This is to certify that the

dissertation entitled

Genetic Engineering of PotyVirus Resistance Using Constructs Derived from the Zucchini Yellow Mosaic Virus Coat Protein Gene

presented by

GUOWEI FANG

has been accepted towards fulfillment
of the requirements for

Doctor degree in Philosophy

Major professor

Date 8-5-92

GENETIC ENGINEERING OF POTYVIRUS RESISTANCE USING CONSTRUCTS DERIVED FROM THE ZUCCHINI YELLOW MOSAIC VIRUS COAT PROTEIN GENE

By

GUOWEI FANG

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Plant Breeding and Genetics Program
and Department of Horticulture

1992

ABSTRACT

GENETIC ENGINEERING OF POTYVIRUS RESISTANCE USING CONSTRUCTS DERIVED FROM THE ZUCCHINI YELLOW MOSAIC VIRUS COAT PROTEIN GENE

By

GUOWEI FANG

Zucchini yellow mosaic virus (ZYMV) is an extremely aggressive potyvirus, it causes major losses in cucurbit crops (cucumbers, squashes and melons), and is capable of out-competing related viruses in establishing infection. This dissertation describes the genetic engineering of resistance to ZYMV and other potyviruses using constructs derived from the ZYMV coat protein gene. The ZYMV coat protein gene was cloned, sequenced, engineered into a form for expression in plants, introduced into muskmelon and tobacco plants, and shown to confer increased resistance to potyvirus infection.

The cDNA sequence of a portion of the putative RNA polymerase gene, the complete coat protein gene and the 3′ untranslated region were determined. The predicted amino acid sequence indicated a ZYMV-unique N-terminal region and potyvirus-characteristic central and C-terminal region. The ZYMV coat protein has 279 amino acids and a calculated Mr. of 31 kd.

In order to produce transgenic plants that express viral capsid protein, an efficient *Agrobacterium tumefaciens* mediated transformation and regeneration system was developed

for melon (*Cucumis melo* L.). The factors affecting transformation efficiency were studied.

Three versions of the ZYMV coat protein gene were engineered for expression in plants: the full length coat protein sequence (CP), the conserved core portion of the gene (Core) and an antisense version (AS). These constructs were introduced into muskmelon and tobacco plants, gene expression was verified by PCR, northern and western analysis. Transgenic R_0 muskmelon plants expressing CP, Core or AS and inoculated with ZYMV showed a lack or delay of systemic symptoms, and little or no virus accumulation as determined by ELISA. Similar resistance was displayed in R_1 transgenic melon plants expressing CP or Core. Furthermore, transgenic R_0 and R_1 tobacco plants expressing the CP, Core or AS constructs of ZYMV, a non-pathogen of tobacco, showed a delay in symptom development and reduced virus titer when inoculated with the heterologous potyviruses, PVY and TEV. The transgenic tobacco plants were not protected against the non-potyvirus, TMV.

ACKNOWLEDGEMENTS

I would like to express sincere appreciation to the members of my guidance committee, Drs. Michael Thomashow, Donald Ramsdell, James F. Hancock, Amy F. Iezzoni, and Rebecca Grumet, for their helpful advice and counsel during the course of my studies here at Michigan State University, and for their efforts in correcting and editing this dissertation. I am especially indebted to Dr. Rebecca Grumet, my major advisor, for her constant academic guidance and technical assistance in the entire program; it would be extremely difficult for me to complete this work without her encouragement and support.

I also wish to thank Ms. Sue Hammer, whose assistance and expert technical support is greatly appreciated.

Special depth of gratitude and love to my wife Hong Wang for her continuous support, understanding and patience; to my parents and Paul, my lovely son, for their help and sacrifices throughout the course of my studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA - abscisic acid;
BAP - 6-benzylaminopurine;
IAA - indole 3-acetic acid;
2,4-D - 2,4-dichlorophenoxyacetic acid;
NPT II - neomycin phosphotransferase II.
CP - coat protein

Potyviruses	
Zucchini yellows mosaic virus	ZYMV
Watermelon mosaic virus 2	WMV-2
Watermelon strain of papaya ringspot virus	PRV-W
Tobacco etch virus	TEV
Tobacco vein mottling virus	TVMV
Potato virus Y	PVY
Plum pox virus	PPV
Johnson grass mosaic virus (formerly Johnson grass JGMV strain of sugar cane mosaic virus)	JGMV
Other viruses	
Tobacco mosaic virus	TMV
Alfalfa mosaic virus	AlMV
Potato virus X	PVX
Brome mosaic virus	BMV
Cowpea mosaic virus	CpMV
Tobacco rattle virus	TRV

LITERATURE REVIEW

INTRODUCTION

The development of molecular biology and plant transformation technologies has made it possible to develop genetically engineered virus resistance in plants. A highly successful strategy has been to use viral coat protein (CP) genes as a source of resistance genes (Beachy et al., 1990; Grumet 1990). My project was to genetically engineer resistance to a group of potyviruses that infect cucurbit plants using viral coat protein genes. The cucurbit family includes some very important vegetable and fruit crops (e.g. melons, cucumbers, squashes), that are subject to serious yield losses due to infection by three members of the potyvirus group, zucchini yellow mosaic virus (ZYMV), the watermelon strain of papaya ringspot virus (PRV-W) and watermelon mosaic virus 2 (WMV-2). WMV-2 and PRV-W have been observed in cucurbits since the 1950's and continue to cause serious outbreaks (Davis and Mizuki, 1987; Nameth et al. 1986). ZYMV, however, is a relatively new and extremely aggressive member of the potyvirus group. To engineer plant virus resistance, two factors are necessary: (1) an efficient transformation and regeneration system for the species of interest and (2) knowledge of the genomic organization of the virus to facilitate cloning and engineering of the CP gene into a form that can be expressed in the target plants. My literature review is therefore focused on plant transformation

technologies, CP-mediated virus protection and potyvirus biology.

A. PLANT TRANSFORMATION

The stable introduction of foreign genes into plants represents one of the most significant developments in a continuum of advances in agricultural technology that includes modern plant breeding, hybrid seed production, farm mechanization, and the use of agrichemicals to provide nutrients and control pests (Gasser and Fraley, 1989). The first transgenic plants expressing engineered foreign genes were tobacco plants produced by use of *Agrobacterium tumefaciens* vectors (Horsch et al. 1984). Transformation was confirmed by the presence of foreign DNA sequences in both primary transformants and their progeny, and by an antibiotic resistance phenotype conferred by a chimeric neomycin phosphotransferase (NPT) gene. The subsequent development of transformation methods has contributed significantly to the facile and routine *Agrobacterium* transformation methods that are used today for many dicotyledonous plant species. In addition to the use of *Agrobacteria* as transformation vectors, a variety of free DNA delivery methods, including microinjection, electroporation, and particle gun technology are being developed for the transformation of monocotyledonous plants such as corn, wheat and rice. In this section, several plant transformation methods are described.

1. *Agrobacterium* mediated transformation

Insertion of foreign DNA into *A. tumefaciens* derived plasmid vectors is currently the most frequently used strategy for generating transgenic plants in a wide variety of species (Potrykus, 1991). *Agrobacterium tumefaciens* is the etiological agent of crown gall disease and produces tumorous crown galls in infected plants (Chilton et al., 1977). The utility of this bacterium as a gene transfer system was first recognized when it was demonstrated that the crown galls were actually produced as a result of the transfer and integration of genes from the bacterial Ti (tumor inducing) - plasmid into the genome of the plant cells (Chilton et al. 1977). Incorporation of the transferred DNA (T-DNA) into the nuclear genome of the plant cell and its resultant expression causes the pathogenic response characteristic of the transformed cell. Since then, a variety of studies have led to the development of Ti based vectors for use in efficient and widely used strategies to generate transgenic plants. One of the most important findings facilitating the practical utilization of the *Agrobacterium*-Ti system was that disarmed T-DNA, that is T-DNA lacking functional oncogenes, can be transferred and integrated into the plant genome, allowing for the regeneration of transgenic plants (Barton et al. 1983). In fact, any DNA fragment (size limitation not known) can be transferred from a Ti plasmid as long as it is between the 25 bp direct repeats, called borders, that flank the wild type Ti-plasmid T-DNA (Slightom

et al. 1986, Binns and Thomashow 1988). The T-DNA insertion appears to be stable. It has been characterized genetically for a number of generations and behaves as a normal Mendelian trait (Wallroth et al. 1986). The virulence (vir) genes of the Ti plasmid are also required for T-DNA transfer, which can function *in trans* to process and transfer T-DNA present on a separate plasmid (Stachel and Nester 1986;, Hoekema et al. 1983). This led to the development of small, easy to handle, binary vectors that can replicate in both *E. coli* and *A. tumefaciens*. These binary vectors carry plant selectable marker(s) (eg. NPT gene), plant scorable marker(s), and various cloning sites, all of which are flanked by the border repeats (Binns 1990). The major advantage of binary vectors is their lack of dependence on a specific Ti plasmid. The vector may be introduced into virtually any *Agrobacterium* host containing any Ti plasmid as long as the vir helper functions are provided.

There are two basic approaches which have been used to obtain transgenic plants: co-cultivation of regenerating protoplasts (Horsch et al. 1984), and the leaf disc procedure (Horsch et al. 1985). Co-cultivation of regenerating protoplasts was the first procedure used successfully to generate a transgenic plant. The procedure involves *Agrobacterium* co-cultivation of protoplasts, followed by selection and regeneration to plants. Because it requires a good regeneration protocol for protoplasts, this procedure is

not useful for many important plant species. In the leaf disc procedure, surface-sterilized leaf pieces, or other axenic explant, are co-cultured on regeneration medium for 2-3 days with *Agrobacterium*. The best choice of explant is usually one that regenerates well in tissue culture for the species of interest, such as stem or petiole segments, shoot, leaf or cotyledon pieces, tuber disks, root pieces or seed (Hooykaas 1989). During co-cultivation, bacteria bind to plant cells around the wounded explant, and T-DNA transfer occurs. A nurse culture of tobacco cells is sometimes used to increase transformation frequency. Following co-cultivation, the explant are transferred to regeneration/selection medium. This medium contains antibiotics to kill the *Agrobacterium* and to select for transformed plant cells. After shoots regenerate, they are excised, rooted on an appropriate medium in presence of the selective agent, and transferred to soil. The leaf disc-based procedures are a great improvement overprotoplasts co-cultivation, because it is not necessary to produce and regenerate protoplast.

Although *Agrobacterium* species are capable of transferring new genes to a wide variety of plant species, their practical use is mainly limited to natural hosts, mostly dicotyledons, even though transgenic plants have been produced in asparagus with *Agrobacterium* vectors (Bytebier et al. 1987). Important cereal grains such as rice, corn, and wheat have not been successfully transformed by *Agrobacterium*. It has been

suggested that monocots are difficult to transform because they do not have the proper wound response (Potrykus 1991). Extensive efforts have consequently been directed toward the development of systems for the delivery of free DNA into these species.

2.protoplasts and direct gene transfer

The systems giving demonstrable transformation of plant cells relied on physical means similar to those used in the transformation of cultured animal cells. Transformation has been achieved in plantprotoplasts through facilitation of DNA uptake by calcium phosphate precipitation, polyethylene glycol (PEG) treatment, electroporation, or combinations of these treatments (Potrykus et al. 1985; Fromm et al. 1986; Uchimiya et al. 1986). Integrative transformation can be very efficient and lead to stable inheritance of predominantly single gene loci of the foreign genes (Negrutiu et al. 1987), but it often leads to multiple integrations (Potrykus, 1991). Transgenic monocots such as rice (Shimamoto et al. 1989), maize (Khodes et al. 1989), and other cereals (Lorz et al, 1985)have been produced by direct gene transfer in several laboratories. Virtually everyprotoplasts system has proven transformable, though with different efficiencies (Potrykus 1991). It would be the ideal experimental system for gene transfer if there were not problems with plant regeneration from protoplasts. Although there has been exciting progress recently in plant regeneration from protoplasts (Roest and Gilissen 1989), and

although it can be foreseen that this progress will continue, this process will probably always be a delicate one (Potrykus 1991).

3. Biolistics or particle gun

The biolistic process is another direct gene transfer method, the process can be defined as the introduction of substances into intact cells and tissues through the use of high-velocity microprojectiles (Sanford 1988,1990). It is primarily a mechanism for breaching cell walls and cell membranes, which are the principal barriers to DNA delivery. By the use of a finely tunable discharge apparatus (eg.particle gun), DNA coated metal particles such as tungsten or gold are used to transform plant tissue (Christou et al. 1988, 1990).

One of the most important features of the biolistic process is that it is of broad utility, and may prove to be something of a "universal" gene delivery mechanism. It has been demonstrated in a very diverse range of plants as well as microbes and animals (for review, see Sanford 1990). There is no apparent difference in the efficiency of biolistic transformation of monocots vs dicots. It is also effective regardless of tissue and cell type, and is a rapid and simple procedure. In addition, the biolistic process appears to be uniquely suitable for organelle transformation. Transgenic plants have been recovered from species that have been difficult to be transformed by other methods, such as maize

and soybean (Fromn et al. 1990, NcCabe et al. 1988). Some expected that this technique would solve all gene transfer problems, however, limitations do remain. When bombarding totipotent tissues, the process often gives rise to chimeric plants, which need to be sorted out and stabilized; and the technique is less efficient than other transformation methods in yielding stable integrative events.

4. Microinjection

Microinjection uses microcapillaries and microscopic devices to deliver DNA into defined cells in such a way that the injected cell survives and can proliferate. This technique has produced transgenic clones from protoplasts (Miki et al. 1987) and from microspore-derived pro-embryos (Neuhaus and Spangenberg 1990). Like biolistics, microinjection delivers DNA into walled plant cells, and is host range independent. The differences from other direct DNA delivery methods are: DNA delivery is precise and predictable, is under visual control, and the quantity of DNA delivered can be optimized. Subsequent individual culture of the microinjected cells in microculture systems offers the possibility of avoiding selection and thus the requirement for selectable marker genes. The limitations of the method are: only one cell receives DNA per injection, handling requires more skill and instrumentation, and it is time consuming.

All of the above four approaches have led to the production of transgenic plants (*Agrobacterium*-mediated gene

transfer, direct gene transfer to protoplasts, biolistics and microinjection); of them *Agrobacterium* mediated gene transfer, direct gene transfer toprotoplasts and the use of biolistics are routine and efficient methods. Other approaches that have potential for integrative transformation are: agroinfection (Grimsley 1990), viral vectors (Ahlquist and Pacha 1990), macroinjection (DelaPena et al. 1987), electrophoresis (Alwen et al. 1990), liposome fusion (Caboche 1990), and the use of microlasers (Weber et al. 1990). Most of these require further optimization and improvement, and stability of the integrative transformation events needs to be proven.

Muskmelon (*Cucumis melo* L.) is a high value and important crop throughout the world. It is subject to severe losses by several viruses (Nameth et al.1985), and so the genetic engineering of virus resistance is a primary goal for this crop (e.g. Grumet and Fang, 1990; Gonsalves et al.1991). As described above, *Agrobacterium tumefaciens* has been the most commonly used vector for the transfer of foreign genes into dicotyledonous plants. Since muskmelon is a suitable host for *A. tumefaciens*, and efficient regeneration from tissue culture has been achieved in several laboratories (eg. Niedz et al. 1989; Kathal et al.1988), one project of my research was to develop a crop specific protocol for *Agrobacterium* mediated transformation in muskmelon.

B. COAT PROTEIN MEDIATED PROTECTION AGAINST VIRUS INFECTION

Dramatic progress in the development of plant transformation and regeneration systems as a mechanism for the expression of novel genes, has allowed for the genetic engineering of plants for crop improvement. One of the most promising applications has been the development of genetically engineered resistance to plant viruses. Virus resistance was first shown for tobacco mosaic virus (TMV) in tobacco plants (Powell et al. 1986); it was found that transgenic tobacco plants expressing the coat protein gene from TMV showed a significant delay in symptom development after inoculation with TMV. Coat protein-mediated protection has now been demonstrated for a number of different viruses from at least eight different virus groups, such as the tobamo-, potex-, cucumo-, tobra-, carla-, poty-, and alfalfa mosaic virus groups (for review see Beachy et al. 1990; Grumet 1990), and more recently the luteovirus group (Kawchuk et al. 1990, 1991). The list is growing rapidly. Virus inoculated leaves of the transgenic plants show fewer chlorotic or necrotic lesions relative to control plants, and systemic spread of infection is either prevented, delayed or reduced (Cuozzo et al. 1988; Hemenway et al. 1988; Hoekema et al. 1989; Hector et al. 1991; Kallerhoff et al. 1990; Kawchuk et al. 1990; Nelson et al. 1987,1988; Powell et al. 1990; Tuner 1987; Van Dun et al. 1988;) . No apparent negative effects on growth, fertility or physical appearance has been observed in the plants

expressing viral coat protein genes. The resistance trait can be stably transmitted over several generations. Practical application of this technology is promising; several transgenic crops have been protected in field experiments under conditions of high infection pressure (e.g. Nelson et al.1988; Gaser and Fraley, 1989).

The coat protein genes have also been introduced into plants in antisense orientation for the purpose of genetically engineering viral resistance (Cuozzo et al. 1988; Hemenway et al. 1988; Kawchuk et al. 1991; Lindbo and Dougherty 1992; Powell et al. 1989). In all these cases, plants expressing antisense RNA were less susceptible to virus infection than were control plants, but the protection levels were variable. In the case of cucumber mosaic virus (Cuozzo et al. 1988), viral coat protein genes were transferred into plants in both orientations for producing sense and antisense RNA. Protection was observed only at low inoculum concentration and to a limited extent in transgenic plants expressing antisense RNA. In contrast all CP expressing plants were significantly protected in all inoculum concentrations. Similar results were obtained with potato virus X (Hemenway et al. 1988) and TMV (Powell et al. 1989). However, in plants producing positive- or negative-sense CP RNA of potato leafroll virus (PLRV), both the pattern and level of protection were the same (Kawchuk et al. 1991). The reason for the difference between PLRV and the above viruses is not known, but unlike PVX, CMV or TMV, which

occur at very high titer, PLRV occurs at low titer and is confined to the phloem of the host plant. Similarly, Lindbo and Dougherty (1992) reported recently that plant lines expressing either sense defective RNA or antisense transcripts of tobacco etch virus (TEV) CP gene were highly resistant and showed greater protection than the plants producing the full-length CP.

Broad spectrum resistances have been observed in CP-mediated protection. Transgenic tobacco plants expressing the coat protein of TMV or alfalfa mosaic virus have a low but significant degree of protection against other tobamoviruses (Nejida and Beachy 1990), or unrelated viruses such as PVX, PVY, and CMV (Anderson et al. 1989). Tobacco plants accumulating coat protein of the potyvirus soybean mosaic virus (SMV), a non-pathogen on tobacco, were resistant to infection by two serologically unrelated potyviruses that are pathogens of tobacco, PVY and TEV (Stark and Beachy 1989). A similar result was reported by Ling et al (1991) in tobacco plants expressing papaya ringspot virus CP gene. The degree of heterologous protection could be of considerable agronomic benefit by enabling a plant to be protected against many viruses using a limited number of different CP genes. It would be especially useful for resistance to potyviruses, which form the largest, most widely distributed and economically important group of plant viruses (Hollings and Brunt 1981). Many hosts are infected by several different potyviruses.

There are several necessary steps to genetically engineer CP-mediated protection in plants (for review see Beachy et al. 1990; Grumet 1990). First, the cDNA that represents the CP open reading frame (ORF) must be cloned. This is relatively straightforward for viruses with CPs that are encoded by subgenomic RNAs. However, for viruses such as those in the potyvirus group, were the CP is part of a large polyprotein (Dougherty and Carrington 1988), defining amino terminals of the CP gene can be somewhat more difficult. Second, the CP gene must be identified, sequenced and isolated. Third, the CP ORF must be linked to an appropriate transcriptional promotor, since viral RNAs do not contain promotors capable of transcription in plant chromosomes. The most commonly used promotor for controlling expression of the CP gene in transgenic plants is CaMV35S promoter, which is a strong, constitutive promoter derived from cauliflower mosaic virus (CaMV) (Guilley et al. 1982). The last step, which can be the most challenging, is the introduction of the gene constructs and regeneration of modified plants. At this time, most examples of resistant plants were produced via *Agrobacterium*-mediated gene transfer (for detail see first section), largely because these plants were responsive to this transformation system; many other plants including the important vegetable and cereal plants are still difficult to be transformed and/or regenerated. For these plants, a model system such as tobacco may be useful for testing CP gene constructs.

Although CP-mediated protection has been demonstrated in several virus groups and plant systems, the mechanism responsible for the protection is not yet well understood. The discovery that expression of viral coat protein(CP) in transgenic plants protects these plants against virus infection is in some ways, a natural extension of work by previous researchers on cross-protection, and it was termed genetically engineered cross-protection (Powell et al. 1986; Turner et al. 1987). For a number of years, agriculturalists have inoculated plants with mild strains of viruses or viroids to prevent more virulent strains from infecting the plant and causing severe disease symptoms. The practice referred to as "cross-protection" has been used to reduce yield loss in some important crops such as tomatoes, potatoes and citrus, due to tomato mosaic virus (ToMV), potato spindle tuber viroid, and citrus tristeza virus respectively (see reviews by Fulton 1986; Urban et al. 1990). There are potential disadvantages to the widespread use of cross protection in agricultural fields. First, the mild strain (protecting strain) might undergo mutation to highly virulent form which could be selected during replication, thereby leading to extensive crop losses rather than protection. Second, the protecting strain might act in synergism with a non-related virus resulting in a disease condition that is more severe than that caused by either virus alone. Third, a protecting virus in one plant might be a severe pathogen to another species in field

situation. Fourth, the protecting strain may itself cause a small but significant loss in yield (Hamilton 1980). Most or all of these objections, however, could be overcome if cross-protection was engendered in plants as the result of expression of a single viral gene, rather that as a result of infection with an intact virus. A single induced gene is unlike a whole virus, it is not capable of self-replication, multiplying to high levels, or spreading from plant to plant. Sanford and Johnston (1985) predicted that a "normal" host-pathogen relationship can be disrupted if the host organism expresses an essential pathogen derived gene in excess amounts, at the inappropriate developmental stage or in a dysfunctional form. This may disrupt the normal replicative cycle of the pathogen and result in an attenuated or aborted infection of the host.

Several types of experiments indicate that CP gene expression in transgenic plants interferes with an early event in virus infection. Studies with CP expressing plantprotoplasts have yielded important information about early events leading to CP-mediated resistance. Loesch-Fries et al(1987) reported that CP expressing protoplasts are resistant to ALMV virus infection, but not to ALMV-RNA. Similarly, Register and Beachy (1988) reported that CP expressing plants and protoplasts were less protected against TMV-RNA, and against TMV virions that were briefly treated at pH 8.0 to swell the virions, than against native TMV virions.

According to the current model of co-translational disassembly for TMV (Wilson 1984; Wilson and Watkins 1986), host ribosomes bind to the 5′ end of TMV, and actively uncoat the virus as they proceed to translate the genomic RNA. The results from the above experiments suggested that the coat protein in transgenic plants interferes with the virus life cycle by preventing uncoating of the virus. However, the mechanism of resistance may not be the same in each case, because the various viruses tested all belong to diverse virus groups with distinct biologies. For example, transgenic plants expressing the coat protein of potato virus X (PVX) are resistant to infection by RNA as well as whole virion (Hemenway et al. 1988). One possible explanation for the difference between TMV and PVX may lie in the difference in coating and uncoating mechanisms. There are also examples that argue against a protein-RNA role in resistance. Dun and Bol (1988) reported that, although the CPs encoded by TCM and PBL strains of TRV could encapsidate the RNA molecules of the other strain, the CP of the TCM strain did not protect plants from infection by the PBL strain. They suggested that it may be due to reduced affinity for RNA or an altered domain involved in a function other than RNA binding.

It is also possible that the coat protein interferes at more than one stage in the viral life cycle. The consistent but low level resistance against infection by TMV RNAs in CP expressing plants (Nelson et al. 1987), and protoplasts

(Register and Beachy 1988) implies another mechanism of resistance that acts after the uncoating event(s). It was suggested that TMV CP may play a role in regulating the replication of TMV-RNA (Osbourn et al. 1989).

