuce leaves. The absorbance was calculated per gm fresh leaf weight. B. LMV multiplication in resistant (cv. Vanguard 75) and susceptible (cv. Vanguard) lettuce leaves. Triplicates were used for each sampling.


A
Absorbance
Calmar
(susceptible)
Kordaat
(resistant)
Time after inoculation (hrs)
0
2
4
6
8
10
12
0
20
40
60
80
100
120
140


B
Absorbance
Vanguard 75
Vanguard
Time after inoculation (hrs)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
20
40
60
80
100
120
140

a hydraulic press. The proteins transferred onto the membrane were detected using India ink staining. The presence of virus coat protein on whole leaf blots was tested serologically. The leaf part which was inoculated showed a background reaction with the anti-TuMV serum because of viral antigen present. No virus antigen was detected on the uninoculated part of the leaf. Originally, Polston *et al.* (1991) developed this technique to detect virus in different parts of a highly infected leaf. In such a leaf there would be enough viral antigen which can be easily detected serologically after transfer from leaf on to the membrane. This technique was not sensitive enough to detect viral antigen in the early stages of virus multiplication. Only highly TuMV-infected *B. perviridis* leaf blot was able to show TuMV antigen when tested serologically.

### 3. Viral induction of the hypersensitive response (HR)

So far none of the defence related proteins has been shown to be the product of a virus disease resistance gene in plants. The protoplast and leaf experiments were done on lettuce cultivars to answer the question that whether resistance to viral infections is offered intra- or intercellularly. Resistance against TuMV infection appears to be constitutive (as discussed above). Results involving the lettuce-LMV interaction indicated that resistant cv. Vanguard 75 allows systemic spread of LMV. Some resistance operated in protoplasts as indicated by lower levels of virus accumulation as compared to susceptible cultivar. To determine whether HR was involved in any of these two plant-virus interactions and whether HR contributed to resistance or not, microscopic

examination of leaves was carried out. For this purpose, four week old leaves of all the four cultivars were inoculated with their respective viruses and examined everyday under microscope. Mock-inoculated (buffer and carborundum only) leaves served as controls.

No hypersensitive response was observed in cv. Kordaat resistant to TuMV. This offers an interesting observation as a common way of action of dominant resistance gene involves localization of virus forming a necrotic lesion (Fraser, 1986). No HR has been previously reported in cv. Kordaat against TuMV infection although a few other reports of resistance conferred by a single dominant gene are available where no visible lesions (HR) were detected in response to virus infection. e. g. *Tm*-2 in tomato and resistance to PVX and PVY in potato (Jones, 1982). It can be suggested that resistance to TuMV is constitutive in nature and the *Tu* resistance gene product is present in sufficient amounts to prevent virus multiplication. This has been deduced from the results of protoplast and leaf experiments (Figures 6 and 9a). However, these experiments do not indicate whether the resistance gene product is acting as an inhibitor of viral genome replication or a protease inhibitor of viral polyprotein processing (as reported by Bruening *et al.*, 1987).

In cases where resistance is controlled by a recessive gene at a single locus, the resistance gene may cause complete suppression of virus multiplication described as immunity (Provvidenti and Schroeder, 1973) but is apparently not very common in nature (Fraser, 1986). In other cases examined, the recessive resistance gene only reduces symptom severity and virus multiplication can be detected in resistant plants showing tolerance (Catherall *et al.*, 1970; Fraser and Loughlin, 1980). In these instances, virus

spreads systemically. Recessive or incompletely dominant genes generally do not operate by localizing virus in necrotic lesions (Fraser, 1986).

It was interesting to observe that LMV-inoculated leaves of cv. Vanguard 75 (resistant to LMV) showed very minute brown spots after 3 days of inoculation. When examined under microscope, this spot (Figure 10) was in fact a cluster of dead cells surrounding an infected hair which was also dead. The hair cell is probably the first cell which would be infected if inoculated mechanically due to its exposure to inoculum. After 6-7 days, the brown spot was surrounded by a multi-layered ring of cells fluorescing under normal light but was more distinct under UV light (Figure 11 and 12a). No such HR was observed in mock-inoculated leaves or leaves of susceptible cv. Vanguard (Figure 12b). In a typical HR virus is not allowed to spread. But the results from protoplasts and leaf experiments showed that LMV spreads systemically. Presence of HR in this case was contrary to the mode of action of recessive gene which allows systemic infection with no HR (Fraser, 1986). This exceptional type of resistance mechanism has also been reported in *Phaseolus vulgaris* cv. Scotia where recessive gene *tm* causes local lesions after inoculation with TMV (Thompson *et al.*, 1962). The resistance to LMV in lettuce appears to be, at least partly, due to induction of defence proteins. This can be confirmed by examining the activity of different defence enzymes like chitinase and glucanase. The data is insufficient to conclude anything about resistance offered at the level of inhibition of cell to cell movement or long range movement.

