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CLONING AND EXPRESSION OF MODIFIED COAT PROTEIN GENES OF ZUCCHINI YELLOW MOSAIC VIRUS TO STUDY THE MECHANISM OF COAT PROTEIN MEDIATED RESISTANCE IN MELONS

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CLONING AND EXPRESSION OF MODIFIED COAT PROTEIN GENES OF ZUCCHINI YELLOW MOSAIC VIRUS TO STUDY THE MECHANISM OF COAT PROTEIN MEDIATED RESISTANCE IN MELONS

By

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ABSTRACT

CLONING AND EXPRESSION OF MODIFIED COAT PROTEIN GENES OF ZUCCHINI YELLOW MOSAIC VIRUS TO STUDY THE MECHANISM OF COAT PROTEIN MEDIATED RESISTANCE

By

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To study the mechanism of coat protein (CP) mediated protection to zucchini yellow mosaic virus (ZYMV), a sense defective version of ZYMV-Ct coat protein (CP-SD) and the variable amino-terminal portion of the coat protein (CP-NT) were engineered for expression in plants. In vitro transcription and translation assays showed that CP-SD and CP-NT are capable of producing a transcript but CP-SD is incapable of producing a protein. Four versions of the ZYMV coat protein: full length, the conserved core portion of the protein, CP-SD, and CP-NT genes were introduced into *Nicotiana benthamiana* plants via *Agrobacterium tumefaciens* mediated transformation. The plants transformed with FLCP, Core, CP-SD, CP-NT constructs produced the mRNAs from the respective transgenes. FLCP and Core transgenics produced detectable amounts of protein. No protein was detected in CP-SD transgenics as expected. Inheritance of the inserted NPT II gene was verified by ELISA in the progeny of the self fertilized transgenic plants.

To

My Mother
Satyavathi Akula

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Name of the virus	Abbreviation
Potyviruses	
zucchiniyellow mosaic virus	ZYMV
watermelon mosaic virus	WMV
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
Johnsongrass mosaic virus	JGMV
Lettuce mosaic virus	LMV
Papaya ringspot virus	PRSV
Soybean mosaic virus	SMV
Bean yellow mosaic virus	BYMV
Clover yellow vein virus	CYVV
Peanut stripe virus	PstV
Pepper mottle virus	PeMV
Pea mosaic virus	PeaMV
Turnip mosaic virus	TuMV
Other viruses	
Tobacco mosic virus	TMV
Alfalfa mosaic virus	AlMV
Potato virus X	PVX
Tobacco rattle virus	TRV
Potato spindle tuber viroid	PSTV
Cucumber mosaic virus	CMV
Rice stripe virus	RSV
Tomato spotted wilt virus	TSWV
Potato leafroll virus	PLRV
Potato acuba mosaic virus	PAMV
Pea early browining virus	PEBV
Tomato mosaic virus	ToMV
Potato virus S	PVS
Potato mop top virus	PMTV

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Viruses can cause serious losses of yield and quality in many crops grown in agriculture, horticulture and forestry (Fraser, 1992). Over the past decade the tools of molecular biology have permitted rapid advances in our understanding of plant viruses and their replicative strategies. The discovery that expression of a viral coat protein gene (CP) in transgenic plants could protect against virus infection, was rapidly followed by several examples of genetically engineered virus resistance in various groups of viruses (Grumet, 1990, 1994, 1995; Beachy et al. 1990; Fitchen and Beachy, 1993; Wilson, 1993; Nelson et al. 1990). However the mechanism of such protection is not clearly understood. It is likely that mechanisms will be different depending on the virus host combination in CP-mediated protection (Lal and Lal, 1993).

Information about the molecular mechanisms is essential to predict the long-term stability of coat protein mediated protection and the possible ecological and biological effects of growing crops engineered to resist viruses (Chasan, 1994). Understanding the mechanism of protection and multiple functions of coat protein may lead to designing of second generation CP genes that improve resistance and extend its applicability to still more viruses and crop plants (Beachy, 1993).

Coat protein mediated protection has been successfully demonstrated for zucchini yellow mosaic virus in melons (Fang and Grumet 1993). It is not known, however which

molecule (CP or CP transcript) or domains of the coat protein are critical in conferring protection. The goal of this study is to clone altered ZYMV CP genes, develop a modelsystem to study the mechanism, and produce transgenic plants expressing altered CPgenes,which can be used to address questions dealing with both homologous and heterologous virus protection

LITERATURE REVIEW

Many crop plants are infected by viruses. Plant viruses have an enormous negative impact on agricultural crop production through out the world (Scholthof et al. 1993). A variety of methods have been used to try to control crop losses due to virus diseases (Hull and Davies, 1992). When possible one of the most successful approaches is traditional plant breeding for virus resistance (Kyle, 1993). In addition, standard techniques of plant pathology, including quarantine, eradication, crop rotation, and certified virus free stock have been important tools to control virus diseases, although each has disadvantages, such as expense, questionable effectiveness, and lack of reliability from year to year (Scholthof et al. 1993). As an additional strategy, cross-protection also has been used (Fulton, 1986).