In most CP expressing plants, systemic infection is either prevented or delayed compared with nontransgenic plants. This could result from interference with the spread of virus from cell to cell in inoculated tissue, the spread from inoculated leaves to uninoculated leaves (Wisniewski et al. 1990), the egress of virus from the inoculated leaf into the vascular tissue, movement through the vascular tissue, and/or initiation of infection in other leaves.

The results from broad spectrum resistance of plant expressing viral CP genes (Stark and Beachy 1989; Anderson et al. 1989; Nejidat and Beachy 1990; Ling et al. 1991) demonstrated that CP-mediated protection is not a one gene/one virus phenomenon, but rather that the expression of a single CP gene can protect a plant against several different viruses. Plants expressing potyvirus SMV CP were resistant to infection by other two potyviruses, PVY and TEV, but not to a tobamovirus, TMV. Protection against the heterologous potyviruses is likely due to amino acid sequence homologies between the "protecting" CP (SMV) and those of the challengers, PVY and TEV (61% and 58% respectively). The failure to protect against TMV may be due to a lack of sequence homology between TMV CP and SMV CP, and/or the

difference in life cycle strategies of potyviruses and tobamoviruses. Stark and Beachy (1989) suggested that CP-mediated protection requires structural as well as sequence homology between the protecting and challenging virus CP. This phenomenon might be also true in antisense and sense-defective CP RNA related protection, but it still needs to be proven.

The mechanism of protection in plants expressing antisense or sense defective CP RNA is not clearly defined. However, several possibilities have been proposed (Cuozzo et al. 1988; Lindbo and Dougherty 1992). One possibility is that the antisense RNA may inhibit viral RNA replication and/or translation through the formation of an antisense-sense RNA hybrid. Another possibility is that the antisense or sense RNA competes directly with viral proteins(e.g. replicase) or host factors essential for replication of virus. These mechanisms could depend on expression levels of antisense RNA and inoculum concentrations. Although the reported results demonstrated that the level of antisense CP RNA related protection was variable, the antisense CP RNA was expected to provide greater protection than sense CP transcripts (Nelson et al. 1989), since they could, in theory, hybridize to both sense genomic and subgenomic viral RNAs to interfere with viral replication. To determine the applicability of the antisense RNA approach, further investigation and more tests on different viruses and plants are required.

It should be indicated that most mechanistic studies to

date have involved TMV-CP, TMV and tobacco and tomato plants; it is very likely that other virus-host-CP combinations will be different from TMV system. It is important that other virus groups and different hosts be studied to develop a more complete understanding of mechanisms of resistance, and to improve and extend the CP-mediated resistance.

C. POTYVIRUS AND ZUCCHINI YELLOW MOSAIC VIRUS BIOLOGY

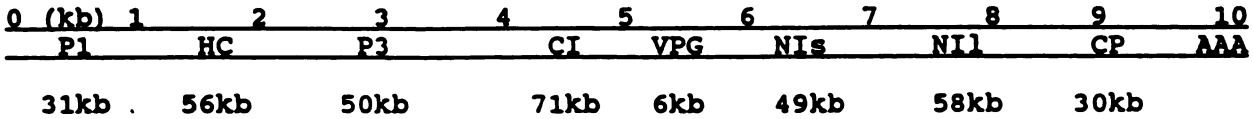
The potyvirus group is the largest known group of plant viral pathogens, there may be over 100 different members in this group (Hollings and Brunt 1981). Potyviruses have the ability to inflict damage to a wide range of crops around world; it is estimated that one quarter of the world's viruses that infect plants come from this group (Hollings and Brunt, 1981; Francki et al., 1985). Frequently, potyviruses are present as part of the natural pathogen population, causing chronic reductions in yield and quality (Lisa and Lecoq, 1984; David and Mizuki, 1986; Nameth et al. 1986).

All members of the potyvirus group share common features. The virus particle has a flexuous rod shape and is usually 700-900nm in length and 12-15nm in diameter. The potyvirus genome is a single-stranded, positive sense infectious RNA molecule that is approximately 10,000 nucleotide in length (Allison et al. 1986; Domier et al. 1986). Potyviral RNA contains a covalently linked protein (VPg) at the 5′ terminus (Murphy et al., 1990) and is polyadenylated at 3′ terminus

(Hari et al., 1979). Most members of the potyvirus group are transmitted by aphids in a nonpersistent, noncirculative manner, which involves a viral encoded helper component protein that is thought to mediate the binding of the virus to the aphid stylet (Thornbury and Pirone 1983; Berger and Pirone 1986); transmission in seed or by mites and dodder has been also reported (Hollings and Brunt 1981). Potyviruses can also be mechanically transmitted.

Based on a compilation of cell-free expression studies, nucleotide sequence data, and biochemical analysis of gene products, the genomic organization of potyviruses has been assessed (Allison et al. 1986; Dougherty and Carrington 1988; Dougherty and Parks, 1991; Marankal and Rhoads, 1991; Verchot et al., 1991). Figure 1 presents the genetic map and putative functions of potyviral proteins. The genes in order from 5′ to 3′ are: I. P1 protein (31kd), a putative cell to cell movement protein based on sequence homologies to other viral movement proteins, and a protease that perform the cleavage between P1 and P2 proteins; II. aphid helper component (56kd), that is required for aphid transmission, and is a protease performing the cleavage between P2 and P3 proteins; III. P3 protein (50kd), with unknown function; IV. cytoplasmic inclusion protein (70kd), it has been hypothesized that this protein is involved in replication as a possible RNA helicase based on sequence analysis; in addition, the cytoplasmic inclusion protein may also be involved in long distance transport and

A. Potyvirus genomic map



B. Putative function of potyvirus gene products

P1	P1 protein ----------	Putative cell to cell movement protein, 5′ protease
HC	Helper component protein---	Aphid transmission, 5′ protease
P3	P3 protein----------	?(specific virus-host interaction)
CI	Cytoplasmic inclution------- protein	? (RNA helicase, replication, viral/host interactions, long distance transportation)
VPg	Viral genome-linked -------- protein	RNA stability, replication, infectivity
NIs	Small nuclear inclusion----- protein	3′ protease
NIl	Large nuclear inclusion----- protein	putative RNA-dependent RNA polymerase
CP	Coat protein ----------	Encapsidation of viral RNA, vector transmission

Figure 1. Potyvirus genomic map and putative functions of gene products

virus/host interaction; V. genome linked protein (VpG, 6kd), the VpG may involved in replication, RNA stability and infectivity; VI. small nuclear inclusion protein (49kd), the N-terminal segment is involved in RNA replication, the C-terminal half is associated with a protease responsible for the majority of proteolytic cleavages ; VII. large nuclear inclusion protein (58kd), a putative viral RNA-dependent, RNA polymerase; and finally, VIII. capsid protein (30kd), which encapsidates the viral RNA and participates in aphid transmission.

The life cycle of potyviruses starts with transmission of the virus to the host cells. Once inside the host plant, the virus is uncoated. Whether particles are uncoated prior to, or after, entering the host cell, is not yet clear (Shaw et al. 1985). The single open reading frame of the viral genome is first translated into a single polyprotein precursor (346kd)(Allison et al., 1986). Rapid autoproteolytic processing occurs after translation, giving rise to intermediate polyprotein cleavage products and finally the eight mature viral proteins. This is accomplished by three proteinases that are encoded by the genome, and autocatalytically released from the polyprotein. These proteins are known as the nuclear inclusion protein (NIa), the helper component and the P1 proteinases. NIa protease is also responsible for the subsequent proteolytic events associated with polyprotein processing (Dougherty and Carrington, 1988;

Carrington,1991).

Minus RNA strands are synthesized using the viral RNA as template, and then plus strands are replicated from minus strands. The plus RNA strands are either translated or encapsidated. Encapsidation is hypothesized to occur from 5′ to 3′ end (Allison et al. 1986; Shulka et al. 1988) in a manner similar to another virus group, the potexvirus group (Lok and Abouhaidar 1986). Virus spread from cell to cell is thought to be via the plasmodesmata (Atabekov and Dorokhov 1984). Characteristic inclusion bodies form during infection. The life cycle is completed by subsequent aphid transmission of progeny virions to other plants.

Studies of potyviral CPs have shown that both the N and C termini of the potyvirus coat proteins are located on the surface, and that the N termini tend to be most variable (Shukla et al. 1988). Comparisons of sequence data from several potyviral capsid genes (such as Shulkla et al. 1987; Allison et al. 1985; Ravelonandro et al. 1988; Grumet and Fang 1990) have shown that although external domains vary, the internal domains are conserved among different potyviruses. The conserved domains have been hypothesized to be responsible for protein-protein or protein-RNA binding (Dougherty et al. 1985).

Zucchini yellow mosaic virus (ZYMV) is a member of potyvirus group. It is a relatively new, but extremely aggressive and destructive virus. The virus was first

described and identified in Italy in 1981 (Lisa et al); since then it has spread rapidly throughout Europe, the Middle East, and United States, and several new strains have been identified (Lisa and Lecoq 1984; Davis 1986; Davis and Mizuki 1987). The virus causes a severe disease in many cucurbit species, such as squash, muskmelon and cucumber. Symptoms include mosaic, leaf distortion, stunting, and fruit and seed deformations. It was reported that the disease caused crop losses of 50-100% in individual fields of summer squash and melons from 1982 to 1986 in eastern, southern, midwestern and west coast states of the U.S. (Mcleod et al. 1986; Davis and Miizuki 1987; Nameth et al. 1986; Provvidenti et al. 1984).

ZYMV is capable of outcompeting the other two cucurbit potyvirus (PRSV-W and WMV-2) in establishing infection in rub inoculation (Davis and Mizuki 1987), aphid acquisition (Lecoq and Pitrat 1985), and field studies (Alderez 1987). The basis for its success is not yet known. Because of its aggressiveness, virulence and aphid transmission, control of this disease will probably be most effectively obtained by the development of resistant cultivars (Davis 1986).

To genetically engineer plant resistance using the viral coat protein gene, I did the following: (1) developed an efficient *Agrobacterium*-mediated transformation system in muskmelon (Fang and Grumet, 1990), (2) sequenced and isolated the ZYMV CP gene (Grumet and Fang, 1990), (3) engineered three versions of CP gene for expression in plants and introduced

them into melon and tobacco plants, and (4) tested the transgenic plants expressing ZYMV CP constructs for protection against ZYMV, PVY and TEV infections. I also developed a simple method for purification of plant genomic DNA to facilitate verification of the inserted gene in transgenic plants (Fang et al., 1992). In this dissertation, the development of the *Agrobacterium*-transformation system in muskmelon, the ZYMV CP sequencing, the engineering of ZYMV CP gene constructs for expression in plants, and tests for the effects of the expression of ZYMV CP constructs against potyviruses infection are described.

LITERATURE CITED

1. Alderz, W.C. 1987. Cucurbit potyvirus transmission by alate aphids trapped alive. J. Economic Entomol. 80:87-92.

2. Allison, R.F., Dougherty,W.G., Parks,T.D., Willis,L., Johnson,R.E. 1985. Biochemical analysis of the capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. Virol. 147:309-316

3. Allison,R.F., Johnston,R. E.,Dougherty,W.G. 1986. The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: evidence for the synthesis of a single polyprotein. Virology 154:9-20

4. Anderson,E.J., Stark,D.M., Nelson,R.S., Powell,P.A., Tumer,N.E., Beachy,R.N. 1989. Transgenic plants that express the coat protein genes of tobacco mosaic virus or alfalfa mosaic virus interfere with disease development of some nonrelated viruses. Phytopathol. 79:1284-1290

5. Barton,K. A., Binns, A.N., Matzke, A.J.M. and Chilton,M.D.1983. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R_1 progeny. Cell 32:1033-1041

6. Beachy,R.N., Loesch-Fries,S., Tumer, N.E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474

7. Berger,P.H., Pirone,T.P. 1986. The effect of helper component on the uptake and localization of potyviruses in *Myzus persicae*. Virology 153:256-261

8. Binns, A.N. 1990. *Agrobacterium*-mediated gene delivery and the biology of host range limitation. Physiol. Plant. 79: 135-139

9. Binns, A. N., Thomashow, M.F. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Annu. Rev. Microbiol. 42:575-606

10. Bytebier, B., Deboeck, F., DeGreve, H., VanMontagu, M., Hernalsteens, J. P. 1987. T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. Proc. Natl. Acad. Sci. USA 84:5345-5349

11. Chilton,M.D., Drummond,M.H., Merlo,D.J., Sciaky,D., Montoya,A.L. Gordon,M.P. and Nester,E.W. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11:263-271

12. Christou,P., McCabe,D.E., Martinell,B.J., Swain,W.F. 1990. Soybean genetic transformation-commercial production of transgenic plants. *Trends Biotechnol*. 8:145-151

13. Cuozzo,M., O'Connell,K.M., Kaniewski,W., Fang,R.-X., Chua,N.-H., Tumer,N.E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. BioTechnology 6:123-143

14. Davis,R.F., and Mizuki,M.K. 1987. Detection of cucurbit viruses in New Jersey. Plant Disease 71:40-44.

15. Domier,L.L., Franklin,K.M., Shahabuddin,M., Hellmann,G.M., Overmeyer, J.H. 1986. The nucleotide sequence of tobacco vein mottling virus RNA. Nucleic Acids Res. 14:5417-5430

16. Dougherty,W.G. and Parks,T.D. 1991. Post-translational processing of the tobacco etch virus 49-kDa small nuclear inclusion polyprotein: identification of an internal cleavage site and delimitation of VPg and proteinase domains. Virol. 183: 449-456.

17. Dougherty, W.G., Willis,L., Johnston,W.E. 1985. Topographic analysis of tobacco etch virus capsid protein epitopes. Virology 144:66-72

18. Dougherty,W.G. and Carrington,J.C. 1988. Expression and function of potyviral gene products. Annu. Rev. Phytopathol. 26:123-143.

19. Fang G. and Grumet R. 1990. *Agrobacterum tumefaciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Reports 9:160-164

20. Fang, G., Hammer, S., Grumet, R. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques 13:52-56

21. Fromn, M. E., Taylor, L. P., Walbot, V. 1986. Stable transformation of maize after electroporation. *Nature* 319:791-793

22. Fromn, M.E., Morrish, F., Armstrong,C., Williams, R., 1990. Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. BioTechnol. 8:833-839

23. Fulton, R.W. 1986. Practices and precautions in the use of cross protection for plant virus disease control. Ann. Rev. Phytophol. 24:67-81.

24. Gasser, C. S., Fraley, R. T. 1989. Genetically engineering plants for crop improvement. Science 244:1293-1299

25. Grumet, R. 1990. Genetically engineered plant virus resistance. Hortscience 25:508-513

26. Grumet, R. and Fang, G. 1990. cDNA cloning and sequence analysis of the 3'-terminal region of zucchini yellow mosaic virus RNA. J. Gen. Vriol. 71:1619-1622.

27. Guilley,H., Dudley,R.K., Jonard,G., Balazs,E., Richards,K.E. 1982. Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization. Cell 30:763-773

28. Hamilton,R.I., 1980. Defenses triggered by previous invaders-viruses. In Plant disease, An Advanced Treatise, (Horsfall,J.G., Cowling,E.R. eds) 5:279-303. New York: Academic Press.

29. Hari,V., Siegel,A., Rozek,C. and Timberlake,W.E. (1979). The RNA of tobacco etch virus contains poly (A). Virol. 92: 568-571

30. Hemenway,C., Fang,R.-X., Kaniewski,J.J., Chua,N.-H., Tumer, N.E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7:1273-1280

31. Hoekema,A. Hirsch, P.R.Hoykaas,P.J.J. and Schilperoort, R.A. 1983. A binary strategy based on separation of vir and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature 303: 179-180

32. Hoekema,A., Huisman,M.J., Molendijk,L., van denElzen, P.J.M., Cornelissen, B.J.C. 1989. The genetic engineering of two commercial potato cultivars for resistance to potato virus X. BioTechnol. 7:273-278

33. Hollings,M., Brunt,A.A. 1981. Potyviruses, In Handbook of Plant Virus Infections and Comparative Diagnosis, ed. E.Kurstak, pp. 732-777. New York: Elsevier/North Holland Biomedical.

34. Hooykaas, P. J. J. 1989. Transformation of plant cells via *Agrobacterum*. Plant Mol. Biol. 13:327-336

35. Horsch,R.B., Fraley,R.T., Rogers,S.G., Sanders, P.R., Lloyd,A., Hoffmann,N. 1984. Inheritance of functional foreign genes in plants. Science 223:496-498

36. Horsch,R.B. Fry, J.E., Hoffman, N.L., Wallroth, M., Eichholz, D., Rogers,S. and Fraley,R.T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231

37. Kallerhoff,J., Perez,P., Bouzoubaa,S., Ben Tahar,S., and Perret,J. 1990. Beet necrotic yellow vein virus coat protein-mediated protection in sugarbeet (*Beta vulgaris L.*) protoplasts. Plant Cell Rep. 9:224-228.

38. Kathal R., Bhatnagar S.P., Bhojwani S.S. 1988. Regeneration of plants from leaf explants of *Cucumis melo* L. Plant Cell Rep. 7:449-451.

39. Kawchuk,L.M., Martin,R.R., McPherson,J. 1990. Resistance in transgenic potato expressing the potato leafroll virus coat

protein gene. Mol. Plant-Microbe Interact. 3:301-307.

41. Kawchuk,L.M., Martin,R.R., McPherson,J. 1991. Sense and antisense RNA-mediated resistance to potato leafroll virus in russet burbank potato plants. Mol. Plant-Microbe Interact. 4:247-253.

42. Lecoq,H., and Pitrat,M. 1985. Specificity of the helper-commponent-mediated aphid transmission of three potyviruses infecting muskmelon. Phytopathol. 75:890-893

43. Lindbo,J.A. and Dougherty, W.G. 1992. Pathogen-derived resistance to a potyvirus: immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.

44. Ling,K., Namba,S., Gonsalves,C., Slightom, J.L., and Gonsalves,D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. BioTechnol. 9:752-758.

45. Lisa,V., Boccardo,G., D'Agostino,G., Dellavalle,G., d'Aquilio,M. 1981. Characterization of a potyvirus that causes zucchini yellow mosaic. Phytopathol. 71:667-672

46. Lisa,V., and Lecoq,H. 1984. Zucchini yellow mosaic virus. No. 282. Descriptions of Plant viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.

47. Loesch-Fries, L.S., Halk,E., Merlo,D., Jarvis,N., Nelson,S. 1987. Expression of alfalfa mosaic virus coat

protein gene and antisense cDNA in transformed tobacco tissue. EMBO J. 6:1845-1851.

48. Lok, S. and Abouhaidar, M.G. 1986. The nucleotide sequence of the 5′ end of papaya mosaic virus RNA: site of *in vitro* assembly initiation. Virology 153:289-296.

49. Lorz,H., Baker,B., Schell,J. 1985. Gene transfer to cereal cells mediated byprotoplasts transformation. Mol. Gen. Genet. 119:178-182.

50. Mavankal, M.G. and Rhoads, R. E. 1991. *In vitro* cleavage at or near the amino terminus of the helper component protein in the tobacco vein mottling virus polyprotein. Virol. 185: 721-731.

51. Murphy,L.F., Rhoads,R.E., Hunt,A.G. & Shaw,J.G. (1990). The VPg of tobacco etch virus RNA is the 49 kDa proteinase or the N-terminal 24 kDa part of the proteinase. Virol. 178:285-288.

52. Nameth,S.T., Dodds,J.A., Paulus,A.O., and Laemmlen,F.F. 1986. Cucurbit viruses of California: An ever-changing problem. Plant Disease 70:8-11

53. NcCabe,D.E., Swain,W.E., Martinell, B.J., Christou, P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. BioTechnol. 6:923-926

54. Negrutiu, I., Shillito, R. D., Potrykus, I., Biasini, GG., Sala, F. 1987. Hybrid genes in the analysis of transformation conditions. I. setting up a simple method for direct gene transfer to protoplasts. Plant Mol. Biol. 8:363-373

55. Nelson,R.S., Powell-Abel,P., Beachy,R.N. 1987. Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virology 172:370-373

56. Nelson, R.S., McCormick,S.M., Delannay,X., Dibe,P., Layton, J. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. BioTechnology 6:403-409

57. Nelson,R.S., Powell,P.A. and Beachy,R.N. 1990. Coat protein-mediated protection against virus infection. *In* Genetic Enginnering of Crop Plants (Lycett,G.W. and Grierson,D. ed) pp. 2-24. Butterworths, Borough Green, Savenaoks, Kent, UK

58. Niedz R.P., Smith S.S., Dunbar K.B., Stephens C.T., Murakishi H.H. 1989. Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo* L. Plant Cell Tissue and Organ Cult. 18:313-319.

59. Osbourn,J.K., Watts,J.W., Beachy,R.N., Wilson,M.A. 1989. Evidence that nucleocapsid disassmbly and a latter step in virus replication are inhibited in transgenic tobacco protoplasts expressing TMV coat protein. Viology 172:370-373

60. Potrykus, I. 1991. Gene transfer to plants: Assessment of published approaches and results. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:205-225

61. Potrykus, I., Saul,M.W., Potuska,J., Paszkowski,J. and Shillito, R.D. 1985. Direct gene transfer to cells of a

Graminaceous monocot. Mol. Gen. Gent. 199:183-188

62. Powell-Abel,P.A., Nelson,R.S., De,B., Hoffman,N., Rogers,S.G., Beachy, R.N.1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743

63. Powell-Abel,P.A.,Sanders,P.R., Tumer,N., Fraley,R.T., Beachy,R.N. 1990. Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequence. Viology 175:124-130

64. Powell,P., Stark,D.M., Sanders,P.R. and Beachy,R.N. 1989. Protection against tobacco mosaic virus in transgenic plants that express TMV antisense RNA. Proc. Natl. Acad. Sci. USA 86:6949-6952

65. Quemada,H.D., Gonsalves,D. Slightom,J.L. 1991. Expression of coat protein gene from cucumber mosaic virus strain C in tobacco: protection against infections by CMV strains transmitted mechanically or by aphids. Phytopathol. 81:794-802.

66. Register,J.C., Beachy,R.N. 1988. Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology 166:524-532

67. Ravelonandro, M., Varveri,C., Delbos,R., Dunez,J. 1988. Nucleotide sequence of the capsid protein gene of plum pox potyvirus. J. Gen. Virol. 69:1509-1516.

68. Rhodes,C.A., Pierce,D.A., Mettler,I.J., Mascarenhas,D. and

Detmer,J.J. 1989. Genetically transformed maize plants from protoplasts. Science 240:204-207.

69. Roest, S., Gilissen, L. J. W. 1989 Plant regeneration from protoplasts: a literature review. *Acta Bot. Neerl*.38:1-23

70. Sanford, J. C. 1990. Biolistic plant transformation. *Physiol Plant*. 79:206-209

71. Sanford,J.C. and Johnston,S.A. 1985. The concept of pathogen derived resistance: Deriving resistance genes from the parasites own geneome. J. Theor. Biol. 113: 395-404.

72. Shimamoto, K., Terada, R., Izawa, T., Fujimoto, H. 1989. Fertile rice plants regenerated from transformed protoplast. Nature 338: 274-276

73. Shulka,D.D., Gough,K.H., Ward,C.W. 1987. Coat protein of potyviruses. 3. Comparision of amino acid sequences of the coat proteins of four Australian strains of sugarcane mosaic virus. Arch. Virol. 96:59-74

74. Shulka,D.D., Strike,P.M., Tracy,S.L., Gough,K.H., Ward,C.W. 1988. The N- and C-termini of the coat protein of potyviruses are surface located and the N-terminus contains the major virus-specific epitopes. J. Gen. Viol.69:1497-1503

75. Slightom,J.L., Durand-Tardif,M., Jouanin,L. and Tepfer,D. 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid: identification of open-reading frames. J. Biol. Chem. 261:108-121.