It is further suggested that there is a need to examine the mechanism of virus

Figure 10. Photomicrograph of LMV inoculated leaf of lettuce cv. Vanguard 75 viewed with bright light illumination. The arrows indicate the necrotic spot showing dead cells after 3 days of inoculation. (2000 x magnification).

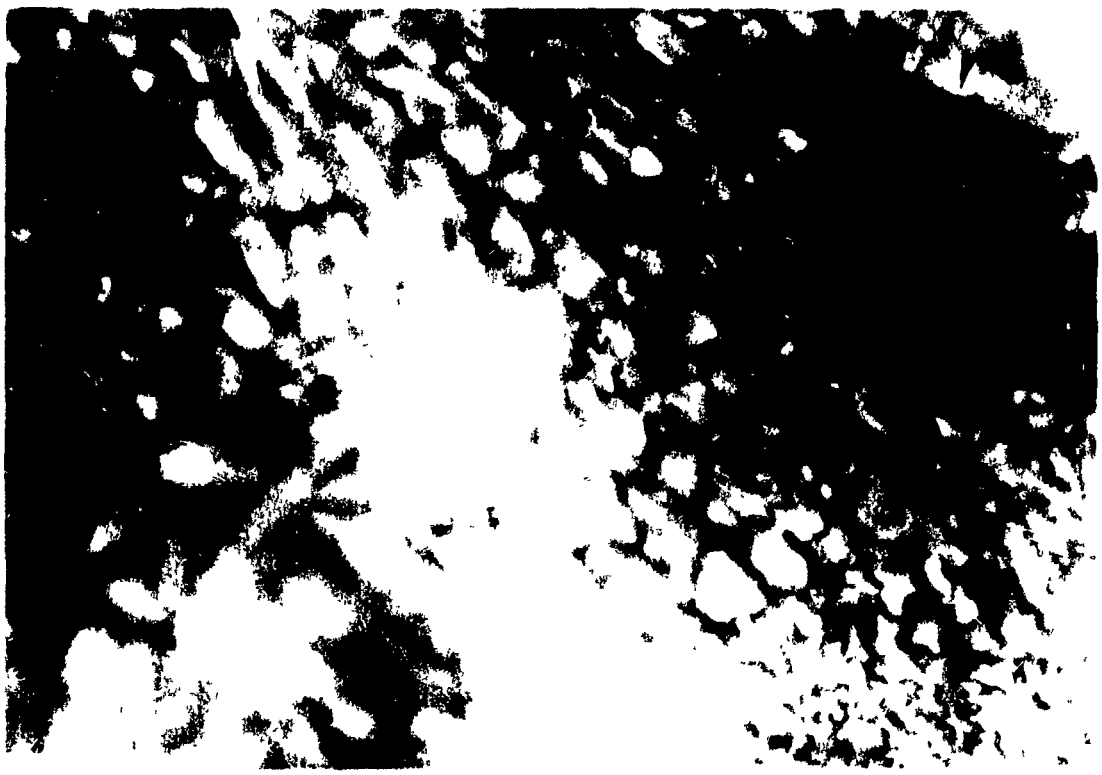
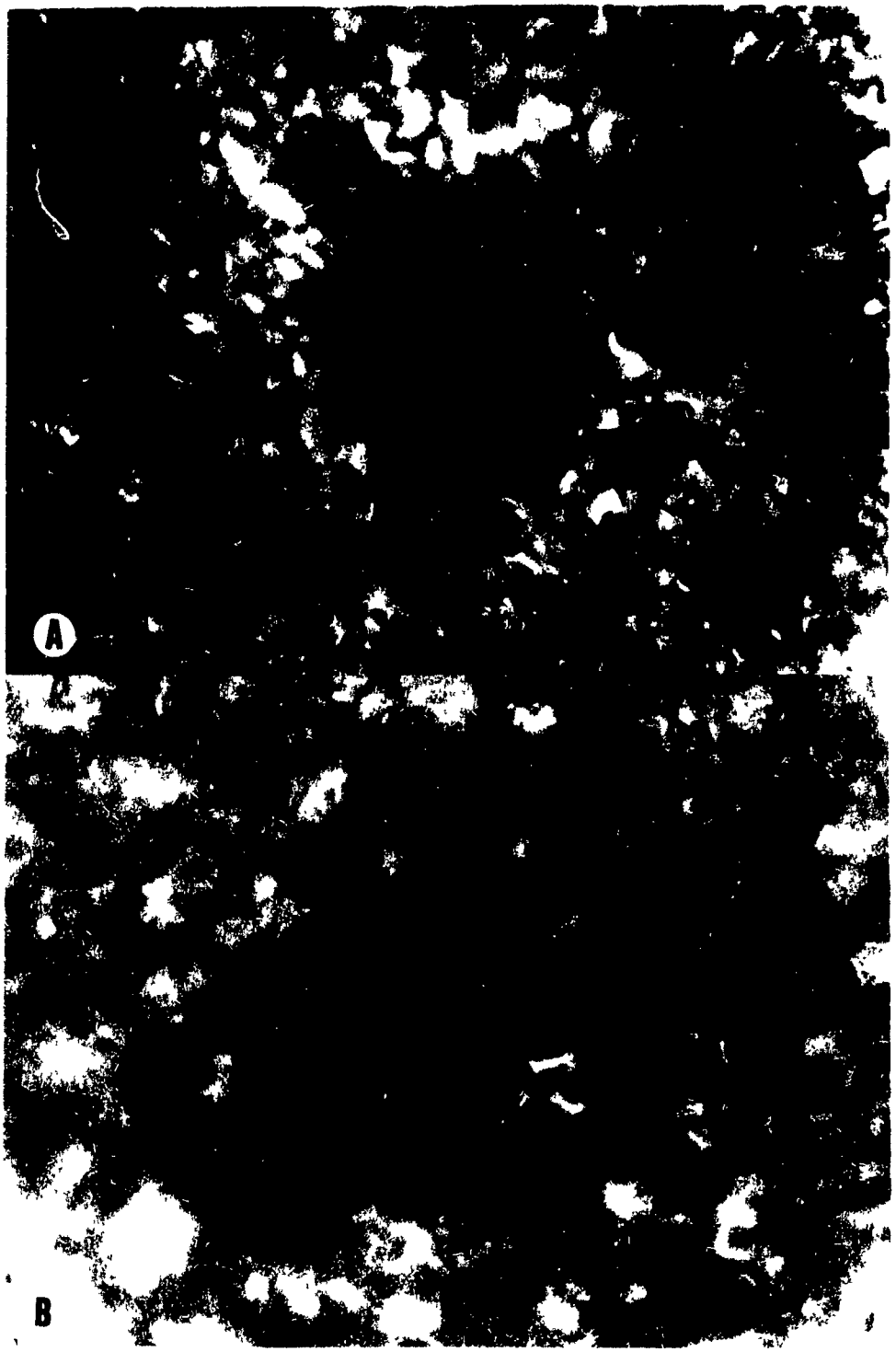