More recently it has been possible to develop virus resistant genotypes via genetic engineering using viral-derived genes as a source of resistance genes [selected reviews: Beachy et al. (1990), Fitchen and Beachy (1993), Gadani et al. (1990), Grumet (1990, 1994), Hull and Davies (1992), Nelson et al. (1990), Scholtof et al. (1993), and Wilson (1993)]. Of the different kinds of genes that have been used to genetically engineer virus resistance, coat protein is the one used most widely (Grumet, 1995). " Coat protein-mediated resistance" is used to refer to the resistance caused by the expression of a virus coat protein (CP) gene in transgenic plants (Beachy et al. 1990). Transgenic plants expressing the CP gene from a given virus are generally protected against infection by the

virus from which the CP gene was isolated (homologous virus). In many cases CP mediated protection extends to strains or viruses that are closely related to the virus from which the CP gene was obtained (heterologous virus) (Beachy et al. 1990; Gadani et al. 1990: Hull and Davis, 1992; Pang et al. 1992; Grumet et al. 1995). This literature review will focus on coat protein mediated resistance, possible mechanisms responsible for conferring resistance and the biology of the coat protein.

2.1 Coat protein mediated resistance

The theoretical bases for the use of coat protein genes have come from two directions:classical cross-protection and pathogen-derived resistance.

2.1.1 Cross-protection

Cross-protection was first observed by McKinney (1929) in tobacco: it is the ability of a mild strain of a virus to protect an infected plant against subsequent infection by virulent strains of the same or a closely related virus (Fulton, 1986). Cross-protection has been used to reduce yield losses in some crops like citrus, tomatoes and potatoes, due to citrus tristeza, tomato mosaic virus (ToMV) and potato spindle tuber viroid (PSTV) respectively (Hamilton 1980). However this labor intensive type of protection is expensive and it necessiates the use of an infectious virus as a control measure (Scholthof et al. 1993).

Although a number of models have been proposed to explain cross-protection, the exact mechanism responsible for cross-protection has not been elucidated. Proposed models include (review, Beachy, 1988): 1. Inhibition of the replication of the challenging virus due to the depletion of a component in the host cell by the inducing virus. 2. Inhibition of the uncoating of the challenger virus by the inducing virus. 3. Sense or antisense RNA of the inducing virus anneals with RNA of the challenger virus, thereby blocking replication of the challenger virus. 4. Capsid protein produced by the first infection encapsidates the RNA of the challenger strain, thereby preventing its replication. In some cases cross-protection can be overcome when challenge inoculum is viral nucleic acid rather than virions (Dodds et al. 1985). From the results of various studies to define the mechanism(s) responsible for cross protection, (review by Beachy, 1988) it appeared that protection in at least some cases occurs prior to the full release of viral RNA from the virion. This led to the suggestion that capsid protein of the protecting strain interferes with the RNA of the challenger during the process of uncoating of the genome. Hamilton (1980), predicted that cross-protection could be induced by introducing cDNAs to various regions of the viral RNA genome into plants.

2.1.2 Pathogen-derived resistance

The theory of pathogen-derived resistance (Sanford and Johnston, 1985) predicts that a "normal" host-pathogen relationship can be disrupted if the host organism expresses essential pathogen-derived genes. It has been proposed that host organisms expressing

pathogen gene products in excess amounts, at the inappropriate developmental stage or in a dysfunctional form, may disrupt the normal replicative cycle of the pathogen and result in an attenuated or aborted infection of the host. This approach is based upon the fact that in any parasite-host interaction, there are certain parasite-encoded cellular functions which are essential to the parasite but not to the host. If one of these functions is disrupted, the parasitic process should be stopped. In the most successful instances, such disruptions would prevent the replication and/or movement of the virus beyond the initially infected cell. Even with less effective interference in the replicative cycle, pathogen-derived resistance might modulate the disease symptoms and result in only a localized infection (Scholthof et al. 1993).