76. Stachel,S.E. and Nester,E.W. 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti

plasmid of *Agrobacterium tumefaciens*. EMBO J 5:1445-1454

77. Stark,D.M. and Beachy,R.N. 1989. Protection against potyvirus infection in transgenic plants: evidence for broad spectrum resistance. BioTechnology 7:1257-1262

78. Thorbury,D.W., Pirone,T.P. 1983. Helper components of two potyviruses are serologically distinct. Viology 125:487-490

79. Tumer,N.E., O'Connell,K.M., Nelson,R.S., Sanders,P.R., Beachy,R.N. 1987. Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. EMBO J. 6:1181-1188

80. Uchimiya,H., Hirochika,H., Hashimoto,H., Hara,A., Masuda, T., Kasumimoto, T., Harada,H., Ikeda, J.E. and Yoshioka,M. 1986. Co-expression and inheritance of foreign genes in transformants obtained by direct DNA transformation of tobacco protoplast. Mol. Gen. Genet. 205:1-8

81. Urban,L.A., Sherwood,J.l., Rezende,J.A.M. and Melcher,U. 1990. Examination of mechanisms of cross protection with non-transgenic plants. In: Recognition and response in plant-virus interactions (ed. R.S.Fraser), Springer-Verlag, Heidelberg.

82. Verchot, S., Kooren, E.V., Carrington, T.C. 1991. The 35 kDa protein from the animo terminus of the potyviral polyprotein function as a third virus encoded proteinase. Virol. 185:527-535.

83. van Dun,C.M.P., Bol,J.F. 1988. Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning

virus. Virology 167:649-652

84. Wai, T. and Grumet, R. 1991. Inheritance of multiple potyvirus resistance from the cucumber TMG-1 line. *In* Abstracts of 1991 APS Annual Meeting, 566.122

85. Wallroth,M., Gerats,A.G.M., Rogers,S.G., Fraley,R.T., Horsch,R.B. 1986. Chromosomal location of foreign genes in *Petunia hybrids*. Mol. Gen. Genet. 202:6-15

85. Wilson,T.M.A. 1985. Nucleocapsid disassembly and early gene expression by positive strand RNA viruses. J. Gen. Virol. 66:1201-1207

CHAPTER ONE

AGROBACTERIUM TUMEFACIENS MEDIATED TRANSFORMATION AND REGENERATION OF MUSKMELON PLANTS

INTRODUCTION

One of the most effective means of gene transfer into dicotyledonous plants is to utilize the natural transformation mechanism of Agrobacterium tumefaciens (Fraley et al. 1986). Genes located between the border sequences of the Agrobacterium's Ti plasmid are inserted into the genome of the host. Utilization of this mechanism for gene transfer requires both susceptibility to infection by A. tumefaciens, and the ability to regenerate plants from individual transformed cells via tissue culture. Although many dicots are suitable hosts for Agrobacteria, only a small number of species have been transformed and regenerated successfully (Gasser and Fraley, 1989). The cucurbit family includes many high value vegetable and fruit crops (cucumbers, melons, squashes). At the time that this work was completed, the only published report of successful transformation and regeneration of a cucurbit species was for cucumber (Cucumis sativus) using the vector A. rhizogenes (Trulson et al. 1986). Several groups have recently reported successful regeneration from muskmelon explants (e.g. Neidz et al. 1989; Dirks and Van Buggenum, 1989). In this chapter, I describe an efficient procedure for the production of transgenic muskmelon (Cucumis melo) plants using the vector A. tumefaciens.

MATERIALS AND METHODS

Plant material and Agrobacteria: Peeled muskmelon seeds (Hale's Best Jumbo) were sterilized in 15% (v/v) Chlorox (5.25% sodium hypoclorite) with a drop of Tween 20 for ten min. then rinsed three times with sterile distilled water and placed on hormone free MS medium (Murashige and Skoog, 1962) with 0.8% agar. The tissue culture growth room conditions were 25 - 26 C with a 16 h photoperiod provided by cool white flourescent lamps (ca. 2500 lux). The Agrobacterium strain was disarmed A. tumefaciens LBA4404 containing the CIBA-GEIGY binary vector pCIB10 (Rothstein et al. 1987) with a transferable selectable marker for kanamycin resistance (the neomycin phosphotransferase gene, NPT II) . Agrobacterium cultures were grown and maintained on AB medium (Chilton et al. 1984) with 50 mg/l kanamycin.

Inoculation and co-cultivation: Excised 4 - 5 day old cotyledons were cut on all edges with a dull scalpel blade, soaked in a fresh overnight culture of LBA4404 + pCIB10 for 10 - 60 min, blotted dry with sterile filter paper to remove excess bacteria, and then transferred to melon regeneration (MR) medium [MR medium = MS medium with 5 uM IAA, 5 uM BAP, 1 uM ABA and 3% sucrose; Neidz et al. 1989] with or without tobacco (Nicotiana tabacum) nurse cultures for a co-cultivation period of 1 - 7 days. In the experiments where tobacco nurse cultures were used, three ml of log stage tobacco suspension cultures grown in MS medium with 2 mg/l

2,4-D and 180 mg/l monobasic potassium phosphate were pipetted onto MR plates three days prior to transformation and kept at 25 - 26 C with 16 h photoperiod. Just before co-cultivation two pieces of sterile #3 filter paper were laid over the tobacco cells, explants were put on the paper.

Plant regeneration and selection: Control melon tissue was tested for sensitivity to kanamycin using MR medium containing 0 - 200 mg/l of kanamycin; based on these results 75 mg/ml was used in transformation experiments.

After co-cultivation with Agrobacteria, explants were washed with sterile distilled water to remove excess bacteria, blotted dry, and transferred onto MR medium containing 75 mg/l kanamycin (to select for transformed tissue) and 100 mg/l cefotoxime (to eliminate bacterial carry over). Developing shoots were transferred to hormone free, root inducing medium (MS salts with 3% sucrose, 100 mg/l cefotoxime) with or without 50 mg/l kanamycin. Regenerated plantlets were transplanted to sterile Bacto soil mix as soon as roots appeared and transferred to the greenhouse.

Genetic analyses: Genomic DNA was extracted from young leaf tissue of putative transformed plants using the procedure of Dellaporta et al. (1983). Southern blot analysis was performed according to Maniatis et al. 1982. Random primer P^{32}-labeled (Feinberg and Vogelstein, 1983), isolated NPT gene was used as probe for hybridization with the genomic DNA.

To test for inheritance of the introduced NPT gene,

transformed melon plants were either selfed, or crossed to control plants, in the greenhouse. The progeny were examined for the NPT gene by dot blot analysis; genomic DNA was extracted from leaves of three week old plants, spotted onto nitrocellulose and probed with labeled NPT fragment. Explants from cotyledons of progeny were also tested for expression of the NPT II gene by culturing on MR medium with kanamycin.

RESULTS AND DISCUSSION

Transformation conditions: Several factors including kanamycin level, Agrobacterium concentration, inoculation time, length of co-cultivation period, and the use of tobacco nurse cultures were tested to optimize the melon transformation system. The sensitivity of non-transformed melon tissue to kanamycin was examined using MR medium containing 0, 10, 25, 50, 75, 100, or 200 mg kanamycin/l. Normal callus and shoots only developed on medium without kanamycin, some callus and shoots were formed on medium with 10 mg/l. At 25 mg/l a few explants produced slowly growing callus and buds, but no shoots were formed. Kanamycin levels at or above 75 mg/l completely inhibited growth of control explants, and so this level was chosen for routine selection of transformed tissue.

Bacterial concentration for inoculation is a critical variable for lettuce transformation (Michelmore et al. 1987), but has little influence on petunia transformation (Horsch et al. 1985). To examine the effect of bacterial titer on gene transfer efficiency for melon, explants were inoculated with concentrations ranging from 10^5 - 10^{10} bacteria/ml. Although there was little influence of bacterial titer on transformation and regeneration, the condition of the bacterial culture was very important. Fresh bacteria grown under optimal conditions (overnight from a fresh inoculum culture, 30 C, well-aerated) gave the highest transformation

efficiency. We routinely used a 15 - 24 h culture at a concentration of 10^7 - 10^8 bacteria/ml.

To study the influence of exposure time to bacteria, explants were soaked in bacteria for either 10, 20, 30, or 60 min (Table 1). There was little difference in transformation efficiency up to 30 min. A 60 minute exposure reduced efficiency by 40% relative to 10 min exposure, probably due to subsequent bacterial contamination that inhibited explant growth. Ten minute soaks were routinely used. Co-cultivation periods of 1, 3, 5 and 7 days were tested (Table 2). Three days of co-cultivation resulted in the highest percentage of shoot regeneration on kanamycin. With longer periods it was not possible to completely eliminate the Agrobacteria.

Tobacco nurse cultures are sometimes used to increase transformation efficiency (Rogers et al. 1986). The effect of nurse cultures on melon transformation was tested after the optimal inoculation and co-cultivation times had been determined. In two of four experiments the presence of tobacco nurse cultures resulted in a 10 - 15% higher rate of shoot regeneration, in the other two experiments there was no obvious difference. Since the nurse cultures did not make a large difference in transformation efficiency they were not routinely included.

Transgenic plants: Explants were either inoculated with LBA4404 + pCIB10, with LBA4404 without plasmid (no NPT gene), or were not exposed to Agrobacteria. Although explants from

Table 1. Effect of *A. tumefaciens* inoculation time on callus and shoot production by melon explants cultured on MR medium with 50 $mg \cdot l^{-1}$ kanamycin.

Inoculation time (min)	Total explants	# Producing callus (%)	Shoots produced (%)
10	79	24 (30.4)	8 (10.1)
20	81	21 (25.9)	9 (11.1)
30	77	19 (24.7)	8 (10.3)
60	73	13 (17.8)	4 (5.5)

Explants were co-cultivated for three days, then transferred to medium with kanamycin, and counted five weeks after inoculation. The data are compiled from two experiments.

Table 2. Effect of co-cultivation period on callus and shoot production by melon explants cultured on MR medium with 75 $mg \cdot l^{-1}$ kanamycin.

Co-cultivation (days without kanamycin)	Total explants	# Producing callus (%)	Shoots produced (%)
0	117	14 (11.9)	3 (2.6)
1	121	29 (23.9)	11 (9.1)
3	119	48 (40.3)	15 (12.6)
5	113	37 (32.7)	12 (10.6)
7	114	17 (14.9)	5 (4.4)

Explants for all treatments were inoculated with *Agrobacterium* for 10 min; counting was done 40 days after inoculation. The data are compiled from three experiments.

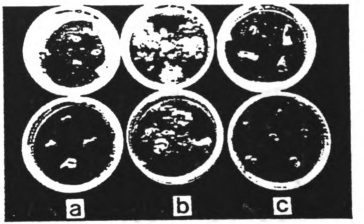


Fig. 2 Cotyledon explants of melon cultured on medium with 75 $mg \cdot l^{-1}$ kanamycin. (a) uninoculated control explants; (b) explants inoculated with *A. tumefaciens* LBA4404 + pCIB10; (c) control explants inoculated with *A. tumefaciens* LBA4404 without plasmid. The cultures were photographed three weeks after inoculation.

all treatments readily formed callus and shoots on medium without kanamycin, only explants inoculated with LBA4404 + pCIB10 produced callus and regenerated shoots on MR medium with 75 mg/l kanamycin (Figure 2). Buds were produced along the cut edges of the explants 2 - 3 weeks after inoculation. In 2 - 3 more weeks approximately 20 - 30% of the buds developed into shoots that were large enough to be transferred to rooting medium (this is an ca. 40% reduction relative to control explants without kanamycin). To further select for transformation, 50 mg/l kanamycin was included in the rooting medium. In another 3 - 5 weeks, about 30% of the shoots produced roots (vs. 80% of control shoots rooted on medium without kanamycin). Plantlets were transferred to soil mix as soon as possible after root initiation, longer growth on medium reduced the survival rate in soil. Although there was some variation in growth rate of the regenerated plants, they appeared to be morphologically normal and were fertile.

In other plant transformation systems, antibiotics were reported to affect development of the regenerated plant (Michelmore et al. 1987) and inhibit rooting of the transformed shoot (Jones et al. 1989). Although the reduced rooting observed for melon may be the effect of the antibiotic, the use of the kanamycin in the rooting medium was helpful in reducing the number of putative transgenic, regenerated plants that did not test positive by Southern blot assay (data not shown).

Genetic analyses and inheritance of kanamycin resistance:

Southern blot analysis was performed to determine if the regenerated plants were transgenic. DNA was isolated from several control melon plants, and from 25 of the ca. 60 regenerated plants that were rooted in the presence of kanamycin. The NPT probe did not hybridize with DNA from the control plants but did hybridize with DNA from 22 of the 25 kanamycin-resistant regenerated plants.

Thus in almost every case there was successful incorporation of the NPT gene into the melon genome. A Bam HI digest of the DNA from eleven of these plants is shown in Figure 3. Nine were positive for the presence of the the NPT gene; there was no hybridization in the control lane. Variable hybridization patterns were observed among the NPT-positive plants indicating the expected random integration of the NPT gene into the melon genome. Most transformed plants appreared to contain a single copy of insert DNA (single band), plant #7 may have two or more integrations into its genome (multiple bands). The DNA from several plants was also double-digested with Bam H1 and Bcl I to release an internal fragment from the transferred DNA. In each case, the expected ca. 1.9 kb band was observed (data not shown).

Four transformants (plant #'s 1, 5, 6, 7) were either self-pollinated, or backcrossed to non-transformed control plants, to produce the next generation. The progeny were examined for the NPT gene by dot blot analysis; the results

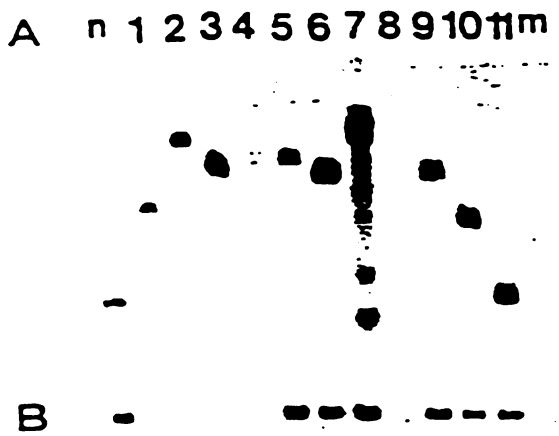


Fig. 3 Southern blot hybridization analysis of genomic DNA from putative transgenic melon plants. (A) The DNA was digested with *Bam* HI and probed with isolated, ^{32}P-labeled NPT fragment. Lane n: one copy reconstruction (50 pg) of 1.9 kb NPT gene isolated from pCIB10; Lanes 1 - 11: genomic DNA extracted from young leaves of individual putative transgenic plants (12 $\mu g \cdot lane^{-1}$); Lane m: 12 μg genomic DNA from untreated control melon plant. (B) A subset of the above samples (#5, 6, 7, 9, 10, 11) were doubly digested with *Bam* HI and *Bcl* I to release the NPT II gene. Other aspects of DNA preparation and probing were as for A.

are summarized in Table 3. The segregation ratios of the progeny of all six families was consistent with predicted Mendelian ratios as tested by X^2 analysis. In each case the P values were much greater than the rejection level of P=0.05. The progeny from self-crosses of plants 1, 5, and 6, gave the expected 3:1 ratio (NPT positive: NPT negative) for the incorporation of a single gene. Similarly, when plants 1 and 6 were backcrossed with the parental genotype, the progeny gave the expected 1:1 ratio for single gene incorporation. The 15:1 ratio observed for the progeny of selfed plant #7 implies that two genes were incorporated. In each case, the number of genes determined by progeny analysis was consistent with the observed Southern blot pattern for the original regenerated plants (Figure 3).

A leaf callus assay was conducted to determine expression of the kanamycin resistance trait. Young leaves from NPT positive progeny were cultured on MR medium with 75 mg/l kanamycin. Explants from NPT-positive progeny produced callus within two weeks; no callus was produced by non-transformed control explants (Figure 4). Thus kanamycin resistance behaved as a dominant trait as reported for other plants (e.g. Chyi et al. 1986, Catlin et al. 1988).

Conclusions We have successfully transformed and regenerated muskmelon plants using Agrobacterium tumefaciens and a modified leaf disk procedure. The best conditions in our

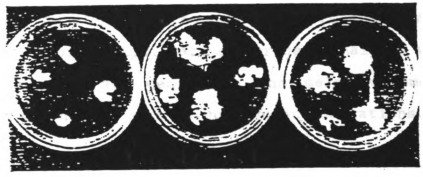
Table 3. Summary of dot blot data of progeny of transgenic regenerants.

Family	NPT[a] +	-	# Plants tested	Probable # of integrations[b]	Expected ratios	X^{2c}	P
1 - self	13	5	18	1	3:1	.073	1.00
1 - cross	9	10	19	1	1:1	.053	1.00
5 - self	13	3	16	1	3:1	.330	0.80
6 - self	15	3	18	1	3:1	.667	0.65
6 - cross	9	11	20	1	1:1	.200	0.90
7 - self	17	1	18	2	15:1	.015	0.75

[a] Genomic DNA was extracted from leaves of three week old plants and probed with ^{32}P-labeled NPT fragment. + hybridized to the NPT probe; - did not hybridize.

[b] Based on number of NPT-positive progeny and Southern blot hybridization patterns.

[c] Calculated as $X^2 = \Sigma [(|o - e| - ½)^2/e]$ using the Yate's correction factor.



a b c

Fig. 4 Sample leaf callus assay for expression of the NPT-II gene. Young leaves from control plants (a) or NPT positive, F_1 progeny (b,c) were cultured on MR medium with 75 $mg \cdot l^{-1}$ kanamycin.

experiments included the use of 4-5 day old cotyledons soaked for ten min in a fresh overnight culture of A. tumefaciens, a three day co-cultivation period, regeneration on MR medium containing 75 mg/l kan, rooting in the presence of 50 mg/l kan and immediate transfer to soil. Efficiency of transformation and regeneration is 3 - 7% (calculated from initial explant to transgenic plant). Time from initiation of the experiment to plants in the greenhouse is approximately three months. Southern blot data and progeny analysis verify integration of the NPT gene into the melon genome and transmission of the NPT gene to subsequent generations.

LITERATURE CITED

1. Catlin D, Ochoa O, McCormick S, Quiros CF (1988) Celery transformation by *Agrobacterium tumefaciens*: Cytological and genetic analysis of transgenic plants. Plant Cell Reports 7:100-103

2. Chilton MD, Currier T, Ferrand K, Bendich J, Gordon M, Nester E (1974). *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672-3676

3. Chyi YS, Jorgensen AJ, Goldstein D, Tanksley SD, Loaiza-Figueroa F (1986) Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. Mol Gen Genet 204:64-69

4. Dellaporta SL, Wood J, Hicks JB (1985) Maize DNA miniprep. *In* Molecular
Biology of Plants. Malmberg, Messing, Sussex (eds.) p. 36-37.

5. Dirks R, Van Buggenum M (1989) In vitro plant regeneration from leaf and cotyledon explants of *Cucumis melo* L. Plant Cell Report 7:626-627

6. Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13

7. Gasser CS, Fraley RT (1989) Genetically engineering plants for crop improvement. Science 244:1293-1299

8. Horsch RB, Fry JE, Heffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229-1331

9. James DJ, Passey AJ, Barbara DJ, Bevan M (1989) Genetic transformation of apple (*Malus pumila* Mill) using a disarmed Ti-binary vector. Plant Cell Reports 7:658-661

10. Maniatis T, Fritsch EF, Sambrook J (1982) "Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

11. Michelmore R, Marsh E, Seely S, Landry B (1987) Transformation of lettue (*Lactuca sativa)* mediated by *Agrobacterium tumefaciens*. Plant Cell Reports 6:439-442

12. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473-498

13. Neidz RP, Smith SS, Dunbar KB, Stephens CT, Murakishi HH (1989) Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo*. Plant Cell Tissue and Org Cult 18:313-319

14. Rogers SG, Horsch RB, Fraley RT (1986) Gene transfer in plants: production of transformed plants using Ti plasmid vectors. Methods in Enzymology 118:627-640

15. Rothstein SJ, Lahners KN, Lotstein RJ, Carozzi NB, Jayne SM, Rice DA (1987) Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. Gene 53:153-161

16. Trulson AJ, Simpson RB, Shahin EA (1986) Transformation of cucumber (*Cucumis sativa* L.) plants with *Agrobacterium rhizogenes*. Theor Appl Genet 73:11-15

CHAPTER TWO

cDNA SEQUENCE ANALYSIS OF THE 3′ TERMINAL REGION OF ZUCCHINI YELLOW MOSAIC VIRUS

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV), is a relatively new and highly agressive member of the potyvirus group. This virus was first described in Italy and France in 1981 (Lisa et al.), since then it has spread throughout the world (Lisa and Lecoq, 1984). It is capable of outcompeting the other two cucurbit potyviruses, the watermelon strain ofpapaya ringspot virus (PRV-W) and watermelon mosaic virus (WMV) in establishing infections in rub inoculation (Davis and Mizuki, 1987), aphid aquisition (Lecoq and Pitrat, 1985), and field studies (Alderez, 1987; Alderez et al., 1985). It also appears to be evolving rapidly. Several new strains have been identified (Lisa and Lecoq, 1984) and a strain capable of overcoming resistance to the dominant Zym gene in muskmelon has been reported (Nameth et al., 1986).

As a group, potyviruses are the most economically important group of plant viruses; they account for approximately one quarter of all plant viral diseases (Shukla and Ward, 1988). Potyvirus virions are flexous filamentous particles 700 - 900 nm long and 11 - 12 nm in diameter (Hollings and Brunt, 1981; Francki et al., 1985). The genome is a positive sense, polyadenylated, single stranded RNA molecule approximately 10 kb in length. The RNA codes for a single, large polypeptide that is subsequently cleaved by viral encoded proteases (Allison et al., 1986; Domier et al., 1986; Hellmann et al., 1988; Carrington and Dougherty,

1987).

As a first step towards determining whether the coat protein of the potyvirus ZYMV can be used to genetically engineer resistance, a portion of the 3′ end of the ZYMV genome was cloned and sequenced (Grumet and Fang, 1990). This chapter presents ZYMV cDNA sequence including part of the putative RNA polymerase gene, the complete coat protein gene, and the 3′ untranslated region. The predicted amino acid sequence is compared with other known potyviral sequences. These data show that the ZYMV coat protein has the highly conserved central and C-terminal regions characteristic of the potyvirus group, and an N-terminal region that is unique to ZYMV. The C-terminal regions of several potyviral polymerase genes were also compared and found to be highly conserved.

MATERIALS AND METHODS

Virus and plant material, ZYMV RNA isolation and cDNA cloning: The Connecticut strain of ZYMV was kindly provided by Dr. R. Davis and used to rub inoculate zucchini (Cucurbita pepo cv. Black Jack) cotyledons in the greenhouse. Characteristic mosaic symptoms were observed within a week. The virus was verified to be ZYMV by reaction with commercial (AgDia) antibodies to ZYMV.

Virus was isolated from infected plant material, RNA preparation and cDNA cloning were performed by Dr. Grumet as descriped in Grumet and Fang (1990). ZYMV cDNA was cloned into Bluescript KS+ plasmid (Strategene) and used to transform competent DH5a Escherichia coli cells. Clones with inserts ranging from 0.3 - 5.5 kb were obtained.

Nested deletions and DNA sequencing. The ca. 1.55 kb clone #187 was chosen for sequencing. To facilitate sequencing of both strands, the insert from clone #187 was also cloned into Bluescript KS-. Nested deletions in both directions were performed according to the Strategene protocol based on Yanish-Perron et al. (1985). The deletion products were transformed into E. coli strain MV1190; clones with inserts of appropriate length to allow for overlapping sequence were used to make single stranded DNA using the M13K07 helper phage (Dotto and Zinder, 1984). Eight overlapping clones were chosen for each strand. Dideoxy sequencing was performed according to Sanger (1981) using

^{35}S-dCTP, both strands of the #187 clone were completely sequenced. Sequence analyses were performed using the DNASIS program (Hitachi Software Engineering) and sequence data from the following potyviruses: watermelon mosaic virus 2 (WMV-2: Yu et al., 1989); tobacco etch virus (TEV: Allison et al., 1985b; Allison et al., 1986); tobacco vein mottling virus (TVMV: Domier et al., 1986); potato virus Y (PVY: Shukla et al., 1986; Van der Vlugt et al., 1989); plum pox virus (PPV: Ravelonandro et al., 1988; Maiss et al., 1989); and Johnson grass mosaic virus (JGMV: Gough et al., 1987).