Figure 11. Photomicrograph of LMV inoculated leaf (after 6 days) of lettuce cv. Vanguard 75 viewed with uv light illumination. The arrows point to fluorescing cells forming a ring around the dead cells. (2800 x magnification).

Figure 12. Photomicrograph of LMV inoculated leaves of lettuce. A. cv. Vanguard 75 viewed with uv light illumination. The arrows point to fluorescing cells forming a ring around the dead cells. B. cv. Vanguard showing no fluorescent surrounding cells around dead cells. (2800 x magnification).


A
B

disease resistance at the level of viral RNA multiplication and inhibition of viral polyprotein processing by protease inhibitor (a putative resistance gene product in case of resistance to TuMV) (Bruening *et al.*, 1987). This will help further in establishing the mechanism of disease resistance at molecular level.

**4. Map-based cloning of resistance gene *Tu* : Screening of $F_3$ population for resistant and susceptible phenotype for use in bulk-segregant analysis.**

The knowledge of resistance gene and its product is necessary to understand the phenomenon of disease resistance and for its subsequent use in crop protection crop protection. Plant viral resistance genes are single dominant or recessive and determine the resistance phenotype (Fraser, 1987) but there is still need to confirm this hypothesis by cloning the resistance gene, identifying its product and determining its involvement in the mechanism of virus disease resistance.

Map-based cloning is a promising approach for the isolation of a resistance gene. Using this approach the human disease cystic fibrosis gene (Rommens *et al.*, 1989) and a bacterial resistance gene in tomato (Martin *et al.*, 1993) have been cloned. So far no virus disease resistance gene has been cloned (Wilson, 1993).