Genetically engineered plant virus resistance using a pathogen derived gene was first demonstrated by Powell-Abel et al. (1986). The coat protein (CP) gene from tobacco mosaic virus (TMV) was inserted into tobacco and the resultant transgenic plants, which constitutively produced viral coat protein, were more resistant to infection by TMV than were control, non transgenic plants. These findings opened new avenues for plant protection in important agricultural crops. Since the initial demonstration, numerous examples of resistance in plants transgenic for viral CP gene constructs have been reported (see reviews by Beachy, 1993; Grumet, 1995). This coat protein mediated resistance approach has broad applicability to a wide range of viruses. CP-mediated resistance has been demonstrated in at least 23 plant viruses from 13 different virus groups including the potyvirus group (see reviews by Grumet, 1995, Beachy, 1990). In addition to coat protein genes several other types of viral genes have conferred resistance, including

replicase genes, movement protein genes and protease genes (for reviews see Grumet 1995; Beachy et al. 1990; Wilson, 1993).

2.1.3 Phenotype of coat protein-mediated protection

The type of resistance that is observed varies among different systems, and can also vary among different lines transformed with the same gene, and even among different individuals within or families derived from the same line and possessing the same gene at the same location in the genome (Grumet, 1994). There are several phenotypes associated with CP-MP, (review by Beachy, 1993) some of which may be reflective of the cellular and molecular mechanisms of resistance. These phenotypes may also vary depending on the host and the environmental conditions of plant growth and testing, and replication and disease strategies of the pathogen. Not all examples of CP-MP exhibit each of the phenotypes listed. In each example of coat protein-mediated resistance described to date, resistance is manifested by several features (See reviews by Beachy et al. 1990 ; Grumet, 1995). **1.** Fewer lesions, in general, upon infection with the virus from which the gene was derived, the inoculated leaves of the transgenic plants show fewer viral lesions (chlorotic, necrotic) than do control plants. **2**. Systemic spread of infection is prevented, delayed or reduced. Virus accumulation is decreased in systemically infected leaves in spite of virus accumulation in inoculated leaves. Severity of disease symptoms is reduced in plants that do become infected. **3**. Infection followed by recovery. The phenotypes of CP-MP are the result of various types of molecular and cellular mechanisms that occur in the host as CP

molecules interact with the virion and/or the replication of its genome (Beachy, 1993) or the host genome and the host cell response as proposed by Dougherty's group (Smith et al. 1994, 1995) .

2.1.4 Broad spectrum resistance

Broad spectrum resistance 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 as many hosts are infected by several different potyviruses (Hollings and Brunt, 1981) and they form the largest, most widely distributed and economically important group of plant viruses. Although in general the highest level of resistance was conferred against the homologous virus, there are many examples where a given CP gene conferred protection against other viral strains and other related viruses. Transgenic tobacco plants expressing coat protein of TMV have a low but significant degree of protection against other tobamoviruses (Nejidat and Beachy, 1990; Anderson et al. 1989). Transgenic tobacco expressing CP of cucumber mosaic virus-O (CMV-O) showed significant level of protection to CMV-Y and to the serologically unrelated chyrsanthemum mild mottle virus (CMMV) , a member of the cucumovirus group (Nakajima et al. 1993). Other examples include resistance to strains of CMV (Quemada et al. 1991), tobacco rattle virus (TRV) (van Dun and Bol, 1989), TMV (Nelson et al. 1988), potato virus-S (PVS) (Mackenzie et al. 1991), and tomato spotted wilt virus (TSWV) (Pang et al. 1992).

In the case of CP-MP against members of the potyvirus group, there is evidence that CP-MP can confer broad resistance. The CP gene of ZYMV-Ct conferred protection against a variety of other ZYMV strains and the closely related potyvirus watermelon mosaic virus (WMV), but not against the less closely related papaya ringspot virus (PRV) (Grumet et al. 1994). Transgenic plants expressing WMV CP gene showed noticeable protection against other potyviruses like bean yellow mosaic virus (BYMV), potato virus-Y (PVY), pea mosaic virus (peaMV), clover yellow vein virus (CYVV), pepper mottle virus (PeMV) and tobacco etch virus (TEV) (Namba et al. 1992). Tobacco plants accumulating coat protein of the potyvirus soybean mosaic virus (SMV), a non pathogen on tobacco, were partially protected from infection by two serologically unrelated potyviruses that are pathogens of tobacco, PVY and TEV (Stark and Beachy, 1989). Similarly Ling et al. (1991) reported that plants that accumulated detectable levels of PRV-CP showed a significant delay in symptom development and the symptoms were attenuated when inoculated with TEV, PVY, and PeMV. Plants expressing the CP of PVY strain N605 showed good resistance to the related strain PVY-O803 (Malnoe et al. 1994).