RESULTS AND DISCUSSIONS

The cDNA sequence including a portion of the putative polymerase gene, the full coat protein gene, and the 3′ untranslated region (excluding the poly A tail) is shown in Figure 5 along with the predicted amino acid sequence. The 211 nucleotide 3′ untranslated region is within the size range of other potyviral untranslated regions. Possible eukaryotic polyadenylation signals AATAA and TATGT (Zaret and Sherman, 1982) 30 and 90 nucleotides upstream from the poly A tail, respectively, are underlined. A putative AATAA polyadenylation signal has also been noted for TVMV (Domier et al., 1986), but not for the other potyviruses with sequenced 3′ terminal regions. However, the possible yeast polyadenylation signal, TATGT, has also been noted for TVMV, TEV and PPV 70-90 nucleotides upstream of the poly A tail (Maiss et al., 1989).

Since potyviral genome are expressed as polyproteins, the beginning and end of genes occur at protease cut sites (review: Dougherty and Carrington, 1988). The proposed N-terminal amino acid based on coat protein size, possible protease cut sites [Q/G, Q/S, or Q/A (Domier et al., 1986; Dougherty and Carrington, 1988)] and the alignment of the putative replicase gene (Figure 6-A), is underlined and marked with a slash (Figure 5). This results in a protein 279 amino acids long, with a predicted Mr of 31,214. Consistent with expression of the potyviral genome as a polyprotein, a single

```
GATCTGATACTTGCAGTCAAAGATGAGGATAGCGGCTTACTTGATAACATGTCATCC   57
D  L  I  L  A  V  K  D  E  D  S  G  L  L  D  N  M  S  S

TCTTTTTGCGAACTTGGACTGAATTATGATTTTTCAGAACGTACGCATAAAAGAGAA  114
S  F  C  E  L  G  L  N  Y  D  F  S  E  R  T  H  K  R  E

GATCTTTGGTTTATGTCCCACCAAGCAATGCTAGTTGATGGAATGTACATTCCAAAA  171
D  L  W  F  M  S  H  Q  A  M  L  V  D  G  M  Y  I  P  K

CTCGAGAAAGAGAGAATTGTTTCAATTCTAGAGTGGGATAGAAGCAAAGAAATTATG  228
L  E  K  E  R  I  V  S  I  L  E  W  D  R  S  K  E  I  M

CACCGAACAGAGGCTATTTGCGCTGCGATGATTGAGGCATGGGGGCACACCGAGCTC  285
H  R  T  E  A  I  C  A  A  M  I  E  A  W  G  H  T  E  L

TTGCAAGAAATCAGAAAGTTTTACCTATGGTTCGTTGAAAAAGAAGAAGTGCGAGAA  342
L  Q  E  I  R  K  F  Y  L  W  F  V  E  K  E  E  V  R  E

TTGGCAGCCCTCGGAAAAGCTCCATACATAGCTGAGACAGCACTTCGTAAGTTATAT  399
L  A  A  L  G  K  A  P  Y  I  A  E  T  A  L  R  K  L  Y

ACTGACAAGGGAGCACATAAAAGTGAACTGGCACGCTACCTACAAGCCCTCCATCAA  456
T  D  K  G  A  H  K  S  E  L  A  R  Y  L  Q  A  L  H  Q

GACATCTTCTTTGAACAAGGAGACACTGTGATGCTCCAATCAGGCACTCAGCCAACT  513
D  I  F  F  E  Q  G  D  T  V  M  L  Q /\ G  T  Q  P  T

GTGTCAGATGCTGGAGCTACAAAGAAAGACAAAGAAGATGACAAAGGGAAAAACAAG  570
V  S  D  A  G  A  T  K  K  D  K  E  D  D  K  G  K  N  K

GACGTTACAGGCTCCGGCTCAGGTGAGAAAACAGTAGCAGCTGTCACGAAGGACAAG  627
D  V  T  G  S  G  S  G  E  K  T  V  A  A  V  T  \K  D  K

GATGTGAATGCTGGTTCTCATGGGAAAATTGTGCCGCGTCTTTCGAAGATCACAAAG  684
D  V  N  A  G  S  H  G  K  I  V  P  R  L  S  K  I  T  K

AAAATGTCATTGCCACGCGTGAAAGGAAATGTGATACTCGATATTGATCATTTGCTG  741
K  M  S  L  P  R  V  K  G  N  V  I  L  D  I  D  H  L  L

GAATATAAACCGGATCAAATTGAGTTATATAACACACGAGCGTCTCATCAGCAGTTC  798
E  Y  K  P  D  Q  I  E  L  Y  N  T  R  A  S  H  Q  Q  F

GCCTCTTGGTTCAACCAGGTTAAGACGGAATATGATTTGAACGAGCAACAGATGGGA  855
A  S  W  F  N  Q  V  K  T  E  Y  D  L  N  E  Q  Q  M  G

GTTGTAATGAATGGTTTCATGGTTTGGTGCATTGAAAATGGCACTTCACCCGACATT  912
V  V  M  N  G  F  M  V  W  C  I  E  N  G  T  S  P  D  I

AATGGAGTGTGGGTTATGATGGACGGAAATGAGCAAGTTGAGTATCCCTTGAAACCA  969
N  G  V  W  V  M  M  D  G  N  E  Q  V  E  Y  P  L  K  P

ATAGTTGAAAATGCAAAGCCAACGCTGCGGCAAATAATGCATCATTTTTCAGATGCA 1026
I  V  E  N  A  K  P  T  L  R  Q  I  M  H  H  F  S  D  A

GCGGAGGCATATATAGAGATGAGAAATGCAGAGGCACCATACATGCCGAGGTATGGT 1083
A  E  A  Y  I  E  M  R  N  A  E  A  P  Y  M  P  R  Y  G

TTGCTTCGAAACCTACGGGATAGGAGTTTAGCACGATATGCTTTCGATTTCTATGAA 1140
L  L  R  N  L  R  D  R  S  L  A  R  Y  A  F  D  F  Y  E

GTCAATTCTAAAACTCCTGAAAGAGCCCGCGAAGCTGTTGCGCAGATGAAAGCAGCA 1197
V  N  S  K  T  P  E  R  A  R  E  A  V  A  Q  M  K  A  A

GCTCTTAGCAATGTTTCTTCAAGGTTGTTTGGCCTTGATGGAAATGTTGCCACCACT 1254
A  L  S  N  V  S  S  R  L  F  G  L  D  G  N  V  A  T  T

AGCGAAGACACTGAACGGCACACTGCACGTGATGTTAATAGAAACATGCACACCTTA 1311
S  E  D  T  E  R  H  T  A  R  D  V  N  R  N  M  H  T  L

CTAGGTGTGAATACAATGCAGTAAAGGGTAGGCCGCCTACCTAGGTTATTGTTTCGC 1368
L  G  V  N  T  M  Q  *

TGCCGACGTAATTCTAATATTTACCGCTTTATTTGATATCTTTAGATTTCCAGAGTG 1425

GGCCTCCCACCTTTAAAGCGTAAAGTTTATGTTAGTTGTCCAGGAGTGCCGTAGTCC 1482

TTTCGGAAGCTTTAGTGTGAGCCTCTCACGAATAAGCTCGAGATTAGACTCCGTTTG 1539

CAAGCCTAAAAAAAAAAA
```

Fig. 5 .The cDNA sequence and predicted amino acid sequence of the terminal 3' 1546 nucleotides of ZYMV. The proposed polymerase–coat protein protease cut site is underlined and marked with a slash. The beginning of the conserved trypsin-resistant core protein is marked with a backslash. Possible polyadenylation signals are underlined.

open reading frame continues for the length of the #187 clone, at least 170 amino acids upstream of the putative cut site for the N-terminus of the coat protein.

It was not possible to directly determine the N-terminal amino acid of the ZYMV coat protein by chemical sequencing because the protein was N-terminally blocked. This problem has been observed for several other potyviral coat proteins (Shukla et al. 1988, Allison et al., 1985a; Domier et al., 1986), particularly those beginning with S rather than G or A (Shukla et al. 1988). The predicted Mr (ca. 31,200) obtained from the proposed Q/S site is consistent with the value estimated from the SDS-PAGE gel (32,700), but is somewhat smaller than the 36,000 estimate of Lisa et al. (1981). Although we cannot be positive about the location of the N-terminus without direct amino acid sequence data, examination of the polymerase gene also aided in assigning the protease cut site. Sequence data from the adjoining putative polymerase gene were aligned (Figure 6-A) with available sequence data from other potyviral polymerases genes beginning at the highly conserved amino acid span, G D D, which is found in viral-encoded polymerases regardless of plant, animal or bacterial origin (Kamer and Argos, 1984). This alignment resulted in assignment of the Q/S dipeptide as the ZYMV polymerase - coat protein junction.

The predicted amino acid sequence of the ZYMV coat protein is compared with the published amino acid sequences

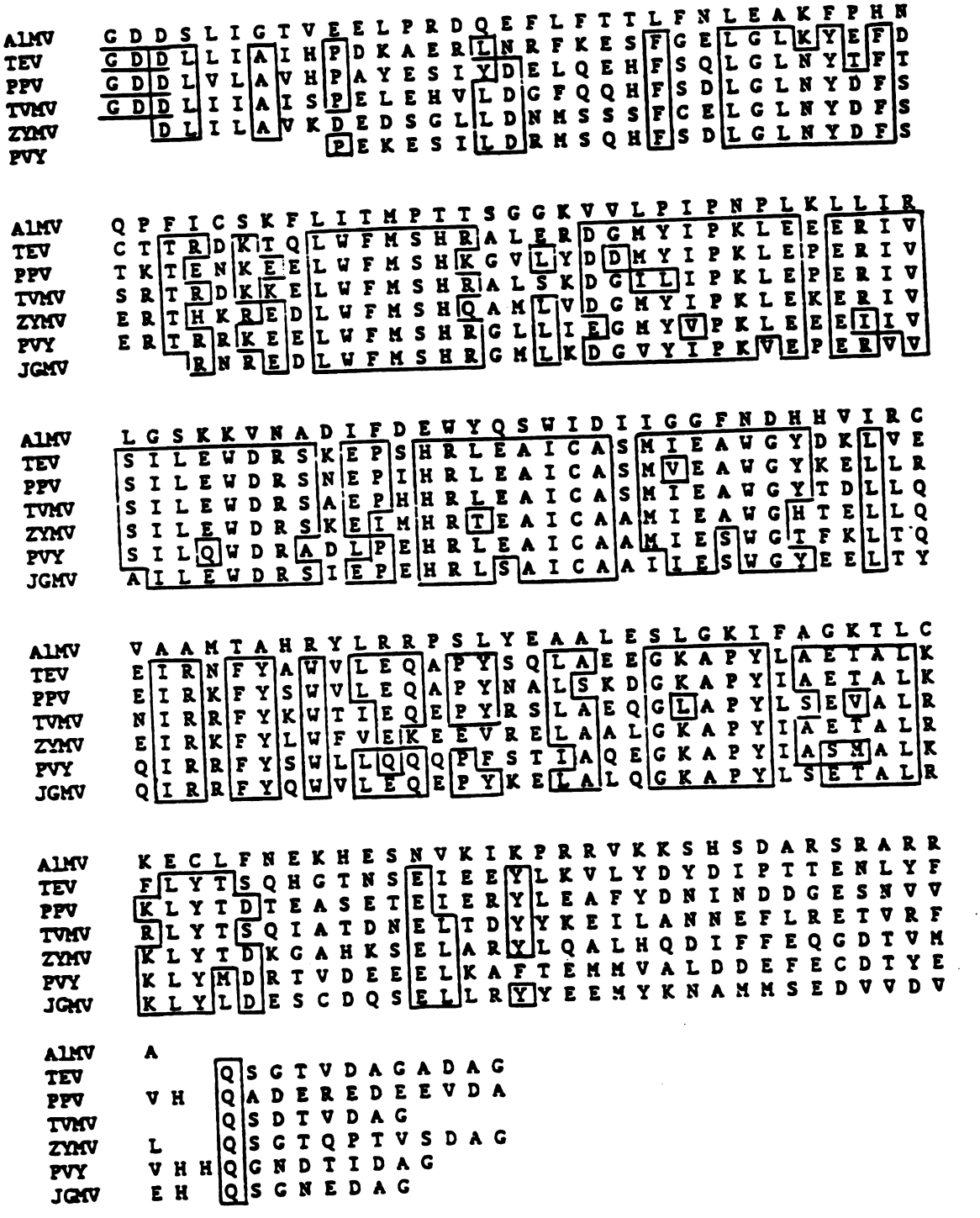


Figure 6-A. Comparison of the C-terminal region of potyviral replicase genes. The source of sequence data are as in Material and methods. Boxes indicate amino acids common to four or more of the viruses. Abbreviations are as in List of abbreviations.

of other potyviral coat proteins in Figure 6-B. The ZYMV coat protein shows very strong homology with other potyviral coat proteins in the highly conserved central and carboxy terminal portions of the protein. The N-terminal region is unique. With the exception of 18.6% homology with the other sequenced cucurbit potyvirus coat protein, WMV-2, (if aligned with deletions to maximize homology, or 11.6% if not aligned with deletions), ZYMV shares less than 14% homology with the other potyviral N-terminal regions (Table 4). Overall homology of the amino acid sequence of the ZYMV coat protein with other potyviral coat proteins ranges from 47.5% to 67.1%. This is in agreement with the 38%- 71% range of homologies observed among distinct potyviruses (Shukla and Ward, 1988; Shukla et al., 1989); different strains of the same virus are greater than 90% homologous (Shukla and Ward, 1988; Shukla et al., 1989).

Potyviral coat proteins can be divided into two general domains. The first region is potyvirus specific (e.g. the N-terminal region of ZYMV shares less than 12% homology with other potyviral N-terminal regions), is subject to removal by limited proteolysis, is externally located, and is the primary antigenic determinant (Shukla and Ward 1989; Dougherty et al., 1985; Allison et al., 1985a). Removal of this region does not interfere with apparent potyviral structure or viral infectivity, and so presumably the RNA binding capacity and coat protein - coat protein interactions involved in viral

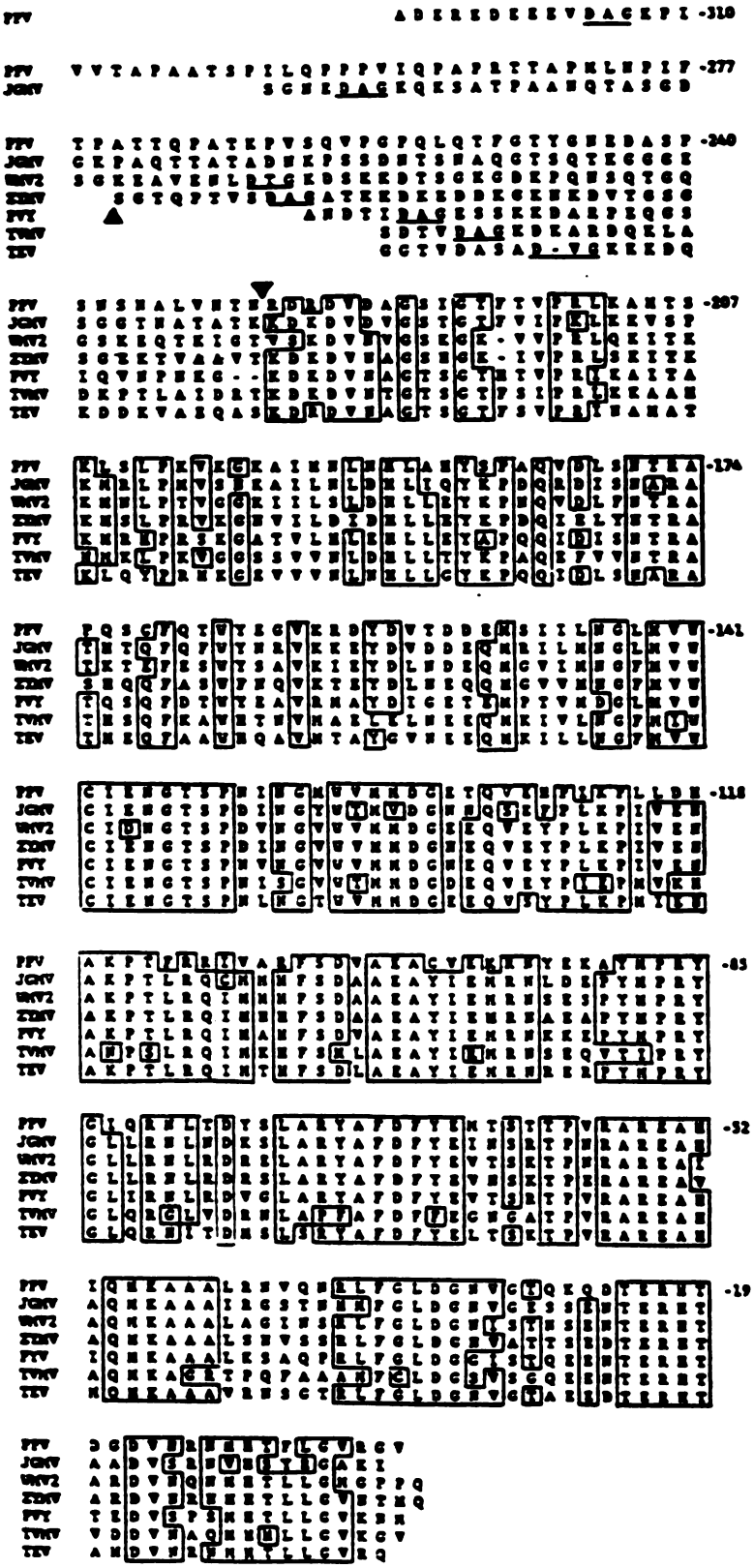


Figure 6-B. Comparison of potyviral coat protein sequences. The sources of sequence data are in Material and method. Boxes indicate amino acids common to five or more of the viruses. Abbreviations are as in Material and methods.

Table 4. *Percentage amino acid sequence homology between ZYMV coat protein and other potyvirus coat proteins*

Virus*	Overall	N terminus	Central and C terminus
WMV-2	67·1	11·6	76·9
PVY-D	59·2	7·0	67·2
TEV-HAT	58·8	4·7	67·7
JGMV	55·0	7·6	65·9
TVMV	50·5	2·3	56·8
PPV	47·5	6·5	61·1

* Abbreviations and sources of sequence data are listed in text.

assembly lie downstream within the highly conserved central and C-terminal regions characteristic of potyviral coat proteins (Dougherty et al., 1985; Shukla and Ward, 1989).

The highly conserved core portion of potyviral coat proteins is of interest in attempting to genetically engineer potyviral resistance. In at least two systems the ability of a viral coat protein to inhibit infection is related to interaction with viral RNA. TMV coat protein is thought to interfere with the TMV uncoating process by interacting with the exposed 5′ end of the viral RNA (Register and Beachy, 1988). In the QB bacteriophage system, coat protein is thought to inhibit viral replication by binding to the initiation domain of the replicase gene and thereby interfering with translation of the viral replicase gene (Grumet et al., 1987). If it is true that the nucleic acid binding domain of potyviruses is located within the highly conserved portion of the protein, and if coat proteins can inhibit viral infection via coat protein - viral RNA interactions, then it may be possible to use a core protein construct to engineer resistance to more than one potyvirus. This possibility will be tested in the work that follows, as well as the possibility that ZYMV coat protein per se can be used to confer virus resistance.

LITERATURE CITED

1. ALDEREZ, W.C. (1987). Cucurbit potyvirus transmission by alate aphids trapped alive. J. Economic Emtomol. 80:87-92. 2.

2. ALLISON, R.F., DOUGHERTY, W.G., PARKS, T.D., WILLIS, L., JOHNSTON, R.E., KELLY, M. & ARMSTRONG, F.B. (1985a). Biochemical analysis of the capsid gene and capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. Virol. 147:309-316.

3. ALLISON, R.F., SORENSON, J.C., KELLY, M.E., ARMSTRONG, F.B. & DOUGHERTY, W.G. (1985b). Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: evidence for synthesis and processing of a polyprotein in potyvirus genome expression. PNAS. U.S.A. 82:3969-3972.

4. ALLISON, R., JOHNSTON, R.E. & DOUGHERTY, W.G. (1986). The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: evidence for the synthesis of a single polyprotein. Virol. 154:9-20.

5. BAULCOMBE, D. (1989). Strategies for virus resistance in plants. Trends in Genetics 5:56-60.

6. DAVIS, R.F. (1986). Partial characterization of zucchini yellows mosaic virus isolated from squash in Turkey. Plant Disease 70:735-738.

7. DAVIS, R.F. & MIZUKI, M.K. (1987). Detection of cucurbit viruses in New Jersey. Plant Disease 71:40-44.

8. DOMIER, L.L., FRANKLIN, K.M., SHAHABUDDIN, M., HELLMANN, G.M., OVERMEYER, J.H., HIREMATH, S.T., SLAW, M.F.E.,

LOMONOSSOFF, G.P., SHAW, J.G. & RHOADS, R.E. (1986). The nucleotide sequence of tobacco vein mottling virus RNA. Nucleic Acids Research14:5417-5430.

9. DOUGHERTY, W.G. & CARRINGTON, J.C. (1988). Expression and function of potyviral gene products. Ann. Review of Phytopathol. 26:123-143.

10. DOUGHERTY, W.G., WILLIS, L. & JOHNSTON, R.E. (1985). Topographic analysis of tobacco etch virus capsid protein epitopes. Virol. 144:66-72.

11. GOUGH, K.H., AZAD, A.A., HANNA, P.J. & SHUKLA, D.D. (1987). Nucleotide sequence of the capsid and nuclear inclusion protein genes from the Johnson grass strain of sugarcane mosaic virus RNA. J. Gen. Virol. 68:297-304.

12. GRUMET, R. (1989). Genetically engineered plant virus resistance. HortScience 25:508-513.

13. GRUMET, R., SANFORD, J.C. & JOHNSTON, S.A. (1987). Pathogen-derived resistance to viral infection using a negative regulatory molecule. Virol. 161:561-569.

14. GUBLER, U. & HOFFMAN, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.

15. HELLMANN, G.M., THORNBURY D.W., HIEBERT E., SHAW, J.G., PIRONE, T.P., & RHOADS, R.E. (1980). Cell-free translation of tobacco vein mottling virus. Virol. 106:207-216.

16. KAMER, G. & ARGOS, P. (1984). Primary structural comparisons of RNA-dependent polymerases from plant, animal

and bacterial viruses. Nucleic Acids Research 12:7269-7281.

17. LECOQ, H. & PITRAT, M. (1985). Specificity of the helper component-mediated aphid transmission of three potyviruses infecting muskmelon. Phytopathol. 75:890-893.

18. LISA, V., BOCCARDO, G., D'AGOSTINO, G., DELLAVALLE, G. & D'AQUILIO, M. (1981). Characterization of a potyvirus that causes zucchini yellow mosaic. Phytopathol. 571:667-672.

20. LISA, V. & LECOQ, H. (1984). Zucchini yellows mosaic virus. CMI/AAB Descriptions of Plant Viruses, no. 282.

21. MAISS, E., TIMPE, U., BRISSKE, A., JELKMANN, W., CASPER, R., HIMMLER, G., MATTANOVICH, D. & KATINGER, H.W.D. (1989). The complete nucleotide sequence of plum pox virus RNA. J. Gen. Virol. 70:513-524.

22. MANIATIS, T., FRITSCH, E.F. & SAMBROOK, J. (1982). Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory.

23. PURCIFULL, D., HIEBERT E. & EDWARDSON, J. (1984a). Watermelon mosaic virus 2. CMI/AAB Descriptions of Plant Viruses, no. 293.

24. PURCIFULL, D., EDWARDSON, J., HIEBERT, E. & GONSALVES D. (1984b). Papaya ringspot virus. CMI/AAB Descriptions of Plant Viruses, no. 292.

25. RAVELONANDRO, M., VARVERI, C., DELBOS, R. & DUNEZ, J. (1988). Nucleotide sequence of the capsid protein gene of plum pox potyvirus. J. Gen. Virol. 69:1509-1516.

26. REGISTER, J. C. & BEACHY, R.N. (1988). Resistance to TMV

in transgenic plants results from interference with an early event in infection. Virol. 166:524-532.