The genetic map of lettuce was constructed using RFLP and RAPD markers (*Kesseli et al.*, 1991). *Tu* was located on the genetic map of *L. sativa* and molecular markers linked to *Tu* gene were identified by bulk-segregant analysis using DNA bulks from $F_2$ plants homozygous resistant (bulk A) or susceptible (bulk B) at *Dm*5/8 locus (Robbins, M.Sc. thesis, 1993). These DNA pools were made on the basis of the *Dm*5/8

phenotype, not *Tu*. DNA bulks were used to find markers around *Tu* locus since the two loci are linked. Though two markers *OPM18/OPLO8* (at 0.4 cM from *Tu*) and *UBC346* (at 0.7 cM) were found closely linked to the *Tu* gene, it is possible that the physical distance between them may be too large for chromosome walking. In that case, new markers closer to the *Tu* gene will be required. Therefore, production of bulks for the *Tu* locus was necessary for further screening for molecular markers linked to *Tu*. To produce bulks around the *Tu* locus, $F_3$ families from $F_2$ individuals of a cross between Calmar and Kordaat (TuMV-resistant), segregating for the *Tu* locus, was screened for resistance to TuMV-infection using ELISA. Eighteen $F_3$ plants from each of 71 $F_2$ individuals were inoculated with TuMV-infected *B. perviridis* (Robbins, M.Sc. thesis, 1993) and tested for TuMV infection twice at 3 and 6 weeks of inoculation. Eleven plants were tested for TuMV infection for the first screening at 3 weeks to reduce the amount of work involved. Also 11 individuals were enough to give 95 % confidence in genotyping. The ratio of resistant and susceptible plants from each $F_2$ individual was determined. Based on this ratio, the genotype of the $F_2$ individual was determined. This exercise was done for each of the 71 $F_2$ individuals. The $F_2$ individuals with all resistant (negative ELISA value) and no susceptible $F_3$ plants were classified as homozygous resistant (*TuTu*), and with all susceptible (positive ELISA value) and no resistant $F_3$ plants were classified as homozygous recessive (*tutu*). Others were designated as heterozygous (*Tutu*). Thirty-one families were found homozygous resistant after 3 weeks of inoculation (Table 1) but 19 of ' families showed TuMV accumulation after 6 weeks of inoculation. The remaining 12 $F_2$ individuals were identified as homozygous resistant

(*TuTu*). One out of every four was expected to be a homozygous resistant. This is based on Mendelian Ratio of 1:2:1 (*TuTu:Tutu:tutu*). Therefore, out of total 71 individuals, 17 or 18 were expected to be homozygous resistant. But only 12 $F_2$ individuals were designated as homozygous resistant (Table 2). Some of the $F_2$ individuals escaped grouping in homozygous resistant bulk because of the death of some $F_3$ plants in the green house. For homozygous recessive genotype, out of total 71 $F_2$, 17 or 18 individuals were expected to be of *tutu* genotype. The recessive genotype was difficult to determine as many of the plants were dead at the time of second screening after 6 weeks of inoculation (Table 2). Ten $F_2$ individuals were chosen where either no resistant plant was detected or the ratio was more in favour of recessive genotype since *Tu* is a dominant allele. The homozygous resistant and susceptible individuals were bulked separately and were subsequently used by A. Joyeux (unpublished) for the preparation of DNA bulks from $F_2$ homozygous resistant (*TuTu*) and susceptible (*tutu*) individuals.

**Table 1. Genotypes of $F_2$ progenitors for *Tu* as determined by using $F_3$ families of a cross between cvs. Calmar and Kordaat. Phenotypic ratios within $F_3$ families were used to determine genotype of the $F_2$ individual. B = homozygous resistant (*TuTu*); A = homozygous susceptible (*tutu*); H = heterozygotes (*Tutu*). The data is based on first screeninig of $F_3$ plants 3 weeks after inoculation.**