The mechanism of this generalized virus resistance is unknown. It is not clear whether the extent of heterologous protection is related to the sequence homology between the challenge virus and the virus from which the CP gene was derived, though in some cases there seems to be a correlation (Grumet et al. 1994; Namba et al. 1992). It is also not known whether the absolute homology in the amino acid sequence between the CP's is important to obtain broad protection or conservation of particular structural domains is

important. The CP gene of the potyvirus lettuce mosaic virus (LMV) conferred complete protection against the heterologous virus PVY (66% amino acid homology to LMV), but did not protect against TEV, which has a similar (60%) percent amino acid homology (Dinant et al. 1993). In the case of TSWV, protection was observed against various strains and isolates of TSWV, but not against two related viruses with about 80% nucleotide sequence identity to TSWV (de Haan et al. 1992). In CMV the degree of amino acid sequence identity between expressed and challenge viruses did not show a direct correlation with the observed level of protection (Quemada et al. 1991). In some lines protection was greater against the heterologous strains than the homologous strains. The heterologous protection seen in CP-MP may also be influenced by the different sequence motifs present in the N-terminal region of the coat protein of the different viruses/strains involved. In the case of classical cross-protection the unidirectional cross-protection between some strains and negative cross-protection against others has been reported to be correlated to different sequence motifs present in the hypervariable coat protein N-terminus of the involved strains (Krstic et al. 1995). In fact the breadth of protection of CP-MP may be related to a number of unknown variables (Beachy, 1993).

2.2 Site and mechanism(s) of coat protein mediated resistance

Normal virus replication requires a subtle blend of host-and virus-coded proteins, present in critical relative concentrations and at specific times and places. An unregulated superimposition of interfering protein or nucleic acid species can result in an apparently

virus-resistant phenotype (Wilson, 1993). As reviewed by Grumet (1994), in the case of coat proteins, which have many roles in the life cycle of the virus, it seems there are many potential points of interference. Several possible mechanisms have been proposed including : prevention of uncoating of the incoming virus, interference with viral translation and /or replication, and interference with cell to cell or long distance movement. Each of these is a potential interference point for the coat protein with the incoming virus, and there is good evidence to indicate that the mechanism of protection is not the same in every virus-CP host combination, because of the differences among viruses and their modes of infection and replication. The resistance derived from a single CP gene in a single plant species may act by more than one mechanism and may inhibit several different stages in the infection or replication process (Fitchen and Beachy, 1993). In spite of the vast literature on coat protein-mediated protection, no unifying hypothesis has been derived to explain the molecular mechanisms of CP-mediated protection.

One of the key features in elucidating the mechanism(s) of protection lies in identifying the site of protection. As reviewed by Nelson (1990), protection by CP expression appears to involve more than one site. There is a decrease in lesion numbers on inoculated leaves of CP expressing plants, thus one site of protection is at the primary infection site. A decrease in virus accumulation in systemically infected leaves in spite of virus accumulation in inoculated leaves equal to that of controls [e.g.: Tobacco/TMV system (Wisniewski et al. 1990)], indicates that a second site exists in CP(+) plants providing protection against virus infection beyond the primary site. Another key feature in elucidating the mechanism(s) of protection lies in identifying the molecule(s) responsible

for the protection. Depending on the individual case, protection might have resulted due to accumulation of coat protein coding sequences (mRNA) or coat protein *per se* (Beachy,1988).

2.2.1 Protection mediated by coat protein *per se*

The majority of the studies on the mechanism(s) of CP-MP have been carried out with TMV and TEV and they may or may not reflect the mechanisms of protection in other systems. In the case of TMV system, there is evidence that CP-MP is a result of a direct protein effect. Accumulation of only CP transcript did not protect the CP transgenic tobacco against the infection of TMV (Powell-Abel et al. 1990). Purified CP was shown to be able to confer transient protection against infection by TMV, in tobacco protoplasts (Register and Beachy, 1989). The effect of elevated temperatures on the accumulation of CP in transgenics was used by Nejidat and Beachy (1989) to demonstrate the requirement of CP accumulation for resistance. Based on studies with tobacco plants carrying the N gene which reacts in a hypersensitive manner to TMV infection, it was concluded that expression of the TMV CP gene in transgenic tobacco plants results in direct interference of the CP with TMV infection rather than in triggering plant defense responses that lead to resistance (Carr et al. 1989).

One manifestation of the CP-mediated protection by TMV CP against TMV infection occurs early in the infection cycle and is overcome partially or fully by inoculation with TMV-RNA, and by TMV virions that were briefly treated at pH 8.0 (Nelson et al. 1987;