27. SANGER, F. (1981). Determination of nucleotide sequences in DNA. Science 214:1205-1210.

28. SHUKLA, D.D. & WARD, C.W. (1989). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. Archives of Virol. 106:171-200.

29. SHUKLA, D.D., INGLIS, A.S., McKERN, N.M. & GOUGH, K.H. (1986). Coat protein of potyviruses. 2. Amino acid sequence of the coat protein of potato virus Y. Virol. 152:118-125.

30. SHUKLA, D.D., McKERN, N.M. & WARD, C.W. (1988). Coat protein of potyviruses. 5. Symptomatology, serology, and coat protein sequences of three strains of passionfruit woodiness virus. Archives of Virol. 102:221-232.

31. VAN DER VLUGT, R., ALLEFS, S., DE HAAN, P. & GOLDBACH, R. (1989). Nucleotide sequence of the 3′ terminal region of potato virus Y-N RNA. J. Gen. Virol. 70:229-233.

32. YANISH-PERRON, C., VIERA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC 19 vectors. Gene 33:103-119.

33. YEH, S.D. & GONSALVES, D. (1985). Translation of papaya ringspot virus RNA *in vitro*: detection of a possible polyprotein that is processed for capsid protein, cylindrical-inclusion protein, and amorphous-inclusion protein. Virol. 143:260-271.

34. YU, M.H., FRENKEL, M.J., McKERN, N.M., SHUKLA, D.D., STRIKE, P.M. & WARD, C.W. (1989). Coat protein of potyviruses 6. Amino acid sequences suggest watermelon mosaic virus 2 and soybean mosaic virus-N are strains of the same potyvirus. Archives of Virol. 105:55-64.

35. ZARET, K.S. & SHERMAN, F. (1982). DNA sequence required for efficient transcription termination in yeasts. Cell 28:563- 573.

CHAPTER THREE

GENETIC ENGINEERING OF THE ZYMV COAT PROTEIN GENE FOR EXPRESSION IN PLANTS

INTRODUCTION

Dramatic progress in the development of plant transformation and regeneration systems as a mechanism for the expression of novel genes has allowed for the genetic engineering of plants for crop improvement. This is most clearly seen by the development of genetically engineered resistance to plant viruses. The resistance was first shown for tobacco mosaic virus(TMV) in tobacco plants (Powell et al. 1986); it was found that transgenic tobacco plants expressing the coat protein gene from TMV showed a significant delay in symptom development after inoculation with TMV. Coat protein-mediated protection has now been demonstrated for a number of different viruses from at least eight different virus groups, such as the tobamo-, potex-, cucumo-, tobra-, carla-, poty-, and alfalfa mosaic virus groups (for review see Beachy et al. 1990; Grumet 1990), and more recently the luteovirus group (Kawchuk et al. 1990, 1991). The list is growing rapidly.

Another approach has been to use viral antisense genes. In several cases, the coat protein genes were introduced into plants in antisense orientation for the purpose of genetically engineering viral resistance, but the protection levels were different in different viruses and plants. In some cases there was virtually no protection, in other cases expression of antisense RNA did result in resistance to virus infection. For example, expression of antisense RNA to the AlMV CP gene in transgenic plants did not inhibit viral replication (Van Dun,

1988). Expression of antisense RNA to the CP gene of CMV and PVX result in limited protection at low inoculum concentrations of the corresponding virus (Cuozzo et al., 1988; Hemenway et al., 1988). In contrast, it was recently reported that for plants producing CP RNA of potato leafroll virus (PLRV), both the pattern and level of protection were the same regardless of whether the positive or negative strand was expressed (Kawchuk et al. 1991). Similarly plant lines expressing either sense defective RNA or antisense transcripts of the potyvirus, tobacco etch virus (TEV) CP gene were highly resistant and showed greater protection than the plants producing the full-length CP (Lindo and Dougherty, 1992).

Zucchini yellow mosaic virus (ZYMV) is a very aggressive member of the potyvirus group. Like other potyviruses, the virus particles are flexuous rods of 750 nm in length; the genome consists of a positive sense, single stranded RNA of about 9.6 kb with a 5′ end genome linked protein (VgP), and a poly (A) tail at the 3′ end. A single open reading frame (ORF) codes for a polyprotein that is proteolytically processed into mature viral gene products (Dougherty and Carrington 1988). The capsid protein is encoded by the sequence present at the 3′ end of the large ORF (Allison et al. 1985).

To genetically engineer plant resistance to ZYMV and other potyviruses, the ZYMV CP gene has been cloned and the sequence has been determined in several laboratories (Grumet and Fang 1990; Gal-On et al. 1990; Quemada et al. 1990). The

sequence encodes a coat protein with 279 amino acids and a calculated Mr of 31214. The sequence comparison shows that ZYMV shares average 50-60% direct amino acid sequence homology in CP with other potyviruses, the majority of which are located in central and C-terminal regions. In our efforts to engineer resistance, we decided to utilize three versions of the ZYMV CP gene: the full length CP gene, the truncated core portion of the CP gene, and an antisense version of the CP gene. The full length CP gene was used because incorporation and expression of viral capsid protein genes has provided the strongest virus resistance in most reported examples of engineered resistance to date (Beachy et al. 1990). A truncated version of the CP gene fragment (Core), including the highly conserved center- and carboxyl- terminal region was also used. Since the RNA binding capacity and coat protein-coat protein interaction involved in viral assembly are contained within the highly conserved core portion of the gene (Dougherty et al., 1983; Shukla and Ward, 1989), the core portion alone might be sufficient to confer resistance. Furthermore, it might be possible that plants expressing the conserved CP gene fragment could be protected from infection by more than one potyvirus. Another potential advantage of using the central and C-terminal region deals with a possible environmental concern, in preventing aphid transmission that might result from transencapsidated viruses (Goeferry, 1991). Deleting the amino terminus of the CP would remove a sequence

that is essential for aphid transmission (Salomon, 1989). The first example of CP mediated protection using truncated viral CP gene has been reported recently (Lindo and Dougherty, 1992). Plants expressing a truncated TEV CP gene demonstrated high level protection against TEV infection, in fact, the resistance was higher than that of plants expressing full length CP.

To express the ZYMV CP gene in plants for the purpose of genetic engineering of resistance to ZYMV and other potyviruses, the intact CP gene sequence and Core fragment were isolated from viral genomic cDNA. Since the ZYMV CP gene is part of a large polyprotein, the CP gene and Core were then given an AUG translational codon to enable translational initiation, and a 5′ untranslated region (UTR) was added to facilitate efficient translation of mRNA. The 5′ UTR has been verified to be the binding site of proteins and/or ribosomes during TEV RNA translation (Carrington and Freed, 1990) and to be a translational enhancer. Since ZYMV is an RNA virus and does not contain a promoter to initiate transcription, a strong transcriptional promoter, the cauliflower mosaic virus (CaMV) 35S promoter, was also added. This promoter is highly effective and has been used in most reported genetically engineered virus resistances. The expression of the CP gene in plants also requires a 3′- end sequence that confers termination and polyadenylation (poly A) to the transcripts; in our case the ZYMV 3′ poly A tail and CaMV 35S terminator

were used. In this chapter, the construction of the three versions of ZYMV chimeric CP genes and the expression of these genes in transgenic muskmelon and tobacco plants are described.

MATERIAL AND METHODS

Plasmid, DNA and bacterial manipulations: The plasmid pTL 37 which contains the TEV 5′ untranslated lead sequence was obtained from Dr. W. Dougherty (Oregon State University). The plasmid pCIB710, which contains the CaMV 35S promoter and the plasmid pCIB10 which includes the T-DNA borders and NPT II gene were provided by CIBA GEIGY Co. A cDNA clone spanning the 3′-terminal 1,550 nucleotide of ZYMV, which includes the coat protein gene sequence, was cloned as described in chapter 2 and Grumet and Fang (1990). All recombinant DNA and bacterial manipulations were carried out using standard methods (Maniatis et al. 1982), unless otherwise indicated. Restriction enzymes were purchased from Bethesda Research Laboratory or Boeringher Manheim (Life Technologies Inc.), and were used according to the suppliers' instructions. *In vitro* mutagenesis was performed using a "Oligonucleotide-directed *in vitro* mutagenesis system version 2" kit (Amersham International lnc.), all reaction conditions followed the protocol supplied by the manufacturer. *In vitro* transcription was performed using the Riboprobe system (Promega Inc.), and the rabbit reticulocyte lysate from Promega was used for *in vitro* translation. Polymerase chain reaction (PCR) was carried out using the GeneAmp PCR Reagent Kit from Perkin Elmer Cetus and following the protocol provided by the manufacturer.

Plant transformation: The pCIB10-derived binary vectors

containing ZYMV-CP gene constructs were mobilized from *E. coli* into the disarmed *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983) by tri-parental mating, using the helper plasmid pRK2013 (Comai et al.1983) in *E. coli* HB101. Cotyledon pieces of muskmelon (*Cucumis melo* L. cv."Hale's Best Jumbo") were co-cultivated with *A. tumefaciens* LBA4404 (Hoekema et al. 1983) containing pCIB10-ZYMV CP constructs for three days. Transformed muskmelon plants were regenerated and selected according to Fang and Grumet (1990) with the following modifications. The transformed cells were selected on 125 mg/l kanamycin in melon regeneration (MR) medium (Neidz et al. 1989) containing 500mg/l carbenicillin. Regenerated shoots were transferred to liquid MS medium containing 150 mg/l kanamycin, 500 mg/l carbenicillin and 0.1 mg/l BAP for two weeks for further selection and elongation. Elongated shoots were transferred to hormone free rooting medium, which contained MS nutrients, 50 mg/l kanamycin, and 400 mg/l carbenicillin. The rooted plantlets were transplanted into soil and grown in the greenhouse.

Tobacco (*N. tabacum* cv."Samsun") plants were transformed using the leaf disk protocol of Horsch et al.(1985). Shoots were regenerated on medium consisting of Murashige and Skoog (1962) salt, sucrose (30 g/l), benzyladenine (1 mg/l), naphthalene acetic acid (0.1 mg/l), kanamycin (300 mg/l) and carbenicillin (500 mg/l). Transformed shoots were subsequently rooted on phytohormone-free medium containing 100 mg/l of

kanamycin prior to transferring to soil.

Enzyme-linked immunosorbent assay (ELISA): Samples of leaf tissue (20-30 mg) from kanamycin-resistant regenerated plants were initially screened for expression of NPT II protein using double-antibody sandwich ELISA. NPT II assay kit was purchased from 5 Prime > 3 prime, Inc., and the assay was performed following the instructions supplied by the manufacturer.

DNA analysis of transgenic plants: Genomic DNA was extracted from young leaf tissue of putatively transformed plants using the procedure of Dellaporta et al. (1985), and purified with the method of Fang et al (1992). The presence of inserted ZYMV CP gene fragments were verified by polymerase chain reaction (PCR) amplification primers, which anneal to the TEV untranslated region (UTR) 5′ end and ZYMV 3′ end respectively.

RNA analysis of transgenic plants: Total RNA was isolated from transgenic plants essentially as described by Nagy et al. (1988). The RNA was separated by electrophoresis in 1.8% agarose gel containing formaldehyde, and was transferred to nitrocellulose (Schleicher and Schuell Inc.). The Northern blots were probed with random primer 32P- labeled ZYMV-CP cDNA fragment isolated from pCIB710-ZCP plasmid using a Random primer DNA labeling kit (United States Biochemical Corp.)

Protein analysis of transgenic plants: Total soluble protein was extracted from leaf tissue of transgenic plants using the method of (Powell et al., 1986), and the

concentration was determined by the method of Bradford (1976). Protein was separated using a 10% SDS-PAGE gel, with a known amount of purified viral CP as a standard for calculating the expression level of CP in transgenic plants. Electrophoresed proteins were transferred to nitrocellulose. ZYMV CP was detected by rabbit anti-ZYMV CP polyclonal antibodies (Hammar and Grumet, unpublished) and alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies (Sigma).

RESULTS

Construction of three versions of chimeric ZYMV CP gene

To introduce an ATG initiation codon and isolate either the full length CP gene (FL CP) or the potyvirus-conserved central- and carboxyl-terminal region (Core) of the CP gene, *in vitro* mutagenesis was performed to introduce a *Nco* I restriction site, which contains an ATG sequence. The specific primers utilized were:

(1) For the full length CP, primer RG-4, 5′ GAGTG CCTGC CATGG GCATC 3′ (position 486 to 505, see Grumet and Fang, 1990) was used. This resulted in three amino acids changes in the sequence, from leucine-glutamine-serine to proline-methionine-alanine. The only change within the CP gene itself is in the first amino acid, from serine to alanine. Both serine and alanine are commonly found in the first position of potyviral coat proteins.

(2) For the core version of the gene, primer RG-5, 5′ TCCTT GTCCA TGGTG ACAGC 3′(position 610 to 629) was used. This resulted in a single amino acid change from lysine to methionine at the first position within the core portion of the protein.

The ZYMV FL CP gene and the core region (840 and 620 nucleotides respectively) were excised from the plasmid ZY187 as *Nco I - Pst I* fragments (Figure 7),which included the ZYMV 3′ untranslated region (226 nucleotide). The fragments were then transferred into the plasmid pTL37, and linked to the ATG

Figure 7.Genetic engineering of the ZYMV CP gene for expression in plants. A *Nco* I restriction sequence which contains ATG codon was introduced upstream of the CP gene in Bluescript KS derived plasmid pZ187 by *in vitro* mutagenesis. The CP gene and ZYMV 3'UTR were isolated from plasmid pZ187 as an *Nco* I - *Pst* fragment, and cloned into the plasmid pTL 37 to obtain the TEV 5'UTR. The fragment containing the TEV 5' UTR and ZYMV CP gene cDNA was amplified by PCR using primers that also introduced *Bgl* II restriction sequence to both ends of the amplified fragments. The amplified fragments were then cloned into the *Bam* HI site of the plasmid pCIB710, between the CaMV 35S promoter and the CaMV 35S terminator. The ZYMV CP gene expression cassette was removed from pCIB710 by *Xba* and *Kpn* digestion, and inserted into the *Agrobacterium* binary vector pCIB10 between the left (L) and right (R) T-DNA borders and adjacent to the selectable marker gene NPT II. The pCIB10-ZCP construct was transferred into *A. tumafuciens* strain LBA4404 by tri-parental mating. The ZYMV Core and ZYMV AS construction followed the same procedure as above, except using truncated ZYMV CP gene or the reverse orientation of the FL CP gene. The restriction sites are: P, *Pst*; N, *Nco* I; B, *Bgl* II; Bm, *Bam* HI; B/Bm, fusion of *BgI* II and *Bam* HI sites; X, *Xba*; and K, *Kpn*.

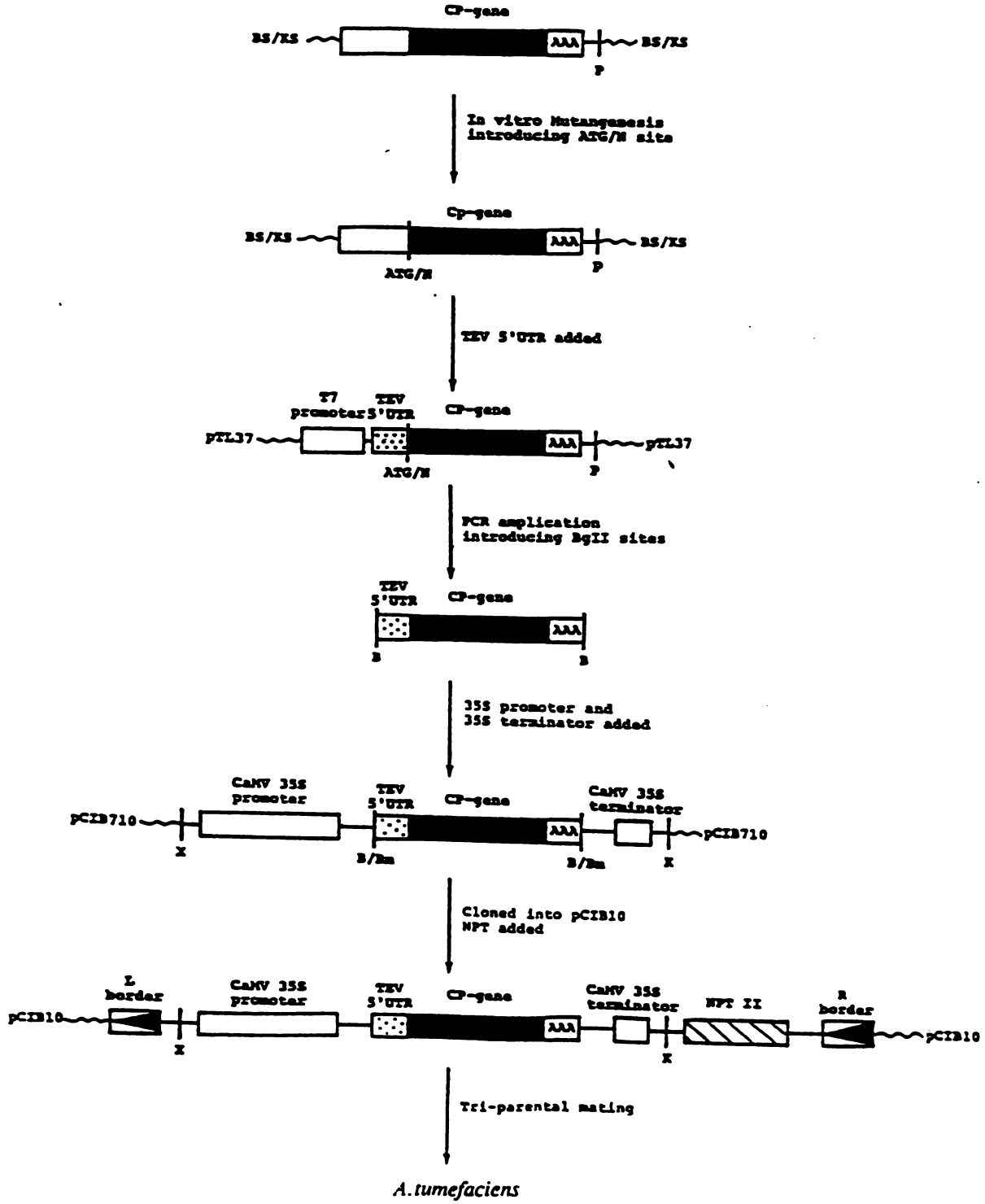


Figure 7. The genetic engineering of ZYMV CP gene for expression in plants

immediately following the tobacco etch virus 5′ untranslated region (TEV 5′UTR), which is a effective translational enhancer (Carrington and Freed, 1990).

To verify the functionality of the translational promotor-CP gene (or Core) construct, the protein products of *in vitro* transcription and translation were examined for correct size by SDS-PAGE. The expected 30 kd CP and 26 kd Core proteins were produced (Figure 8).

The fragments containing the chimeric TEV 5′UTR-ZYMV CP (or core)-ZYMV 3′UTR were then amplified and isolated by polymerase chain reaction (PCR). Two PCR primers were designed for generating *Bgl* II restriction sites at both ends of the fragments during PCR amplificaation:

RG-6: 5′ AGATC TAAAT AACAA ATCTC AACAC 3′ (the 5′ end of TEV UTR), and

RG-7: 5′ AGATCTCTGC AGCCC TTTTT TTTT (ZYMV 3′ end).

The PCR generated fragments were then ligated into the *Bam* HI site of plasmid pCIB710 (Rothstein et al . 1987) between the cauliflower mosaic virus (CaMV) 35S promoter and the CaMV 35S terminator. The resultant clones were analyzed by restriction enzyme digestion to determine the orientation of the inserts with respect to the CaMV 35S promoter and terminator. Constructs containing the FL ZYMV CP gene inserted in both sense and antisense orientations were selected to make the sense and antisense RNAs. Three constructs were generated (Figure 9): ZYMV-CP, which contains the FL CP gene sequence in

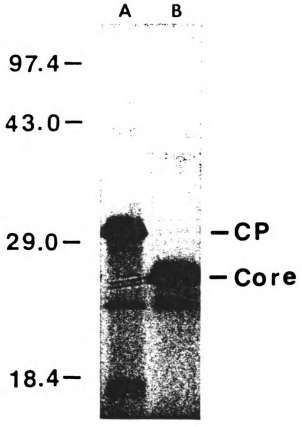


Figure 8. *In vitro* transcription and translation of the ZYMV CP and Core genes. ZYMV CP and ZYMV Core cDNA in plasmid pTL 37 were used as the templates for *in vitro* transcription, and then translated *in vitro* using rabbit reticulocyte lysate (Promega). The protein products were labeled with ^{35}S-methionine. Lane A, translational product when ZYMV FL CP RNA was used as template, lane B, the protein product using ZYMV Core RNA as template.

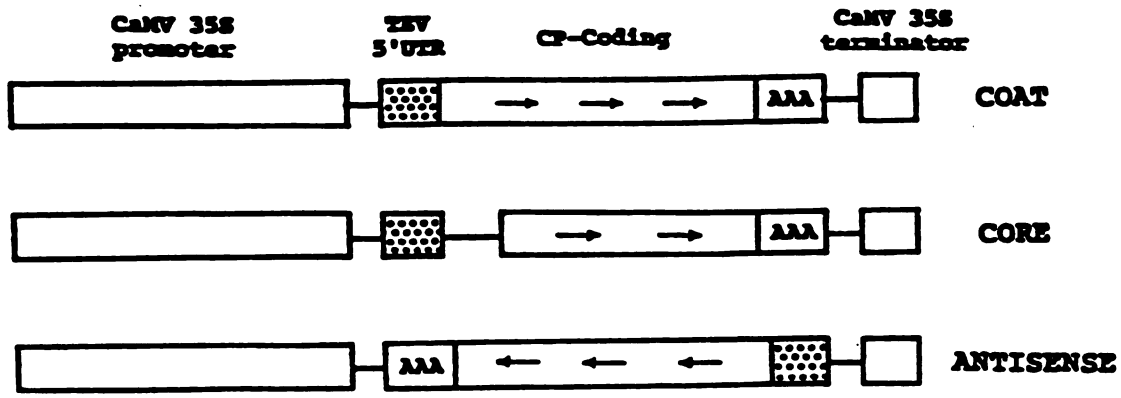


Figure 9. Three versions of the ZYMV CP gene. The "Coat" construct contains the CaMV 35S promoter, the TEV 5′ UTR, the full length ZYMV CP coding sequence, the ZYMV 3′UTR and the CaMV 35S terminator. The "Core" construct contains the truncated ZYMV CP central- and carboxyl- terminal region instead of the FL CP. The "Antisense" (AS) construct includes the same components as "Coat" construct, except that the TEV 5′ UTR, ZYMV CP coding sequence and 3′UTR are inserted in reverse orientation.

the sense orientation; ZYMV-Core, which includes only the 620 nucleotide of the conserved region of the CP gene; and ZYMV-Antisense (AS), which contains the FL CP sequence but in an antisense orientation. All three constructs contain the CaMV 35S promoter, TEV 5′ UTR, ZYMV 3′ UTR and CaMV 35S terminator.

These ZYMV CP gene expression cassettes were excised from plasmid pCIB710 by digestion with the restriction enzymes *Xba* and *Kpn*, and then ligated into the binary vector pCIB10 (Rothstein et al. 1987), adjacent to the transferable plant-selectable marker for kanamycin resistance, the NOS/NPT II chimeric gene. Both ZYMV gene constructs and NPT II gene were located within left and right *A. tumefaciens* T-DNA borders.

Plant transformation

The *A. tumefaciens* binary transformation system was used for introducing ZYMV CP constructs into muskmelon and tobacco plants. Kanamycin resistant plants were initially screened for expression of NPT II protein by ELISA. Approximately 45% of the muskmelon plants regenerated in the presence of kanamycin were NPT II test positive. More than 80% of the tobacco regenerants were found to be NPT II positive.

Most of the regenerated melon and tobacco plants were healthy, morphologically normal, and produced typical flowers and seeds. No symptoms typical of ZYMV or other viruses, such as mosaic or etching leaves were observed on non-inoculated regenerated plants.