| $F_2$ individual | Resistant $F_3$ progeny | Susceptible $F_3$ progeny | Inferred Genotype of $F_2$ individual |
|---|---|---|---|
| 50 | 6 | 5 | H |
| 77 | 11 | 0 | B |
| 78 | 8 | 3 | H |
| 85 | 4 | 6 | H |
| 72 | 4 | 7 | A |
| 84 | 6 | 5 | H |
| 83 | 11 | 0 | B |
| 62 | 11 | 0 | B |
| 63 | 9 | 2 | H |
| 68 | 2 | 9 | A |
| 82 | 11 | 0 | B |
| X | 10 | 1 | H |
| 74 | 11 | 0 | B |
| 03 | 11 | 0 | B |
| 06 | 4 | 7 | A |
| 33 | 11 | 0 | B |
| 34 | 5 | 6 | H |
| 64 | 11 | 0 | B |
| 18 | 11 | 0 | B |
| 19 | 10 | 1 | H |
| 16 | 9 | 2 | H |
| 76 | 11 | 0 | B |
| 60 | 11 | 0 | B |
| 58 | 9 | 2 | H |
| 67 | 8 | 3 | H |
| Z | 3 | 3 | H |
| 24 | 10 | 1 | H |
| 44 | 10 | 1 | H |
| 14 | 11 | 0 | B |
| 42 | 11 | 0 | B |
| 12 | 11 | 0 | B |

(contd. ...)

**Table 1 (....continued from last page) The data is based on first screeninig of $F_3$ plants 3 weeks after inoculation.**

| $F_2$ individual | Resistant $F_3$ progeny | Susceptible $F_3$ progeny | Genotype of $F_2$ individual |
|---|---|---|---|
| 21 | 1 | 9 | A |
| 27 | 11 | 0 | B |
| 29 | 11 | 0 | B |
| 22 | 11 | 0 | B |
| 55 | 11 | 0 | B |
| 01 | 11 | 0 | B |
| 54 | 11 | 0 | B |
| 45 | 11 | 0 | B |
| 57 | 11 | 0 | B |
| 02 | 8 | 3 | H |
| 88 | 8 | 3 | H |
| 52 | 7 | 4 | H |
| 46 | 11 | 0 | B |
| 75 | 11 | 0 | B |
| 81 | 11 | 0 | B |
| 35 | 11 | 0 | B |
| 80 | 7 | 4 | H |
| 65 | 11 | 0 | B |
| 41 | 2 | 9 | A |
| 20 | 5 | 6 | H |
| 51 | 1 | 10 | A |
| 36 | 9 | 2 | H |
| 40 | 11 | 0 | B |
| 87 | 8 | 3 | H |
| 39 | 8 | 3 | H |
| 08 | 11 | 0 | B |
| 09 | 9 | 2 | H |
| 10 | 8 | 3 | H |
| 48 | 5 | 6 | H |
| 25 | 9 | 2 | H |
| 13 | 6 | 5 | H |
| 15 | 11 | 0 | B |
| 11 | 2 | 9 | A |
| 28 | 8 | 3 | H |
| 32 | 9 | 2 | H |
| 31 | 3 | 8 | A |
| 38 | 5 | 6 | H |
| 37 | 3 | 8 | A |
| 23 | 8 | 1 | H |

**Table 2. Genotypes of $F_2$ progenitors for *Tu* as determined by using $F_3$ families of a cross between cvs. Calmar and Kordaat. Phenotypic ratios within $F_3$ families were used to determine genotype of the $F_2$ individuals. B = homozygous resistant (*TuTu*); A = homozygous susceptible (*tutu*); H = heterozygotes (*Tutu*). The data is based on the second screeninig of $F_3$ plants 6 weeks after inoculation.**

| $F_2$ individual | Resistant $F_3$ progeny | Susceptible $F_3$ progeny | Inferred Genotype of $F_2$ individual |
|---|---|---|---|
| 50 | 1 | 8 | A |
| 77 | 11 | 0 | B |
| 78 | -- | -- | -- |
| 85 | 4 | 4 | H |
| 72 | 2 | 7 | A |
| 84 | -- | -- | -- |
| 83 | 15 | 2 | H |
| 62 | 16 | 0 | B |
| 63 | -- | -- | -- |
| 68 | 4 | 5 | H |
| 82 | 12 | 2 | H |
| X | -- | -- | -- |
| 74 | 14 | 0 | B |
| 3 | 2 | 11 | A |
| 6 | 1 | 1 | H |
| 33 | 15 | 0 | B |
| 34 | 1 | 2 | H |
| 64 | 10 | 1 | H |
| 18 | 8 | 2 | H |
| 19 | -- | -- | -- |
| 16 | -- | -- | -- |
| 76 | 11 | 1 | H |
| 60 | 14 | 0 | B |
| 58 | -- | -- | -- |
| 67 | -- | -- | -- |
| Z | 1 | 5 | A |
| 24 | -- | -- | -- |
| 44 | -- | -- | -- |
| 14 | 8 | 2 | H |
| 42 | 12 | 0 | B |
| 12 | 11 | 3 | H |

(contd. ...)