Register and Beachy, 1988). The decreased level of protection to infection by TMV-RNA shows that CP-MP involves the inhibition of an event prior to the release of the viral RNA from the virion. Treatment of TMV at pH 8.0 greatly enhances the translation of TMV *in vitro,* but does not cause structural changes (Wilson 1984a,b). It was postulated that the treatment at pH 8.0 results in removal of small number of CP molecules from the 5' end of the viral RNA exposing it to binding by ribosomes and subsequent co-translational uncoating (Wilson, 1984). In *in vitro* translation assays, the addition of TMV CP reduced the efficiency of translation of pH 8.0 treated virions (Wilson and Watkins, 1986); this suggests that CP bound to the 5' end of the viral RNA prevents ribosome binding. The lack of resistance to infection by pH 8.0 treated TMV (Register and Beachy, 1988) suggests that virion stabilization in the CP-expressing plants occurs by exchange of low numbers of CP molecules (less than 20) from the virion rather than re-constitution of the virions. Such translationally active complexes, called 'striposomes' were isolated from infected leaf tissue (Shaw et al. 1986). Striposomes are characterized by the attachment of 80s ribosomes to an exposed 5' portion of the viral genome, while the 3' end remains encapsidated (Wilson and Watkins, 1986). Wu et al. (1990) reported that the number of striposomes in transgenic CP(+) protoplasts after electroporation with TMV was greatly reduced compared to CP(-) protoplasts. It was suggested that the modification of TMV that leads to co-translational virion disassembly is affected in CP accumulating transgenic plants (Reimann-Philip and Beachy, 1993a). Further evidence that whole plant resistance is the result of inhibition of an early event of TMV infection was obtained by tissue specific gene expression studies where it was shown that CP must accumulate in the

initially infected tissue in order to interfere with TMV infection (Reimann-Philip and Beachy, 1993b).

Although the exact molecular mechanism of CP-MP is not clear, many studies in TMV/tobacco system, as described above, indicate that inhibition of virion disassembly plays a major role. The process which leads to the loss of CP molecules in the initial stage of TMV infection is not yet known. The chemical environment in the cell might be sufficient to cause disassembly of CP from RNA (Reimann-Philip and Beachy , 1993a). Results of protoplast assays indicate that disassembled virions of TMV at pH 8.0 are not reencapsidated, this indicates that protection can be due to inhibition of uncoating. There are two current hypotheses to explain this. The first suggests that there are receptor sites for uncoating that are blocked by the expressing coat protein. The second proposes that local intracellular conditions which favor virus disassembly may be involved. TMV CP present in transgenic cells could shift these kinetics (TMV CP preferentially associates with itself), causing a fully assembled virus structure to be maintained (Register and Beachy 1988; review by Wilson, 1985). If this involved limited exchange of CP subunits on the virion with endogenous CP in the transgenic cell, then the endogenous CP would be required to be competent to assemble with CP subunits present on the virion (Clark et al. 1995). Interestingly, the capacity of the CP to assemble into normal virion structures was shown to be dispensible with respect to CP-MP in the TMV system (Clark et al. 1995).

The second stage of manifestation of CP-MP is during local and systemic spread of the virus. Movement of the TMV virus from the infected leaves to the upper leaves of

infected CP+ plants is delayed, even when an equal number of infection sites are present on the inoculated leaves of transgenic and control plants (Wisniewski et al. 1990; Anderson et al. 1989). This indicates that protection against systemic spread in CP+ plants is caused by one or more mechanisms that, in correlation with protection against initial infection upon infection, results in a phenotype of resistance to TMV (Wisniewski et al. 1990; Wu et al. 1990; Osbourn et al. 1989). However experiments with tissue specific promoters (Reimann-Philip and Beachy, 1993a) show that a major component of sytemic resistance is a reiteration of interference during the infection process.

In support of the inhibited-uncoating model, CP-MP is sensitive both to the level of expression of the transgene and the concentration of the challenge virus (Powell-Abel et al. 1986; Nejidat and Beachy, 1988). The idea that the protein and not its mRNA is responsible for the resistant phenotype, was supported by observations that higher levels of CP gene expression would lead to higher levels of resistance [e.g. AlMV (Loesch-Fries. 1987; Hill et al. 1991); PVX (Hemenway et al. 1988; Hoemkema et al. 1989); TMV (Powell Abel et al. 1990); rice stirpe virus (RSV) (Hayakawa et al. 1992)]. Another model proposed to explain the molecular mechanisms underlying this resistance suggests that, the high levels of intracellular coat protein accumulating in the transgenic plants disregulates transcription and replication of incoming viral RNA by altering the mode of the viral polymerase (de Haan et al. 1992). For reasons that are not understood, the accumulation of large amounts of coat protein is not always necessarily correlated with the most effective protection. Protection is also obtained in conjunction with very low accumulation of coat protein in transgenic plants. For instance, protection against ZYMV