The regenerated, NPT-positive melon and tobacco plants were verified for the presence of the inserted ZYMV CP gene sequences by PCR amplification from plant genomic DNA. The expected 1,200 base pair FL CP or 1,000 base pair Core fragments were generated from most (more than 90%) of the 30 tested plants (Figure 10).

Transcriptional analysis of transformants

The transcripts of ZYMV CP constructs were examined by Northern analysis. The results are shown in Figure 11. Hybridization with a labelled ZYMV CP fragment revealed strongly hybridizing bands in transgenic melon (M) and tobacco (T) plants (lane M1, M2, M3, T1, T2 and T3), which were each individually transformed with one of the three versions of ZYMV CP gene. The control, vector transformed plants did not give any signal (lane Cs). The estimated size of the specific transcripts produced by plants transformed by FL ZYMV CP gene (both sense and antisense) was 1,200 bases (lane M1, M2, T1 and T2). This compares well with the size of the RNA that was expected, which is composed of 150 bases of TEV 5′ UTR, 840 bases of ZYMV FL CP sequence and 226 bases of ZYMV 3′ UTR. The ZYMV Core construct transformed plants showed the expected bands of 1,000 bases (lane M3 and T3), including 150 bases of TEV 5′UTR, 627 bases of CP sequence and 226 ZYMV 3′ UTR.

Fourteen of fifteen tested kanamycin resistant, PCR-positive tobacco plants and all eight NPT positive, PCR positive melon plants produced detectable bands of the

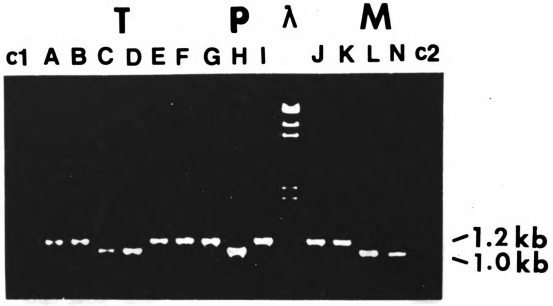


Figure 10. PCR amplified ZYMV CP DNA fragments from transgenic tobacco (T) plants, melon (M) plants and plasmid pCIB710 containing ZYMV CP DNA fragments. The samples from left to right are vector transformed tobacco plant (lane C1); lane A and B, FL CP transformed tobacco plants; lanes C and D, Core transformed tobacco plants; lanes E and F, AS tobacco plants; lanes G, H and I, pCIB710 plasmid containing FL CP, AS and Core respectively; lane J, ZYMV AS transformed melon plant; lane K, FL CP melon plant; lane L and N, Core melon plants; and vector transformed melon plant (lane C2). The primers used in PCR were RG-6 and RG-7. The ZYMV FL CP and AS fragments were approximately 1,200 base pairs, and the ZYMV Core fragment was about 1

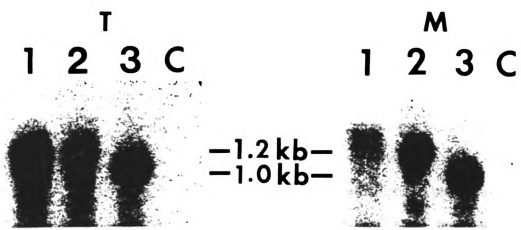


Figure 11. Accumulation of transcripts of CP gene constructs in transgenic plants. The Northern blot was loaded with 10 ug of total RNA isolated from leaves of transgenic melon (M) and tobacco (T) plants. Lane T-1, AS transformed tobacco plant; lane T-2 FL CP tobacco plant; lane T-3 Core tobacco plant; lane T-C, vector transformed control plant; lane M-1 AS transformed melon plant, lane M-2 FL CP melon plant, lane M-3, Core melon plant, and lane M-C, vector transformed melon plant. The blot was hybridized to 32P-labeled cDNA corresponding to ZYMV CP gene.

expected size, even through the expression level was variable (data not shown). These plants had also shown the expected ZYMV CP DNA amplified from total genomic DNA.

Expression of ZYMV coat protein in transformed plants

A total of 22 NPT-positive tobacco plants were tested for presence of ZYMV coat protein by Western analysis. Accumulation of detectable amounts (detection limit = 5 ng) of viral protein of the expected size was found in 3 of 8 tobacco plants transformed with the sense full-length CP gene, and 5 of 8 plants transformed with core fragments. None of 8 plants transformed with the ZYMV AS construct produced detectable ZYMV coat protein. The accumulated level of ZYMV coat protein in transgenic tobacco plants ranged from below 0.01% (detection limit) up to 0.05% of total extracted protein. In a total of 13 transgenic melon plants tested by Western analysis, 2 of 5 plants transformed with sense FL CP, and 4 of 5 plants transformed with Core fragment produced detectable amounts of viral protein. The range of protein level was similar to that of the transgenic tobacco plants, from below 0.01% up to 0.05%. No viral protein was found in all three tested melon plants transformed with ZYMV CP AS constructs.

Figure 12 shows a western blot containing protein from different transgenic melon plants and control vector-transformed plants. The antiserum to ZYMV CP clearly binds to protein extracted from plants transformed with viral sense cDNA (lanes 5 and 6) and viral Core fragment (lanes 3 and 4),

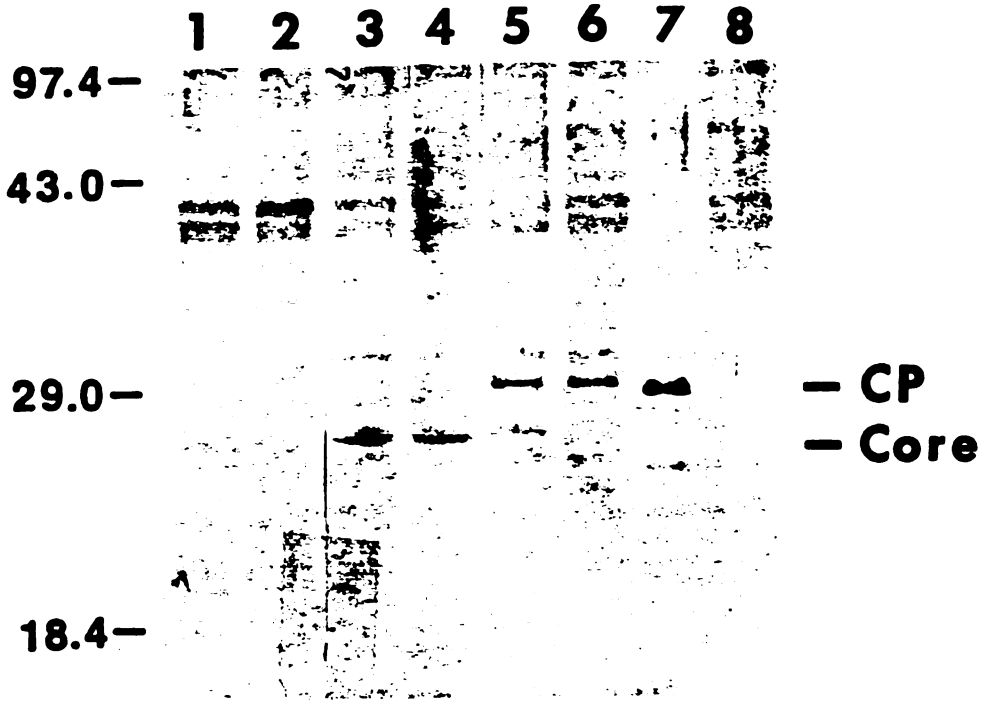


Figure 12. Detection of ZYMV CP and Core proteins in transgenic melon plants. Total soluble protein was isolated from leaf samples of transgenic plants. 50 ug total protein isolated from each of six individual plants transformed with three versions of ZYMV CP gene constructs was separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and treated with rabbit antibody against ZYMV CP , followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Lane 1 and 2 contain protein from AS transformed plants R-103 and R-106, lane 3 and 4 are Core plants, Co-310 and Co-323, lane 5 and 6 are FL CP transformed plants, Cp-401 and Cp-207; lane 7 contains 20 ng of purified ZYMV CP; lane 8 is vector transformed plant. The FL CP is approximately 30 kd, the Core protein is about 26 kd.

TABLE 5. SEGREGATION RATIO IN R_1 TRANSGENIC TOBACCO PLANTS

Plant line[1]	Total No.	NPT+	NPT-	+/_Ratio	X^2_2
CP TO-1	33	25	8	3:1	0.011_{ns}[3]
CP T1-1	72	54	18	3:1	0.000_{ns}
CP T5-3	30	22	8	3:1	0.044_{ns}
CP T8-3	33	24	9	3:1	0.091_{ns}
CP F-7	51	48	3	15:1	0.013_{ns}
CP T3-1	22	0	22	-	
CO B-7	56	47	9	3:1	2.381_{ns}
CO B-2	53	41	12	3:1	0.157_{ns}
CO E-6	22	4	18	-	
AS R-3	45	35	10	3:1	0.185_{ns}
AS A-1	48	44	4	15:1	0.352_{ns}
AS G-1	45	24	21	-	

[1] Plants labeled CP are transformed with the full length CP construct; CO, construct of truncated core portion of the gene; and AS, antisense version of the gene.

[2] $X^2$2 values calculated as $X^2 = \sum[(Io-eI-{}^1/_2)^2/e]$ using the Yate's correction factor

[3] $_{ns}$ - Ratios not significantly different from predicted ratios by X^2 analysis at P+0.05.

but not to the protein from plants transformed with antisense CP cDNA (lanes 1 and 2) or from the control vector transformed plant (lane 8).

Segregation analysis of the inserted genes in the progeny of transgenic plants. To study the inheritance of the inserted genes in transgenic plants, progeny of self-fertilized transgenic tobacco plants were analyzed for the presence of the NPT II gene by ELISA. The results of the progeny analysis of transgenic tobacco plants are shown in Table 5. Progeny from plants CP TO-1, CP T1-1, CP T5-3, CP T8-3, CO B-2, CO B-7 and AS R-3 segregated with a ratio of 3:1 (NPT + :NPT -), indicating that the NPT genes was inserted at a single locus. The segregation ratio of progeny from plants CP F-7 and AS A-1 were 15:1 (NPT +: NPT-), suggesting that the NPT II gene was inserted at two loci. The reason(s) for the aberrant segregation ratios in the progeny from plants CO E-6, AS G-1 and CP T3-1 are unknown.

DISCUSSION

Three versions of ZYMV CP gene, FL CP, truncated central- and carboxyl- terminal of the CP gene (Core), and AS CP have been constructed and successfully expressed in muskmelon and tobacco plants. ZYMV CP and Core fragments were detected in more than 90% of kanamycin resistant plants by PCR amplification. This indicated that the constructs in the T-DNA region between the right and left borders remained intact during transformation. Northern blot analysis further verified the co-integration of the NPT II gene and the ZYMV CP gene. The Western blots for coat protein products revealed the presence of the 30 kd FL ZYMV CP polypeptide and the 26 kd core protein products in transgenic plants transformed with FL CP or Core constructs, but did not detect any similar protein in AS expressing plants. The northern and western results verified that the engineered ZYMV CP gene constructs were functional for expression in plants.

It has been noticed that the protein level of FL CP or Core in different transformants was different, some plants produced higher level of coat protein, which were detected by western analysis, while other plants produced either no protein, or amounts that were below the detection level. On average, levels of protein expression were at least as high, or higher, for Core than FL CP plants. Higher portion of Core plants produced detectable protein than did FL CP plants. The coat protein (or core) in transgenic plants ranged from under

0.01% to 0.05% of the total soluble protein. This is within the range of 0.01% to 0.1% of total plant protein, the expression level that has been reported for engineered viral coat protein in different transgenic plants (e.g. Powell et al., 1986; Van Dun et al., 1987). Transgenic melon plants produced comparable amounts of ZYMV CP protein as transgenic tobacco plants. The plant to plant variability for coat protein level maybe caused by positional effects due to insertion in different chromosomal locations.

The segregation studies of the progeny of transgenic tobacco plants indicated that the ZYMV CP gene constructs were transmitted to the next generation. In most lines the segregation ratios of the progeny were 3:1 (NPT positive: NPT negative), indicating the incorporation of a single gene.

With these verified transgenic, CP expressing R_0 and R_1 melon and tobacco plants, it was then possible to initiate virus testing for resistance against ZYMV and other potyviruses as described in chapter four.

LITERATURE CITED

1. Allison,R.F., Johnston,R. E.,Dougherty,W.G. 1986. The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: evidence for the synthesis of a single polyprotein. Virology 154:9-20

2. Bradford,M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

3. Beachy,R.N., Loesch-Fries,S., Tumer, N.E. 1990. Coat protein-mediated resistance against virus infection. Annu. Rev. Phytopathol. 28:451-474

4. Carrington,J.c., and Freed,D.D. 1990. Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J. Virol. 64:1590-1597.

5. Cuozzo,M., O'Connell,K.M., Kaniewski,W., Fang,R.-X., Chua,N.-H., Tumer, N.E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. BioTechnol. 6:123-143

6. Dellaporta,S.L., Wood,J., Hicks,J.B. 1985. Maize DNA miniprep. *In* Molecular Biology of Plants. Malmberg, Messing, Sussex (eds.) p. 36-37.

7. Dougherty,W.G. and Carrington,J.C.1988. Expression and function of potyviral gene products. Annu. Rev. Phytopathol. 26:123-143.

8. Fang, G.W. and Grumet, R. 1990. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants.

Plant Cell Report 9:160-164

9. Fang, G.W., Hammer, S., and Grumet, R. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques 13:52-56

10. Gal-On,A., Antignus,Y., Rosner,A. and Raccah,B. 1990. Nucleotide sequennce of zucchini yellow mosaic virus capsid-encoding gene and its expression in *Escherichia coli*. Gene 87:273-277

11. Grumet,R. 1990. Genetically engineered plant virus resistance. Hortiscience 25:508-513

12. Grumet, R., Fang,G.-W. 1990. cDNA cloning and sequence analysis of the 3'-terminal region of zucchini yellow mosaic virus RNA. J. Gen. Vriol. 71:1619-1622.

13. Guilley,H., Dudley,R.K., Jonard,G., Balazs,E., Richards,K.E. 1982. Transcription of cauliflower mosaic virus DNA: detection of promoter sequences, and characterization. Cell 30:763-773

14. Hemenway,C., Fang,R.-X., Kaniewski,J.J., Chua,N.-H., Tumer,N.E. 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7:1273-1280

15. Hoekema,A. Hirsch, P.R.Hoykaas,P.J.J. and Schilperoort, R.A. 1983. A binary strategy based on separation of vir and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature 303: 179-180

16. Horsch,R.B. Fry, J.E., Hoffman, N.L., Wallroth,

M.,Eichholz, D., Rogers,S. and Fraley,R.T. 1985. A simple and general method for transferring genes into plants. Science 227:1229-1231

17. Kawchuk,L.M., Martin,R.R., McPherson,J. 1990. Resistance in transgenic potato expressing the potato leafroll virus coat protein gene. Mol. Plant-Microbe Interact. 3:301-307.

18. Kawchuk,L.M., Martin,R.R., McPherson,J. 1991. Sense and antisense RNA-mediated resistance to potato leafroll virus in russet burbank potato plants. Mol. Plant-Microbe Interact. 4:247-253.

19. Lindbo,J.A. and Dougherty, W.G. 1992. Pathogen-derived resistance to a potyvirus: immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:144-153.

20. Ling,K., Namba,S., Gonsolves,C., Slightom, J.L., and Gonsalves,D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. BioTechnol. 9:752-758.

21. Maniatis T, Fritsch E.F., Sambrook, J. 1982. "Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

22. Murashige T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant 15:473-498

23. Nagy,F., Kay, S.A., Chua,N.H. 1988. Analysis of gene expression in transgenic plants. *In* Plant Molecular Biology Manual. B4 1-29. Gelvin, S.B. and Schilperoort, R.A. (eds.) Kluwer Academic Publishers, Dordrecht, Belgium.

24. Niedz R.P., Smith S.S., Dunbar K.B., Stephens C.T., Murakishi H.H. 1989. Factors influencing shoot regeneration from cotyledonary explants of *Cucumis melo* L. Plant Cell Tissue and Organ Cult. 18:313-319.

25. Powell-Abel,P.A., Nelson,R.S., De,B., Hoffman,N., Rogers,S.G., Beachy, R.N.1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743

26. Powell,P., Stark,D.M., Sanders,P.R. and Beachy,R.N. 1989. Protection against tobacco mosaic virus in transgenic plants that express TMV antisense RNA. Proc. Natl. Acad. Sci. USA 86:6949-6952

27. Quemada,H., Sieu,L.C., Siemieniak,D.R., Gonsalves,D., Slightom,J.L. 1990. Watermelon mosaic virus II and zucchini yellow mosaic virus: cloning of 3′-terminal regions, nucleotide sequences, and phylogenetic comparisions. J. Gen. Virol. 71:1451-1460.

28. Rothstein,S.J., Lahners,K.N., Lotstein,R.J., Carozzi,N.B., Jayne, S.M., Rice, D.A. 1987. Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. Gene 53: 153-161

29. Stark,D.M. and Beachy,R.N. 1989. Protection against

potyvirus infection in transgenic plants: evidence for broad spectrum resistance. BioTechnol. 7:1257-1262

30. Van Dun,C.M.P., Bol,J.F. 1988. Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. Virology 167:649-652

CHAPTER FOUR

PLANTS EXPRESSING THE ZYMV COAT PROTEIN GENE CONSTRUCTS ARE PROTECTED AGAINST INFECTION BY ZYMV AND HETEROLOGOUS POTYVIRUSES

INTRODUCTION

Genetically engineered coat protein protection has been used to develop resistance to viruses in several virus groups (for review see Beachy et al., 1990; Grumet 1990). In most of the examples, transgenic plants expressing the CP gene from a given virus were protected against infection by the virus from which the CP gene was isolated (homologous virus). Virus inoculated leaves of the transgenic plants show fewer chlorotic or necrotic lesions relative to control plants, and systemic spread of infection is either prevented, delayed or reduced. CP-mediated protection has also been demonstrated to extend to strains or viruses that are closely related to the virus from which the CP gene was obtained (heterologous viruses). For example, the CP of tobacco mosaic virus (TMV) U1 strain protects against infection by the severe strain of TMV, PV230 (Nelson et al. 1987), and against some other tobamoviruses (Nejidat and Beachy 1990). Expression of the CP genes of the potyviruses soybean mosaic virus (SMV) and papaya ringspot virus (PRV) in transgenic tobacco plants conferred protection against infection by two other potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY, Stark and Beachy, 1989; Ling et al. 1991).

Cucurbit plants are subject to severe losses due to infection by three potyviruses, the watermelon strain of PRV (PRV-W), watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV). Among these ZYMV is a relatively new but

very aggressive member of the potyvirus group that has spread rapidly throughout the world since it was first described in 1981 (Lisa et al. 1981; Davis and Mizuki, 1987). In this project I sought to engineer resistance to ZYMV, to test for both homologous and heterologous protection, and to gain insight into possible mechanisms of protection by using a set of three different CP-derived constructs.

Both melon and tobacco plants were transformed with: (1) the full length ZYMV CP gene; (2) a fragment coding for only the conserved central and C-terminal regions of the protein; and (3) an antisense version of the gene. This chapter describes the effect of these genes on increasing the resistance of transgenic melon and tobacco plants to infection by ZYMV and two heterologous potyviruses, TEV and PVY.

MATERIALS AND METHODS

Preparation of inocula: The Connecticut strain of ZYMV was maintained and increased in zucchini (*Cucurbita pepo* cv. Black Jack) plants. TEV, TMV and PVY are propagated and maintained in tobacco (*Nicotiana tobacum* cv. Samsun) plants. Inocula were made from infected zucchini or tobacco plants by homogenizing leaf tissue in 20 mM KPO_4 buffer, pH 7.0. The homogenate was diluted to a specific concentration (gm leaf tissue : ml buffer volume). PVY, TEV and TMV, were diluted to 1:20, 1:100 and 1:100 respectively. For ZYMV, a 1:75 dilution was used.

Plant material: Muskmelon (*Cucurbita melo* cv. Hale's Best Jumbo) and tobacco (cv. "Samsun") plants transformed with FL-CP, Core or AS of the ZYMV CP gene were used for the virus infection tests. R_1 plants were produced by self pollinating R_0 plants. To produce a set of replicate individuals for virus testing of the transgenic muskmelon R_0 plants, shoots were cut from R_0 transgenic plants and rooted in rooting cubes of the Oasis growing medium (Smithers-Oasis Co.), Plants at 20 days after rooting were used for the infection test. The parent plants were used for seed production. R_1 melon plants at 16 days after seed germination were inoculated with ZYMV (3-4 leaf stage). R_0 tobacco plants with 4-5 leaves, R_1 tobacco plants at 22-25 days or 32-35 days after seed germination were inoculated with PVY, TEV or TMV.

Virus inoculation and observation: Plants were dusted with 400 mesh carborundum on the two youngest expanded leaves

and rubbed with viral inocula. The inoculated plants were observed daily for systemic disease symptom development. Leaf tissues were sampled periodically after inoculation to determine virus level in plants by indirect ELISA. The antibodies used were: anti-ZYMV antibody raised against ZYMV virion (Hammer and Grumet, unpublished), antibody against PVY (Agdia Inc.), and antibody against the potyvirus group (Agdia Inc.), which was used for detecting TEV.

Leaf disk samples were placed in microtiter plates, and then frozen (at -80° C) and thawed twice. The leaf tissue samples were then incubated in coating buffer at 4° C for 16 hours. The samples were reacted with 1:1000 (ZYMV or PVY) or 1:100 (TEV) dilution of antibodies for two hours at 37° C, prior to the addition of alkaline phosphatase-goat anti-rabbit antibody conjugate (Sigma) at 37° C for another two hours. After washing plates, *p*-nitrophenyl-phosphate substrate was added and absorbance at 405 nm was monitored using a Datatech plate reader.

Experimental design for determining protection level in transgenic plants. To test for protection against ZYMV infection in R_0 transgenic melon plants, cuttings from 5-6 independently transformed plants from each group (CP, Core, AS, vector only and non-transformed control) were inoculated with ZYMV by rub inoculation. Symptom development was observed and recorded daily. Samples of the uppermost second and third leaves were collected periodically after inoculation to

quantitate virus titer by ELISA. The protection test was performed on two separate sets of independently transformed R_0 plants from each genotype (a total of 9-16 plants/genotype). The first experiment included vector-only transformed plants as controls in addition to the non-transformed controls. In the resistance test of R_1 melon plants, seventeen NPT+ progeny plants from each transgenic line or non-transformed control were inoculated with ZYMV, symptom development was recorded daily.

In the heterologous protection tests of transgenic tobacco plants, R_0 plants were first screened for resistance against virus infection. Twenty to twenty-five independently transformed plants from each group (CP, Core, AS and Controls) were inoculated with PVY. The R_0 PVY experiments were performed three times, each with different sets of independently transformed plants. A total of 60-70 independent Ro's per constructs were tested. Symptom development was recorded, and virus titer determined by ELISA. Several transgenic plants were allowed to self pollinate to produce progeny. The progeny seedlings of each transgenic line were first verified for the presence of NPT II gene by ELISA (see chapter three). Approximately 10-20 NPT+ plants from each line, 10-20 NPT- plants, and 10-20 non-transformed control plants were inoculated with PVY, TEV or TMV. The PVY and TEV experiments were performed three times, different lines of R_1 progeny were included. The TMV experiment was performed once.

The number of plants showing disease symptoms was recorded daily. Leaf samples were collected 20 and 50 days after inoculation and virus titer was determined by ELISA. The virus level in each line was calculated as the mean of the ELISA readings of individual plants in the group. The data for both melon and tobacco plants were analyzed by t-tests and/or analysis of variance.

RESULTS

1. Protection in transgenic melon plants against ZYMV infection. Transgenic and control melon plants were inoculated with ZYMV, and monitored daily for the appearance of disease symptoms. All of the non-transformed control plants showed symptom ten days after inoculation (Figure 13). Infected leaves exhibited severe mosaic symptoms (Figure 14), and the growth of infected plants was greatly reduced. All younger leaves and shoots displayed the disease symptoms. Symptom development in vector transformed control plants, which had only the NPT II gene, was equivalent to wild type plants in both time to appearance and severity of symptoms (data not shown).