**Table 2. (....continued from last page) The data is based on the second screeninig of $F_3$ plants 6 weeks after inoculation.**

| $F_2$ individual | Resistant $F_3$ progeny | Susceptible $F_3$ progeny | Genotype of $F_2$ individual |
|---|---|---|---|
| 21 | 5 | 1 | H |
| 27 | 18 | 0 | B |
| 29 | 8 | 2 | H |
| 22 | 13 | 0 | B |
| 55 | 13 | 1 | H |
| 01 | 9 | 2 | H |
| 54 | 14 | 2 | H |
| 45 | 12 | 0 | B |
| 57 | 14 | 0 | B |
| 02 | -- | -- | -- |
| 88 | -- | -- | -- |
| 52 | -- | -- | -- |
| 46 | 12 | 4 | H |
| 75 | 13 | 1 | H |
| 81 | 13 | 1 | H |
| 35 | 14 | 0 | B |
| 80 | -- | -- | -- |
| 65 | 14 | 0 | B |
| 41 | -- | -- | -- |
| 20 | 0 | 4 | A |
| 51 | 1 | 1 | H |
| 36 | -- | -- | -- |
| 40 | 14 | 1 | H |
| 87 | -- | -- | -- |
| 39 | -- | -- | -- |
| 08 | 5 | 6 | H |
| 09 | -- | -- | -- |
| 10 | -- | -- | -- |
| 48 | 0 | 2 | A |
| 25 | -- | -- | -- |
| 13 | 2 | 4 | A |
| 15 | 5 | 9 | A |
| 11 | 0 | 2 | A |
| 28 | -- | -- | -- |
| 32 | -- | -- | -- |
| 31 | 4 | 0 | B |
| 38 | 10 | 4 | H |
| 37 | 3 | 8 | A |
| 23 | -- | -- | -- |

## V. Conclusion

Not much is known about the mechanism of virus disease resistance in plants. The aim of this research was to answer whether resistance to viruses is offered within the cell or at the intercellular level.

Conditions were optimized to isolate viable protoplasts (section IV.1) from the leaves of *Lactuca sativa* cultivars for viral infection. Protoplasts (section IV. 2.1) and leaves (section IV. 2.2) from *Lactuca sativa* cultivars were inoculated separately with two different viruses (TuMV and LMV, potyvirus group) and virus multiplication was compared in resistant and susceptible cultivars.

TuMV multiplication (section IV. 2.1.1) was supported neither in leaves nor in protoplasts of resistant lettuce cv. Kordaat suggesting that resistance to TuMV is conferred intracellularly and not at the level of inhibition of cell to cell movement of the virus. A common way of expression of resistance is through the hypersensitive response (HR) where resistance is conferred by a single dominant gene (Fraser, 1986). No HR (section IV. 3) was observed in the case of resistant cv. Kordaat further suggesting that resistance is available within the cell and seems to be constitutive. Potyviruses are known to express their genome through its translation into a polyprotein which is processed by a viral protease. The resistance may be at the level of inhibition of polyprotein processing by a protease inhibitor. The resistance may also be because of the inhibition of viral genome replication. These speculations need to be confirmed at the RNA and protein levels.

In similar experiments with LMV, protoplasts (section IV. 2.1.2) and leaves (section IV 2.2) from both resistant (Vanguard 75) and susceptible (Vanguard) lettuce cultivars supported virus multiplication. Results from the protoplast experiment suggest that though resistance to LMV is offered within the cell but to a lesser extent. Results from the leaf experiments suggest that the resistant cultivar allows systemic infection indicating that resistance to LMV is not offered either at the level of inhibition of cell to cell movement of virus across plasmodesmata or inhibition of long range movement of virus. A single recessive gene *mo* confers resistance to LMV. A HR was observed in resistant cv. Vanguard. It may be speculated that lettuce is tolerant to LMV infection and a HR seems to be at least partly responsible for controlling symptoms.

In order to contribute towards mapping of resistance gene *Tu*, a $F_3$ population from 71 $F_2$ individuals of a cross between Calmar and Kordaat was screened for resistance to TuMV infection. This data was used to determine the genotype of $F_2$ individuals. The homozygous resistant (*TuTu*) and susceptible (*tutu*) $F_2$ families were selected and used for production of bulks around the *Tu* locus. These bulks will be used to find new molecular markers linked to gene *Tu*.

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