was not correlated with the level of protein expression (Fang and Grumet, 1993), and field protection to PVY occurs in transgenic plants producing undetectable levels of PVY coat protein (Kaniewski et al. 1990) [other examples potyviruses (Lawson et al. 1990; Namba et al. 1992; Regner et al. 1992; Ravelonandro et al. 1993; Farneilli and Malnoe 1993; van der Vlugt et al 1992); luteoviruses (Kawachuck et al. 1990); tospoviruses (Gielen et al. 1992 ; MacKenzie and Ellis, 1992; Pang et al. 1992)]. In the case of peanut stripe mosaic virus (PstV) and tomato spotted wilt virus (TSWV) (Cassidy and Nelson, 1995; Pang et al. 1994) higher levels of CP accumulation were associated with lower levels of resistance Suggested explanations for the lack of correlation have included cell specific accumulation, differential subcellular localization of the CP, or that RNA rather than CP is responsible for conferring protection. Possibility of RNA-mediated protection will be discussed in the following section (2.2.2).

While blockage of virus uncoating is also consistent with the characteristics of engineered cross-protection against AlMV (van Dun et al. 1988; Tumer et al. 1987; Taschner et al. 1994; Loesch-Fries et al. 1987), it is not universal; transgenic plants expressing the coat proteins of some potex-, carla-, and nepoviruses were protected against infection even when inoculated with viral RNA (Hemenway et al. 1988; MacKenzie and Tremaine, 1990; Bertioli et al. 1992; Brault et al. 1993). In the case CMV, whole plants were protected against systemic infection by both virions and RNA, but protoplasts were protected against virions and not against RNA (Okuno et al. 1992). These observations show that the incoming viral RNA is able to replicate in the primary inoculated cells, but that subsequent spread is limited. However in some cases high levels

of CP might also interfere with the replication of viral RNA, since there was some protection against TMV RNA in plants transgenic for the CP of the virus (Nelson et al. 1987; Register and Beachy, 1988)

Several hypotheses concerning the mechanism of CP action, such as interference with uncoating, translation, or replication depend on interaction between the transgene expressing CP and the viral RNA (Grumet 1994). There are some instances, however, where the ability to interact with the RNA may not be sufficient to confer resistance. In potyviruses the conserved core portion of the coat protein contains information necessary for polymerization, and is capable of forming virions of normal appearance (Dolja et al. 1994). However, expression of just the Core portion of the ZYMV CP in melons did not give high levels of protection when compared to full length CP (Fang and Grumet, 1993). In AlMV a mutant CP with a single nucleotide change was unable to offer CP-MP, even though it was capable of binding to RNA *in vitro* (Tumer et al. 1991). The CPs of two strains of tobacco rattle virus (TRV) are capable of reciprocal encapsidation, but the CP's only confer protection against the homologous strain (van Dun and Bol, 1988) and for TMV, disruption of assembly functions did not eliminate CP-mediated protection (Clark et al. 1995).

Some experiments suggest that the amino-terminal portion of the coat protein of certain types of viruses is critical for CP-mediated protection, possibly via interaction with a host factor. Deletion of the amino terminus of ZYMV CP reduced the levels of protection conferred in melons when compared to full length CP (Grumet and Fang, 1993). Changing the second amino acid in the AlMV amino terminal portion, eliminated

the ability of CP to confer resistance to infection (Turner et al. 1991). Similarly, removal of the amino-terminus of the CP of the potexvirus, potato acuba mosaic virus (PAMV) eliminated the ability to confer protection against virus infection, while mutation in core domain which is thought to be essential for assembly, offered the same amount of resistance as observed with the full length CP (Leclerc and AbouHaider, 1995). It was hypothesized that a plant component interacts with the viral CP, and that the interaction may be blocked by an expressed protein in transgenic plants containing the N terminus of the viral CP leading to the attenuation of the viral infection (Leclerc and AbouHaider, 1995). When the inoculum concentration is increased, the virions compete for the available sites, leading to a successful infection of such plants. However, the nature of such a plant component is unknown.

2.2.2 Protection conferred by the accumulation of CP-transcript

In some cases it seems that the CP-transcript is involved in the protection observed in the transgenic plants, because protection was observed even when the coat protein was not detected. The mRNA resistance was in some cases as effective as that conferred by translationally competent constructs. In plants transformed with potato leaf roll virus (PLRV) coat protein, titer of PLRV has been greatly reduced, however transgenic plants accumulated PLRV coat protein transcripts, but the PLRV coat protein was not detected (Kawchuck et al. 1991). Resistance to PLRV replication in the transgenic lines was broadly related to the amount of PLRV CP transcript detected, protection seemed to be

based on interference with PLRV multiplication at the RNA level (Barker et al. 1993). The non translatable RNA of the PVY CP was as efficient as the translatable construct in protection against PVY. However the PVY CP could not be detected in the translatable construct (van der Vlugt et al. 1992). Transgenic tobacco plants carrying a translationally defective TSWV coat protein gene exhibited levels of resistance similar to those reported in experiments with translationally competent gene constructs (de Haan et al., 1992). Since the mRNA resistance in some cases is as effective as that conferred by translationally competent constructs, the RNA may be the active entity, even when the protein does accumulate (Kawachuck et al. 1991; de Haan et al. 1992; Gielen et al. 1991). In fact, it has been suggested that transcript mediated protection may be preferable to CPMP because this precludes the need for accumulation of a foreign protein in crop plants (de Haan et al. 1992).