Plants expressing ZYMV core protein, showed a 3-10 day delay in symptom appearance, eventually, however, all become infected (Figure 13). The symptoms on the core-protein expressing plants were milder than for the control plants in most cases. Furthermore, after 3-4 weeks, several of the plants appeared to recover; the newly emerging leaves were either asymptomatic or showed very mild symptoms. Similar results were obtained in both experiments. Five of the six plants expressing ZYMV AS transcripts showed a delay in symptom appearance (3-9 days), the sixth plant, however, did not develop symptoms for at least 90 days post inoculation. The pattern and degree of severity of disease symptoms in three of the five symptom-expressing plants were not

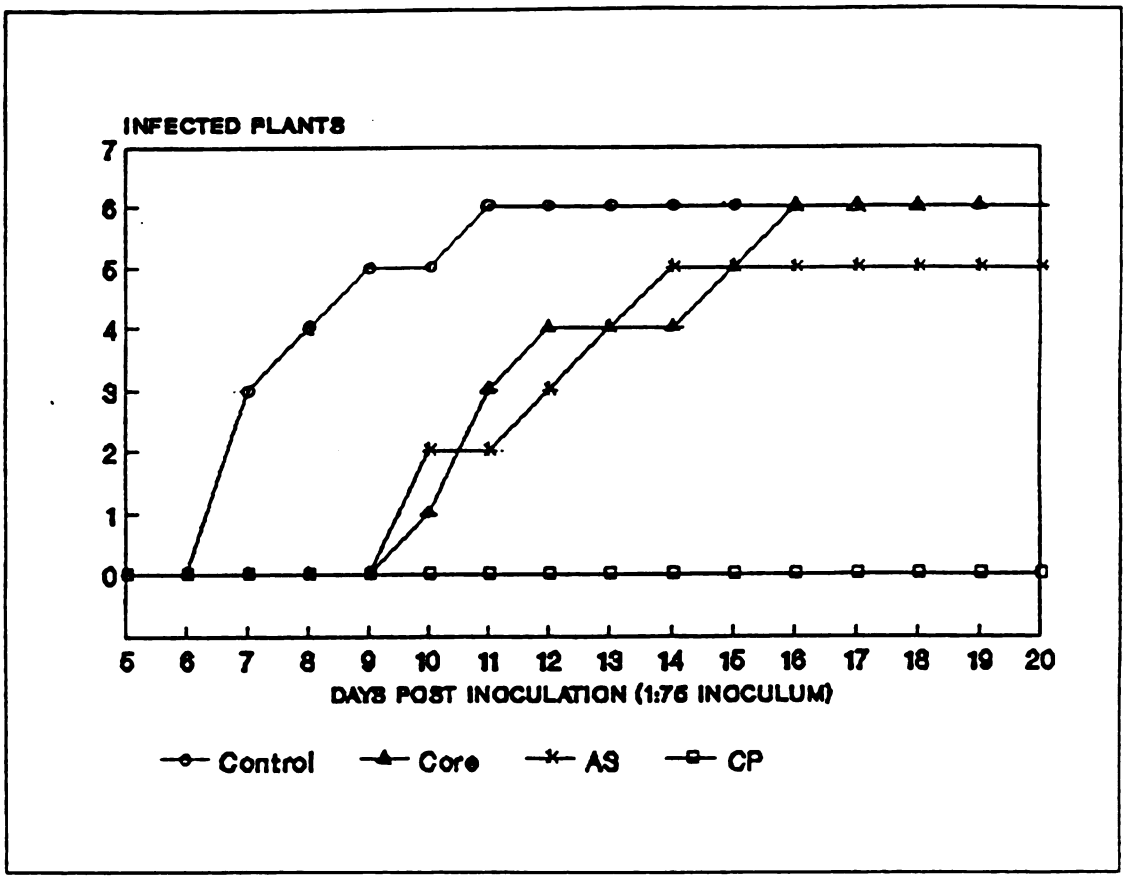


Figure 13. Zucchini yellow mosaic virus (ZYMV) symptom development in Ro transgenic melon plants. The plants were inoculated with a 1:75 (W/V) homogenate of ZYMV infected leaf tissue. CP, plants transformed with ZYMV FL CP construct; Core, plants transformed with ZYMV Core construct, and AS plants transformed with ZYMV antisense CP construct, the control plants are non-transformed plants. Each genotype includes 6 plants. The symbol used to designate each genotype is shown at the bottom of the frame.

Figure 14. Comparison of ZYMV disease symptom in non-transformed control and CP+ transgenic melon plants. Plants were mechanically inoculated with a 1:75 homogenate prepared from leaves of zucchini yellow mosaic virus (ZYMV) infected plant. By three weeks post inoculation, severe classic symptoms (mosaic) of systemic ZYMV infection manifested in the leaf of the control plant (right), whereas the transgenic plant expressing ZYMV CP showed no visible disease symptoms.

obviously different from that of the control plants at two weeks post inoculation; two plants, however, showed milder symptoms. With increasing time (greater than three weeks post inoculation), the symptoms in all four plants were attenuated in younger leaves relative to control plants.

In striking contrast to the core protein and antisense expressing plants, all of the plants producing the full length CP, a total of eleven independently transformed R_0 plants tested in two experiments showed a much higher protection level against ZYMV infection. In both experiments, symptom appearance in all CP+ plants was delayed for at least 30 days. Eight of the eleven plants did not show any disease symptom for at least 90 days post inoculation, three of the eleven plants produced some very mild, modified symptoms 30 days post inoculation.

To compare the extent of virus replication and spread in the transgenic plants expressing constructs of the ZYMV CP gene and the non-transformed control plants, leaf disks were sampled from systemic leaves of each inoculated plant. Virus levels were quantitated by ELISA analysis using antibody against ZYMV. Consistent with the lack of symptoms, the FL CP expressing plants did not accumulate measurable virus levels (Figure 15); the ELISA values were not significantly different from non-inoculated control plants (ANOVA; P=0.05). The virus titer in transgenic plants expressing the Core construct was intermediate to the inoculated controls and the FL CP+ plants.

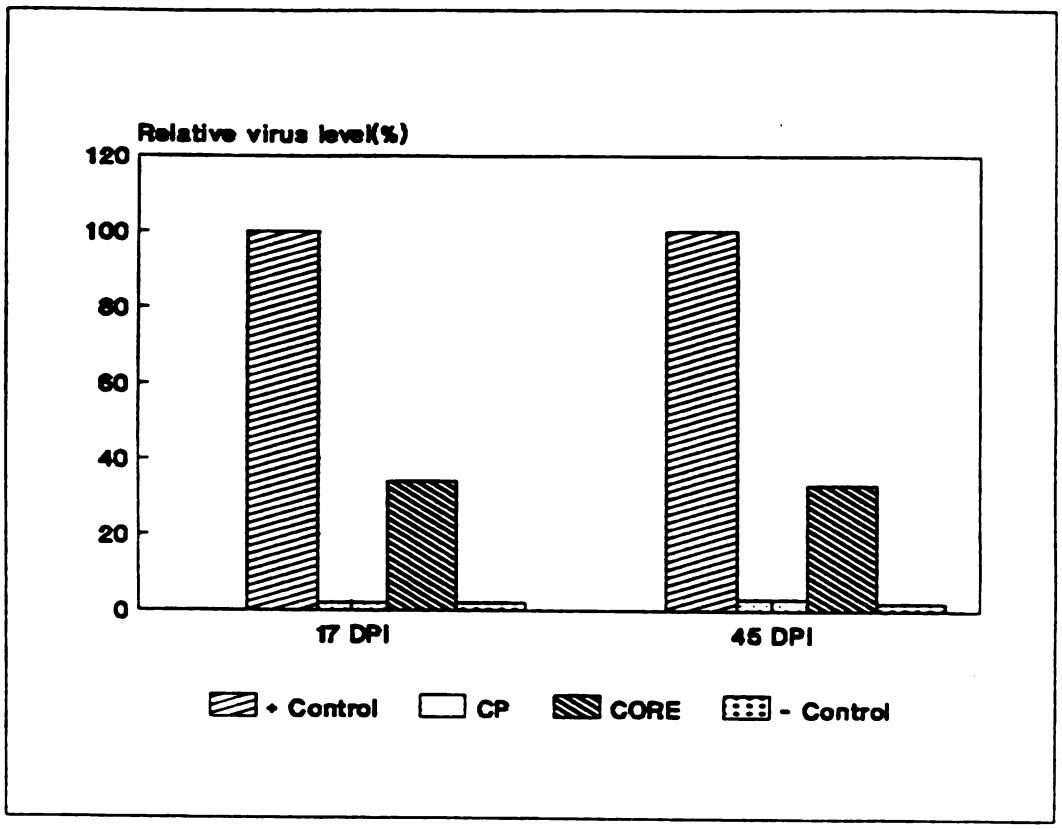


Figure 15. Zucchini yellow mosaic virus (ZYMV) accumulation in R_0 transgenic melon plants and non-transformed control plants. The transgenic plants were transformed with full length ZYMV CP gene (FL CP), Core construct (Core) or antisense construct (AS). The plants were mechanically inoculated with a 1:75 homogenate of ZYMV infected tissue. Duplicate leaf disk samples were obtained from upper non-inoculated leaves 17 days post inoculation (DPI) and 45 DPI, and assayed for presence of ZYMV by ELISA as described in Materials and methods. The relative virus titer in non-transformed control plants was defined as 100%.

The virus levels in the AS plants were highly variable (44% ± 70%) and reflected the variability in the reaction of these plants to infection; e.g. one plant did not have symptoms or measurable virus levels, another had virus levels as high as controls (data not shown). The difference in virus levels among transgenic melon plants and the control plants persisted throughout the observation period (Figure 15). At 45 DPI, as at 16 DPI, the mean values for the transgenic groups was significantly less than that of the controls (ANOVA, P=0.05).

The resistance observed in the R_0 plants was also displayed in their R_1 progeny. The parental lines (CP 401 and Core 310) used to make R_1 progeny both had comparable high levels of viral protein expression. All 17 NPT+ progeny from a FL CP line (CP401) were asymptomatic at 25 days post inoculation (Figure 16), whereas the average time for symptom appearance in control plants is 10 DPI. Several R_1 Core plants (line 310) showed a delay of several days in symptom appearence, but all of them were infected by 21 DPI. These experiments are still in progress to extend the observation period and test additional R_1 lines.

2. Heterologous protection of transgenic R_0 tobacco plants against PVY infection. To determine if transgenic plants expressing ZYMV CP gene constructs could confer protection against heterologous potyviruses, R_0 transgenic tobacco plants were inoculated with PVY. Most of the transgenic plants

displayed enhanced resistance to PVY infection. Vector transformed controls and wild type plants showed disease symptoms at a similar rate (Table 6-A), all controls were infected 7-9 days after inoculation, depending on the experiment. Transgenic tobacco plants expressing full length CP showed a delay in symptom development from several days to a few weeks when compared to the control plants; a few plants did not exhibit a delay in symptom , whereas several other plants were asymptomatic for the whole growth period. A several days delay of symptom appearance was also observed in plants expressing Core or AS. Virus levels in systemic leaves were determined by ELISA assay using antibody against PVY. Those plants transformed with vector only had similar virus titer to the non-transformed controls (Table 6-B). The virus titer for the transgenic plants expressing the different CP constructs, however, was significantly reduced relative to the controls at 25 DPI (Table 6-B). This reduction in virus titer persisted for at least 45 days (data not shown). Those plants that did not become infected had no measurable virus titer; when several asymptomatic R_0 plants were tested as a virus source by rub inoculation, they did not transmit virus.

3. Protection of R_1 transgenic tobacco lines against PVY infection. The R_1 progeny of several transgenic lines were rub inoculated with PVY at 1:20 or 1:100 inoculum, and observed for symptom development. All "Samsun" non-transformed controls became infected within 6 days after inoculation with 1:20

TABLE 6-A. PVY SYMPTOM DEVELOPMENT IN Ro TRANSGENIC TOBACCO PLANTS[1]

Genotype	#	PVY infected plants (%) DPI 5	6	7	8	9	10	11	12	13	14
Control	23	0	26	74	91	100	100	100	100	100	100
Vector only	19	10	67	84	95	100	100	100	100	100	100
CP	25	0	0	0	16	78	84	88	88	88	88
Core	22	0	0	18	68	73	77	86	100	100	100
AS	23	0	0	0	13	22	69	74	74	83	83

TABLE 6-B. PVY VIRUS TITER IN TRANSGENIC Ro TOBACCO PLANTS AT 25 DAYS POST INOCULATION

Genotype	plants with symptoms/ total plants	mean Abs	relative amount	range
Control	16/16	1.012	1.00	0.591-1.373
Vector only	16/16	0.914_{NS}[2]	0.90	0.668-1.251
CP	21/24	0.344**	0.34	0.750-0.920
- symptoms	3/24	0.088**	0.09	0.075-0.107
+ symptoms	21/24	0.379**	0.37	0.101-0.920
Core	13/13	0.685**	0.68	0.328-0.917
AS	20/24	0.219**	0.22	0.600-0.708
- symptoms	4/24	0.085**	0.08	0.060-0.110
+ symptoms	20/24	0.245**	0.24	0.088-0.708

[1] The plants were inoculated with a 1:100 homogenate of PVY infected tissue

[2] $_{NS}$.= value not significantly different from control by t-test; **, significantly less than control by t-test,

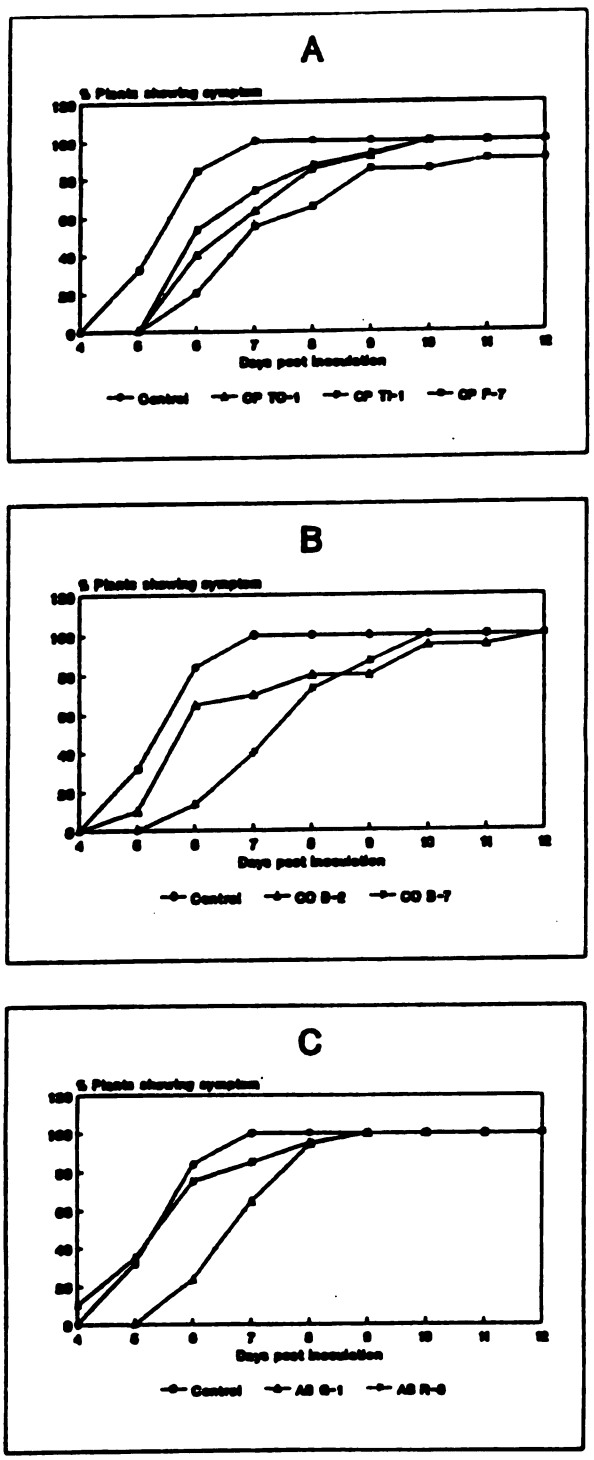


Figure 17. Potato virus Y (PVY) symptom development in tobacco R1 transgenic lines. Percentage of plants showing symptoms at daily intervals post inoculation with a homogenate of 1:20 (W/V) of PVY infected leaf tissue. (A). Seedlings of transgenic lines of CPTO-1 and CPF-1, which were the progeny of the plants transformed with ZYMV FL CP construct, and seedlings of non-transformed plant. (B). R_1 progeny of the lines transformed with ZYMV Core construct. (C). R_1 progeny of the lines transformed with ZYMV antisense CP construct. The symbol used to designate each plant line is shown at the bottom of the frame. Results were obtained from the infection of about 20 R_1 plants from each line.

inoculum (Figure 17).

Segregating R_1 progeny were tested for presence or absence of the introduced NPT gene by NPT II ELISA (see chapter three, Table 5). Those that did not express the NPT II gene showed the same rate of symptom appearance as the wild type controls (data not shown). In the NPT positive individuals that had been transformed with CP constructs, disease symptom appearance was delayed for 2-5 days in the majority of the plants compared to the controls (Figure 17-A), a few plants from the CPF-7 remained asymptomatic for 3-4 weeks. The disease symptoms in most plants were milder, and often younger leaves were devoid of disease symptoms. Virus accumulation as determined by ELISA was correlated with the degree of visual symptoms (Table 7). NPT positive plants in line CoB-2 and line CoB-7, which were transformed with the ZYMV core gene, also showed a 1-4 day delay in symptom appearance (Figure 17-B); the disease symptoms were milder than for the control plants. Plants expressing antisense CP transcripts, line ASG-1 and line ASR-3, displayed a short delay in symptom development, from 1-2 days (Figure 17-C), the symptoms were slightly attenuated. The difference in symptoms and virus titer between most transgenic lines and the controls were persistent during the observation period of 50 days post inoculation (data not shown).

4. TEV protection in transgenic R_1 tobacco plants: The R_1 transgenic tobacco lines were further tested by inoculation

with another potyvirus, TEV. Symptom appearance for most of the transgenic individuals expressing ZYMV CP constructs was delayed for 2-4 days relative to the controls (Figure 18-A). All of the non-transformed control plants and the NPT II negative plants were infected 7 days after inoculation with 1:100 inoculum of TEV, in contrast, only 40-80 % of the transgenic plants within a given line showed diseased symptoms at the same time. Symptoms were milder in most transgenic plants than the control plants. Virus titer was also significantly reduced relative to the controls (Table 7). All of the transgenic lines (CP, Core or AS) displayed similar patterns and protection levels (Figure 18).

5. ZYMV CP constructs did not confer protection against TMV infection. To determine if protection in transgenic plants expressing the different forms of the ZYMV CP gene was specific to members of the potyvirus group, R_1 progeny were challenged with a tobamovirus, TMV. There was no obvious difference between transgenic plants expressing any of three forms of the ZYMV CP gene and the control plants in time to symptom appearance (Figure 18), or symptom severity in systemic leaves post TMV inoculation.

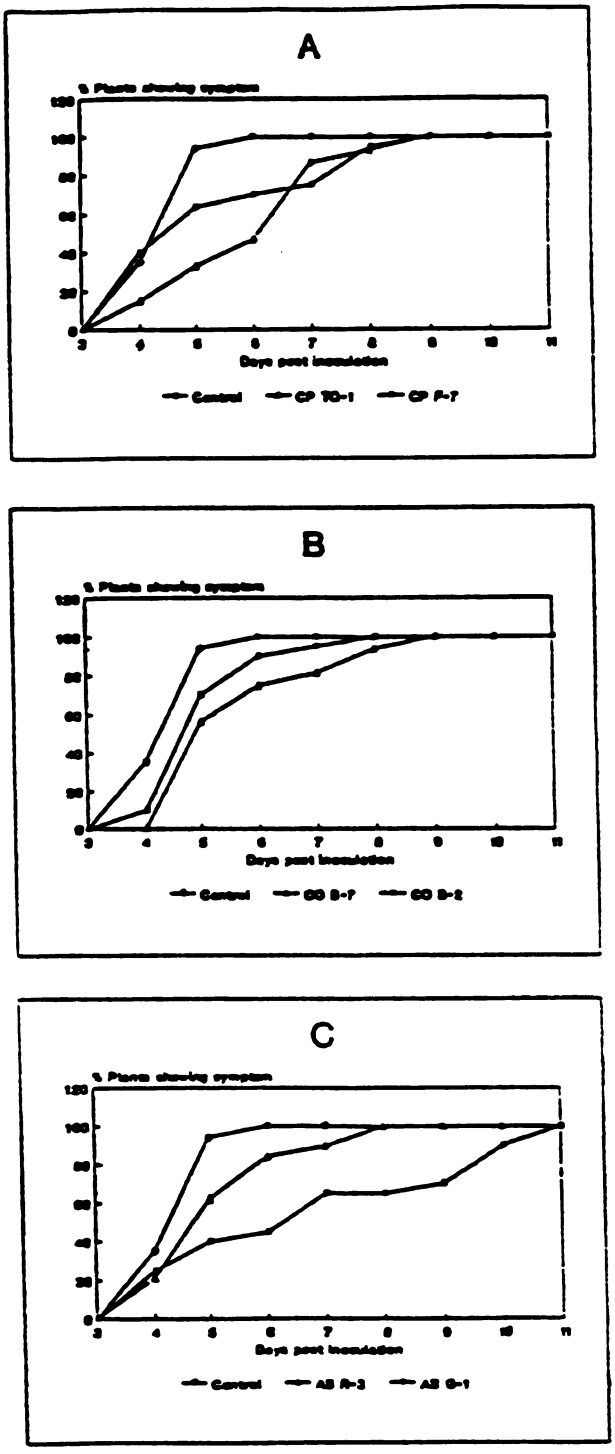


Figure 18. Tobacco etch virus (TEV) symptom development in tobacco R1 transgenic lines. Plants were inoculated with a homogenate of 1:100 (W/V) of TEV infected leaf tissue. (A). Seedlings of transgenic lines of CPTO-1 and CPF-1, which were the progeny of the plants transformed with ZYMV FL CP construct, and seedlings of non-transformed plant. (B). R1 progeny of the lines transformed with ZYMV Core construct. (C). R1 progeny of the lines transformed with ZYMV antisense CP construct. The symbol used to designate each plant line is shown at the bottom of the frame. Results were obtained from the infection of about 20 R1 plants from each line.

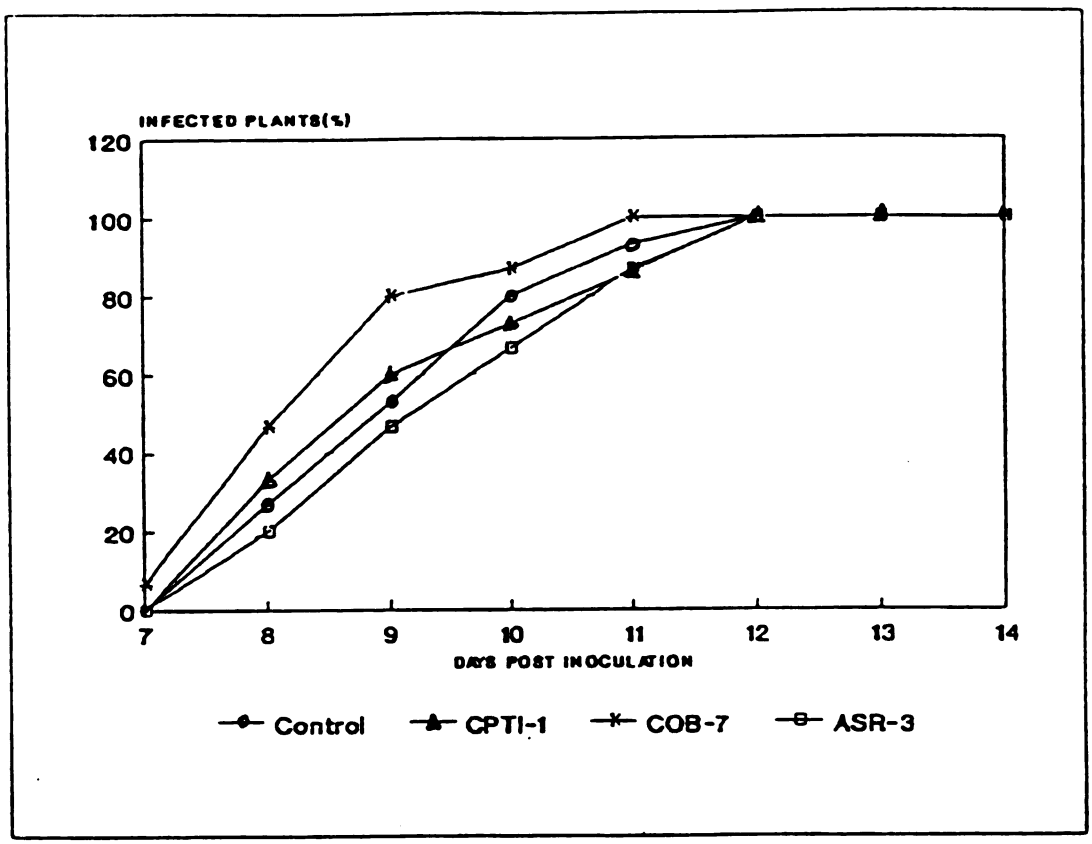


Figure 19. Tobacco mosaic virus (TMV) symptom development in tobacco R1 transgenic lines. Plants were ninoculated with 1:100 (W/V) of TMV infected leaf tissue. The symbol used to designate each plant line is shown at the bottom of the frame. Results were obtained from the infection of about 20 R1 plants from aech line.

DISCUSSION

These results demonstrate that transgenic melon plants expressing the full length ZYMV coat protein gene are highly resistant to infection by ZYMV. The majority of the CP-expressing R_0 melon plants remained asymptomatic for a period of at least two to three months. These plants also had no measurable virus accumulation, while all of the inoculated control plants had a high level of virus. The striking resistance observed in the R_0 plants was also evident in their R_1 progeny. As of 25 days post inoculation the progeny did not exhibit symptoms, whereas the average time to symptom development in the control plants was 10 DPI. The R_1 experiments are still in progress.

The potyvirus group is the largest and most agriculturally important group of plant viruses, but at present, reported examples of genetically engineered resistance to potyviruses are limited. Three reports of CP-mediated protection in potyviruses used PVY, TEV and plum pox virus (PPV) CP genes to protect transgenic plants against their corresponding viruses respectively (Lawson et al., 1990; Lindo and Dougherty 1992; Regner et al., 1992). Our experiments extended genetically engineered virus resistance to an additional economically important potyvirus, ZYMV. Furthermore, with the exception of alfalfa plants that have been engineered for resistance to AlMV, all of the examples of engineered plant virus resistance to date have been limited to Solanaceous species, tobacco,

tomato or potato plants. Demonstration of resistance in additional non-Solanaceous species further verifies the general applicability of this approach as a possible method to develop virus resistant plant cultivars.

Virus infection tests in transgenic tobacco plants showed that expression of different forms of the ZYMV CP gene also resulted in limited protection against two heterologous, Solanaceous potyviruses, TEV and PVY. Heterologous CP-mediated protection has been reported for two other members of the potyvirus group, SMV and PRV (Stark and Beachy 1989, Ling et al. 1991) against TEV and PVY. Compared to the delay conferred by the SMV and PRV coat proteins, the heterologous protection levels in our experiment are not as strong. The differences in effectiveness against heterologous viruses may related to the individual relationships among the different viruses. Despite the limited protection, these results contribute support to the hypothesis that it may be possible to protect against several viruses by using a limited number of different CP genes (Stark and Beachy 1989). Since many crops are often infected by several members of the potyvirus group, this phenomenon would make it much easier to develop resistance to the potyviruses. Of particular interest is to determine whether the ZYMV-CP gene will confer protection against the other potyviruses which infect cucurbit plants, e.g. PRV-W and WMV.

In all but one case of genetically engineered, coat

protein-mediated resistance, full length coat protein genes were used. To gain insight into the mechanism of protection, and to determine whether it would be possible to confer more broad scale protection, we also tested an amino terminal truncated coat protein construct. Potyviral coat proteins are noted to have highly conserved central and carboxyl terminal regions, the 'trypsin resistant core' portion of the protein, and highly variable amino terminal portions (Shukla and Ward, 1989). Since trypsin treated virion still appear intact in the electron microscope, and are still infectious by rub inoculation, the domains within the CP that are responsible for assembly, CP-RNA interaction and CP-CP interaction, are thought to reside within the core portion of the protein (Shukla et al., 1988; Dougherty et al., 1985). It has been hypothesized for several system that CP-mediated protection involves CP-RNA or CP-CP interaction (Beachy et al., 1990; Grumet, 1990). If these processes are critical for protection against potyvirus infection, then the core portion of the protein would be expected to confer resistance.

Interestingly, although the core portion was expressed at levels comparable to the FL CP (Core310 and CP401, see chapter three) and did confer some protection (several days delay and reduced virus titer), it was not as effective as the FL-CP construct which resulted in near immunity. Possibly the core and amino terminus of the protein interfere with virus infection at different stages of the process, or the full

length CP may have higher affinity for viral RNA or other CP molecules than does Core. In the case of protection against the heterologous viruses TEV and PVY, both the FL-CP and Core constructs performed similarly. It may be that the function provided by the core portion or its RNA, which results in a delay in infection and reduction in virus titer, is capable of acting on more than one potyvirus. In contrast, the effect of the amino terminus, which is highly virus specific, may be limited to the virus from which the CP gene was derived.

These results are somewhat different from the report by Lindo and Dougherty (1992), which is the only other example using a truncated CP gene to engineer virus resistance. In their experiments, unlike those with PVY, PPV and ZYMV, the full length TEV CP gene provided little or no protection against TEV infection. On the other hand, tobacco expressing truncated TEV CP were more protected from TEV infection than were transgenic plants expressing the FL TEV CP. The constructs lacking C-terminal amino acids or the constructs lacking both C- and N-terminal amino acids resulted in much better protection against TEV infection than constructs lacking N-terminal amino acids only, which did not show obvious protection against TEV. The reason (s) for the difference between these experiments is unclear.

The heterologous protection experiments also give insight into the possible role of the TEV 5′ UTR. All three forms of ZYMV CP gene constructs resulted in relatively similar

protection against both heterologous viruses TEV and PVY. This indicates that the 5′ UTR from TEV may not be a critical factor in conferring protection. Although it may have contributed to protection overall, either by increasing the translational efficiency of the CP constructs, or by interacting with the viral RNA, there did not appear to be a virus specific effect in its interaction with TEV vs. PVY.

Plants expressing antisense RNA of the ZYMV CP plus the TEV 5′UTR resulted in variable protection levels in transgenic melon plants against potyvirus infection. On average the AS constructs were similar to the core constructs, raising the possibility that it was the core RNA rather than the protein that was important. In most virus system example to date (e.g. CMV, PVX, TMV or AlMV) antisense CP genes have resulted in little or no protection (Cuozzo et al., 1988; Hemenway et al.,1988; Powell et al.,1989; and Van Dun et al., 1988). However, there were two exceptions published recently, where it was hypothesized that it was the RNA that conferred protection. Potato plants expressing antisense CP RNA (including 5′ UTR) of potato leafroll virus (PLRV) displayed a high level of resistance to PLRV infection, the pattern and level were similar to plants producing sense RNA (Kawchuk et al. 1991). In transgenic tobacco plants, expression of antisense or translationally-deficient sense TEV CP RNA resulted in much higher protection against TEV infection than that by expression of the sense version of FL coat protein

gene (Lindo and Dougherty, 1992). To understand the mechanism of antisense RNA related protection, and to determine the effectiveness of the strategy, further investigations are required.

In summary, we have demonstrated that transgenic melon plants expressing three forms of ZYMV CP gene were protected against ZYMV infection. The best protection, near immunity was observed for melon plants that express the full length ZYMV CP gene and were inoculated with ZYMV. Heterologous protection against two other potyviruses was also displayed in transgenic tobacco plants expressing ZYMV CP constructs. Melon plants expressing FL CP displayed higher protection levels than Core or AS expressing transgenic plants. To further evaluate the potential usefulness of this resistance in cucurbits, future experiments with the ZYMV CP expressing plants would include test against other ZYMV strains and against other cucurbit potyviruses, tests using aphid inoculation and tests of field performance.

LITERATURE CITED

1. Alderz, W.C. 1987. Cucurbit potyvirus transmission by alate aphids trapped alive. J. Economic Entomol. 80:87-92.

2. Anderson, E.J., Stark, D.M., Nelson, R.S., Powell, P.A., Tumer, N.E. and Beachy, R.N. 1989. Transgenic plants that express the coat protein genes of TMV and AIMV interfere with disease development of some non-related viruses. Phytopathol. 79:1284-1290.

3. Beachy, R.N., Loesch-Fries, S., Tumer, N.E. 1990. Coat protein mediated resistance against virus in fection. Annu. Rev. Phytopathol. 28:451-474.

4. Carrington, J.C. and Freed, D.D. 1990. Cap-independent enhancement of translation by a plant potyvirus 5′ nonotranslated region. J. Vriol. 64:1590-1597

5. Cuozzo, M., O′connell, K.M., Kaniewski,W., Fang,R.-X., Chua, N.-H., Tumer,N.E. 1988. Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat proetin or its antisense RNA. BioTechnol. 6:123-143.

6. Davis, R.F. and Mizaki, M.K. 1985. Detection of cucurbit viruses in New Jersey. Plant disease 71:40-44.

7. Dougherty, W.G., Willis, L. and Johston, R.E. 1985. Topographic analysis of tobacco etch virus capsid protein epitopes. Virol. 144:66-72

8. Grumet, R. 1990. Genetically engineered plant virus resistance. Hortiscience 25:508-513.

9. Grumet, R. and Fang, G. 1990. cDNA cloning and sequence

analysis of the 3'-terminal region of zucchini yellow mosaic virus RNA. J.Gen. Virol. 71:1619-1622.

10. Hemenway, C., Fang, R.-X., Kaniewski, J.J., Chua, N.-H., Tumer, N.E. 1988. Analysis of the mechansim of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7:1273-1280.

11. Kawchuk, L.M., Martin, R.R., McPherson, J. 1991. Sense and antisense RNA-mediated resistance to potato leafroll virus in russet burbank potato plants. Mol. Plant-Microbe Interact. 4:247-253.

11. Lawson, C., Kaniewiski, W., Haiey, L., Rozman, R., Newell, C., Sanders, P., and Tumer, N.E. 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. BioTechnol. 8:127-134.

12. Lecoq, H. and Pitrat, M. 1985. Specificity of the helper-component-mediated aphid transmission of three potyviruses infecting muskmelon. Phytopathol. 75:890-893.

13. Lindo, J.A. and Dougherty, W.G. 1992. Pathogen-derived resistance to a potyvirus: immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol.Plant-Microbe Interact. 5:144-153.

14. Ling, K., Namba, S., Gonsolves, C., Slighton, J.L. and Gonsalves, D. 1991. Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing

the papaya ringspot virus coat protein gene. BioTechnol. 9:752-758.

10. Loesch-Fries, L.S., Halk, E., Merlo, D., Jarvis, N., Nelson, S. 1987. Expression of alfalfa mosaic virus coat protein gene and anti-sense cDNA in transformed tobacco tissue.

11. Miller, W.A., Bujarski, J.J., Dreher, T.W. and Hall,T.C. 1986. J. Mol. Biol. 187:537-546.

12. Nejidat, A and Beachy, N. 1990. Transgenic tobacco plants expressing a coat protein gene of tobacco mosaic virus are resistant to some other tobamoviruses. Mol. Plant-Microbe Interactions 3:247-251

13. Nelson, R.S., Powell-Abel, P., and Beachy, R.N. 1987. Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virol. 158:126-132.

14. Powell-Abel, P.A., Stark, D.M., Sanders, P.R. and Beachy, R.N. 1989. Protection against tobacco mosaic vitus in transgenic plants that expree TMV antisense RNA. P.N.A.S. USA 86:6949-6952.

15. Regner, F., Machado, A.C., Machado, M.L.C., Steinkellner, H., Mattanovich, D., Hanzer, K., Weiss, H., and Katinger, H. 1992. Coat protein mediated resistance to Plum Pox Virus in *Nicotiana clevelandii* and *N. benthamiana*. Plant Cell Report 11:30-33

16. Shulka,D.D., Strike,P.M., Tracy,S.L., Gough,K.H.,

Ward,C.W. 1988. The N- and C-termini of the coat protein of potyviruses are surface located and the N-terminus contains the major virus-specific epitopes. J. Gen. Viol.69:1497-1503

17. Shukla, D.D. and Ward, C.W. 1989. Structure of potyvirus coat protein and its application in the taxonomy of the potyvirus group. Adv. In Virus Res. 36:273-314.

18. Stark, D.M. and Beachy, R.N. 1989. Protection against potyvirus infection in transgenic plants: evidence for broat spectrum resistance. BioTechnol. 7: 1257-1262.

19. Van Dan, C.M.P. and Bol, J.F. 1988. Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. Virol. 167:649-652.

A Quick and Inexpensive Method for Removing Polysaccharides from Plant Genomic DNA

ABSTRACT

A quick and inexpensive method has been demonstrated to remove polysaccharide contamination from plant DNA. Isolated plant genomic DNA with polysaccharide contaminants was dissolved in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) with NaCl ranging from 0.5–3.0 M, then precipitated with two volumes of ethanol. Most of the polysaccharides were removed effectively in a single high-salt precipitation at 1.0–2.5 M NaCl. At 3.0 M NaCl, the salt precipitated out of solution. Purified DNA was easily digested by either HindIII *or* EcoRI *and was satisfactory as a template for PCR. The results show that high-salt precipitation effectively removed polysaccharides and their inhibitory effects on restriction enzyme and* Taq *polymerase activity.*

INTRODUCTION

Polysaccharide contamination is the most common problem affecting plant DNA purity (10). These carbohydrates can inhibit the activity of many molecular biological enzymes, such as polymerases, ligases and restriction endonucleases (1,9,12,13), and can interfere with concentrating the DNA sample. Unfortunately, most plant DNA isolation and purification methods do not efficiently separate polysaccharides from DNA (3,6,7,10,12). Phenol-chloroform extractions are only partially helpful, and the use of CTAB (cetyltrimethylammonium bromide) often results in DNA degradation (unpublished results and personal communications). It was recently reported that Elutip-D (RPG-5 type resin) (Schleicher & Schuell, Keene, NH) columns can be used to remove polysaccharides from DNA (5). Although effective and simple, this procedure is volume-limited, time-consuming and relatively expensive. We sought to adapt the column procedure to a batch method using ion-exchange resins. In doing so, we found that the high-salt buffer used to elute the DNA from the resin was by itself sufficient to remove the polysaccharide.

In this report we show that by dissolving polysaccharide-contaminated plant DNA samples in TE buffer with high salt (1.0–2.5 M NaCl) and then precipitating with ethanol, the polysaccharide remains in solution with the ethanol rather than precipitating with the DNA. This quick and inexpensive method can be used with any DNA isolation and purification protocol. We tested the efficiency of different concentrations of salt (NaCl) in separating polysaccharides from DNA samples. The DNA quality was evaluated by restriction enzyme digestion and by using the DNA as a substrate for PCR.

MATERIALS AND METHODS

Greenhouse-grown muskmelon (*Cucumis melo*) and cucumber (*C. sativus*) leaves were chosen for DNA isolation because the DNA from these plants is often contaminated with large amounts of polysaccharides. DNA was also isolated from potato (*Solanum tuberosum*), soybean (*Glycine max*) and geranium (*Pelargonium*) leaves. Young leaves were quickly frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Genomic DNA was isolated according to the protocol of Dellaporta et al. (4). Isolated DNA was then extracted three times with phenol:chloroform:isoamyl alcohol (24:24:1) and precipitated with two volumes of 95% ethanol. The DNA pellet was redissolved in TE, the appropriate amount of 4 M NaCl was added to give a final concentration ranging from 0.5–3.0 M, and the sample was reprecipitated with ethanol. For effective polysaccharide removal, it was very important to completely dissolve the DNA in TE prior to the addition of salt. Alternatively, NaCl was added to the sample directly after phenol extraction thereby eliminating the need for two ethanol-precipitation steps. The resulting DNA pellet was washed twice with 75% ethanol and dissolved in TE.

Polysaccharide contamination was determined visually (noticeably viscous pellets) by refractometry (ABBE-3L; Milton Roy, Rochester, NY) or by the phenol-sulfuric acid method of Ashwell (2). Pure salmon sperm DNA gave a negligible refractometric reading (0.01 for 1 µg DNA/µl) and a negligible carbohydrate reading as estimated by the phenol-sulfuric acid assay (0.06 µg/µg DNA). Melon and cucumber DNA samples that were precipitated with or without high salt (2 M NaCl) were digested with *Hin*dIII and *Eco*RI and examined on a 0.8% agarose gel. Genomic DNA isolated from transgenic melon plants (8) was also used as template for PCR. PCR was performed according to the manufacturer's protocol (Perkin Elmer, Norwalk, CT) using 100 ng of DNA template for each reaction and two 18-mer primers specific for the neomycin phosphotransferase (NPT) gene [NPT sequence data from (11) and an annealing temperature of 50°C]. The products were examined on a 1% agarose gel.

RESULTS AND DISCUSSION

Polysaccharide contamination is a common problem when isolating DNA from many plant species (10). For example, a typical DNA sample prepared from young melon leaves, extracted with phenol and chloroform, and precipitated with ethanol was found to contain about 3 µg carbohydrate (assayed by the phenol-sulfuric acid method) per µg DNA. This polysaccharide contamination is readily apparent by DNA pellets that are extremely viscous. The refractive index of the polysaccharide-contaminated sample was 4.0 (the refractive index of salmon sperm DNA at a comparable concentration [1 µg/µl] was 0.01; i.e., the DNA itself does not contribute significantly to the refractive index readings).

If the DNA was precipitated in the presence of the proper concentration of salt (NaCl), the polysaccharide remained in solution and was discarded with the ethanol supernatant. With increasing NaCl concentrations up to 2.5 M, there was a visible decrease in polysaccharide content. This was evident as a decrease in total dissolved solids as measured by the refractive index (Figure 1). Although the refractive index varied, the amount of DNA in each

sample as determined by spectrophotometry and/or ethidium bromide staining was equivalent (approximately 1 µg/µl).

One molar of salt could remove most of the polysaccharide in a single precipitation. For DNA containing very high levels of polysaccharide, however, 1.5 M or 2.0 M NaCl were more effective. Refractometry readings indicated that as much as 90% of the total dissolved solids were removed by precipitation with 1–2.5 M NaCl. Higher salt concentrations resulted in salt precipitation that was difficult to remove. The efficiency of DNA recovery was above 90% with all salt levels tested.

Salt (2 M NaCl) was also effective in removing polysaccharide from DNA samples of potato, soybean and geranium (data not shown). For these species as well as cucumber and melon, the procedure was most successful when DNA was prepared from young leaves. In some cases a second precipitation with high salt was helpful in removing additional polysaccharide.

The quality of DNA following precipitation in the presence of high salt (TE + 2 M NaCl) was evaluated by digestion with the restriction enzymes *Hin*dIII and *Eco*RI. Melon DNA precipitated from TE (containing polysaccharide) was digested only partially, whereas the high-salt treatment resulted in polysaccharide-free DNA that was digested completely (Figure 2A). Digestion of cucumber DNA showed similar results (data not shown). Precipitation in the presence of high salt also improved the quality of the DNA as a template for PCR. DNA samples isolated from transgenic melon plants were precipitated in TE or TE + 2 M NaCl, washed in 70% ethanol and used as template for amplifying the introduced NPT-coding DNA fragment. Expected fragments were found only in the reaction using high salt precipitated DNA as template (Figure 2B). Therefore, precipitation in the presence of high salt is useful in removing polysaccharides that inhibit restriction enzyme and *Taq* DNA polymerase activity. Overall, the high-salt procedure to remove polysaccharides from DNA is quick, convenient, inexpensive and is not volume limited.

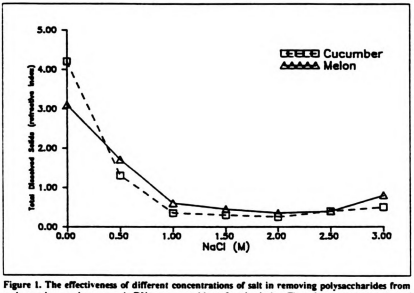


Figure 1. The effectiveness of different concentrations of salt in removing polysaccharides from melon and cucumber genomic DNA as assayed by refractive index. The refractive index reading of 1 µg/µl salmon sperm is 0.01.

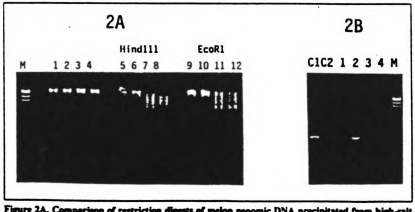


Figure 2A. Comparison of restriction digests of melon genomic DNA precipitated from high-salt TE (TE + 2 M NaCl) or TE only. Lane M, *Hin*dIII-cut lambda DNA; lanes 1 and 2, uncut DNA with polysaccharide contamination; lanes 3 and 4, uncut high-salt-precipitated DNA (most polysaccharides are removed); lanes 5, 6, 9 and 10, polysaccharide-contaminated DNA digested with *Hin*dIII or *Eco*RI; lanes 7, 8, 11 and 12, high-salt-precipitated DNA digested with *Hin*dIII or *Eco*RI. Figure 2B. PCR product comparison using melon genomic DNA that was precipitated from TE or TE + 2 M NaCl. Lane M, lambda DNA marker; lane C1, plasmid containing the NPT gene (positive control); lane C2, non-transformed genomic DNA (negative control); lanes 1 and 2, high-salt-precipitated transgenic plant DNA; lanes 3 and 4, DNA isolated from the same plants as lane 1 and 2, but containing polysaccharides.

REFERENCES

1. **Aoki, Y. and H. Koshihara.** 1972. Inhibitory effects of acid polysaccharides from sea urchin embryos on RNA polymerase activity. Biochim. Biophys. Acta 277:33–43.
2. **Ashwell, G.** 1966. New colorimetric methods of sugar analysis. Methods Enzymol. 8:85–95.
3. **Bendich, A.J., R.S. Anderson and L.B. Ward.** 1979. Plant DNA: long, pure and simple. p. 31–33. *In* C.J. Leaver (Ed.), Genome Organization and Expression in Plants. Plenum Press, New York.
4. **Dellaporta, S.L., J. Wood and J.B. Hicks.** 1985. Maize DNA miniprep. p. 36–37. *In* R. Malmberg, J. Messing and L. Sussex (Eds.), Molecular Biology of Plants. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
5. **Do, N. and R.P. Adams.** 1991. A simple technique for removing plant polysaccharide contaminants from DNA. BioTechniques *10*:163–166.

6. **Doyle, J.J. and J.L. Doyle.** 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. *19*:11-15.
7. **Edelman, M.** 1975. Purification of DNA by affinity chromatography: Removal of polysaccharide contaminants. Anal. Biochem. *65*: 293-297.
8. **Fang, G. and R. Grumet.** 1990. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Rep. *9*:160-164.
9. **Furukawa, K. and V.P. Bhavanani.** 1983. Influences of amniotic polysaccharides on DNA synthesis in isolated nuclei and by DNA polymerase: correlation of observed effects with properties of the polysaccharides. Biochim. Biophys. Acta *740*:466-475.
10. **Murray, M.G. and W.F. Thompson.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. *8*:4321-4325.
11. **Oka, A., S. Hiroyuki and T. Mituru.** 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. *147*: 217-226.
12. **Richards, E.** 1988. Preparation of genomic DNA from plant tissue. p. 2.3.1-2.3.3. *In* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Struhl (Eds.), Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.
13. **Shioda, M. and K. Marakami-Murofushi.** 1987. Selective inhibition of DNA polymerase by a polysaccharide purified from slime of *Physarum polycephalum*. Biochem. Biophys. Res. Commun. *146*:61-66.

We would like to thank Dr. Mike Thomashow and Dr. Julia Bell for helpful reviews of the manuscript. Acknowledgment is also made to the Michigan Agricultural Experiment Station for its support of this research. Address correspondence to R. Grumet.