Various hypotheses that have been put forward to explain the transcript mediated protection include: (1) The high levels of CP gene transcripts in the transgenic plants give rise to antisense inhibition of viral replication., (2) The expressed coat protein mRNA may bind to and compete for virus and / or host associated replicase proteins or otherwise shift transcriptional or replicative events so that virus multiplication is reduced, (Kawachuck et al. 1991; Hemenway, 1990; Cuozzo et al. 1991; de Haan et al. 1992).

Lindbo and Dougherty (1992) postulated that protection sometimes results from coat protein mRNA accumulation and is independent of a requirement for coat protein expression *per se*. They showed that the use of non translated RNA and its antisense were more efficient in protecting than full length or truncated versions of the TEV CP. It was

suggested that the resistance is mediated through a defective RNA species and not the expected translation product. Their further investigation of their transgenic lines have led to some interesting findings. Transgenic tobacco expressing the full length form of TEV coat protein or a form truncated at the N-terminus of the coat protein were susceptible to TEV infection initially, but 3 to 5 weeks after a TEV infection was established, transgenic plants "recovered" from the TEV infection, and new stem and leaf tissue emerged symptom and virus free (Lindbo et al. 1993). The recovered tissue also was not susceptible to reinoculation. When transgenic tobacco plants expressing an untranslatable version of TEV CP were analyzed for resistance to TEV infection, three different responses were noted: 1. some were highly resistant, no viral replication occurred, 2. some were susceptible but able to recover from systemic TEV infection; and 3. some were susceptible to TEV infection (Dougherty et al. 1994). Recovered tissue could not be infected with TEV. Steady-state transgene mRNA levels in recovered tissue were lower than those of unchallenged transgenic plants. Nuclear runoff assays suggested a post-transcriptional reduction in specific RNA levels. An inverse correlation between transgene transcript accumulation and virus resistance was observed. The resistance was virus specific, and functional at the single cell level. Similar results were noted in experiments with homozygous double haploid tobacco plants (Smith et al. 1994) and in transgenic potato plants expressing an untranslatable version of PVY coat protein. They suggest that virus resistant phenotype is likely mediated by a host cell response and not from the transgene product competing with viral encoded product (Smith et al. 1995). The same

kind of recovery and immunity results were observed in case of potato spindle tuber viroid (PstV), but only with a translatable sense CP (Cassidy and Nelson, 1995).

A working model has been proposed (Lindbo et al. 1993; Smith et al. 1994) to explain the lack of correlation between degree of protection and steady state levels of the transgene transcript. It has been proposed that the molecular basis of the recovered phenotype is a cytoplasmic event in which plant cells are able to (1) sense elevated or aberrant RNA levels in a manner not understood, these specific RNA sequences are then (2) targeted, and (3) inactivated by a cellular factor that may be a protein or nucleic acid. The complex formed between the target RNA sequence (host and /or viral) and the cellular factor will direct cellular enzymes to (4) degrade the RNA, resulting in its elimination from the cytoplasm. Mechanistically, the model suggests that a protein or nucleic acid factor binds to a specific RNA sequence, rendering this RNA functionally inactive and targeting it for elimination. Stimulation of this system apparently results in a highly resistant phenotype to TEV, because TEV RNA sequences are inactivated (Smith et al. 1994; Lindbo et al. 1993).

To explain the occurrence of recovered phenotypes and variable responses in transgenic lines, even those derived from a single transformation event of a common haploid plant and isogenic for transgenes, Smith et al. (1994) suggested that plant cells have an undefined "threshold" below which they can accommodate a specific RNA species. Transcription of the transgene within the nucleus produces varying amounts of RNA among the different transgenic lines. Plants with high transcription rates generate RNA levels that exceed a certain threshold level, which activates a cytoplasmic-based,

cellular process that specifically targets this RNA for elimination and results in low steady state levels of the transgene mRNA. If the transgene shares nucleotide homology with a plant virus, the plants are phenotypically resistant to the challenging virus. Alternatively, in lines where transcription rates are lower and the threshold level is not exceeded, the putative cytoplasmic degradation mechanism is not induced. Steady state levels of the mRNA will be proportional to the transcription rate of the gene. The cytoplasmic regulatory system is off and a susceptible phenotype will be manifested. In the case of "inducible" resistance, also referred to as recovery phenotype (as in TEV), the additive level of transgene mRNA and the viral RNA containing the target sequence exceed the threshold level, resulting in an activation of the cytoplasmic system, which is manifested as a lowering of the transgene mRNA steady state levels and the concomitant establishment of the resistant phenotype.

Different mechanisms seem to be operating in different plants. Translation product may only be one of a number of components involved in establishing the virus-resistant state (Silva-Rosales et al. 1994). In the case of TSWV, resistance against homologous isolates and closely related isolates, seems to be RNA-mediated, in the presence of low levels of transcript, while partial protection to homologous isolate and distantly related isolate, is protein mediated, in the presence of high amounts of CP protein (Vaira et al. 1995; Pang et al. 1993; Pang et al. 1994).

2.3 Genetic engineering of potyvirus resistance

Of the 28 plant virus groups or families, the potyvirus group is the largest and accounts for about 30% of all viruses known to infect plant species around the world (Shukla and Ward 1989. Francki et al. 1985). Potato virus Y (PVY) is the type member of the potyvirus group. Most members of the potyvirus group are transmitted by aphids in a non persistent, non circulative 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, dodder, and fungus has been also reported (Hollings and Brunt 1981; Ward and Shukla, 1991). Potyviruses also can be mechanically transmitted.

CP-mediated protection has been demonstrated for various potyviruses like PVY, TEV, SMV, PRV, WMV, ZYMV, PPV etc. (review by Beachy 1993). In majority of potyviruses [e.g. PVY (Lawson et al. 1990), PPV (Regner et al. 1992), PRSV (Fitch et al. 1990) and ZYMV (Fang and Grumet 1993)] full length potyviral CP genes were very effective, whereas in case of TEV, truncated coat proteins, untranslated version and antisense version of CP were also effective (Lindbo and Dougherty, 1992). The reason(s) for the difference between these experiments is unclear. Mechanism(s) of protection appears to be different even among the viruses within the same group.

CP-mediated resistance against potyviruses has several important features (review, Beachy et al. 1990). First it is possible to produce plant lines that are highly resistant to infection by mechanical or aphid inoculation. Second, a CP gene from a potyvirus that is

not a pathogen (SMV) can protect transgenic tobacco plants against pathogenic potyviruses (PVY and TEV). Third there is no correlation between the level of expression and the level of resistance observed. There are some exceptions and other features in addition to these characteristics. In case of TEV CP+ plants the resistance was not conferred against infection by other potyviruses. Resistance to PPV infection in some transgenic *N.benthamiana* plants lines, does not depend on the concentration of the challenge virus. This virus concentration independent resistance is a novel observation for potyviruses (Ravelnandro et al. 1993). A similar observation has been made in tetraploid transgenic muskmelon plants (Grumet, 1995; personal communication). Though coat protein mediated protection has been successfully demonstrated in potyviruses, there is no unifying hypothesis to explain the phenomena and varying responses in each case, it may be that the mechanism of protection varies among virus-host combinations.

2.3.1 Potyvirus biology-Coat protein

To study the mechanisms responsible for coat protein mediated resistance, knowledge of potyvirus biology is important, of particular relevance are structural characteristics, immunological properties, and functional roles of the coat protein in the virus life cycle. Information about coat protein structure and its role in virus host interactions may reflect upon the various features associated with CP-MP.

All members of the potyvirus group share common features. The virus particle has a flexuous rod shape and is usually 680-900nm in length and 12-15nm in diameter (Shukla

et al. 1991). The potyvirus genome is single stranded, positive sense infectious RNA molecule that is approximately 10,000 nucleotides 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 the 3' terminus (Hari et al. 1979). A single open reading frame (ORF) codes for an approximately 350,000-Da (350-kDa) polyprotein that is proteolytically processed into mature viral gene products (Dougherty and Carrington 1988; Allison et al. 1985). The RNA is encapsidated by approximately 2,000 copies of a CP monomer to form a virion (Holling and Brunt 1981). The capsid protein is encoded by the sequence present at the 3' end of the large ORF (Allison et al. 1985). A tertiary structure proposed for potyvirus CP monomers (Shukla et al. 1988 ; Shukla and Ward, 1989) is composed of seven helices and three loops with N and C termini projecting towards the surface.

Distinct potyviruses exhibit coat protein sequence homologies of 38-71% with major differences in the N-terminal portion of their coat proteins. The amino terminal region of the coat protein of different potyviruses is highly variable in sequence and length (29-95 residues) and is virus specific (Allison et al. 1986 ; Shukla et al. 1988). In contrast, the C terminal regions of the different coat proteins vary in length by only one or two amino acid residues (Shukla et al. 1988) and there is high sequence homology (65%) in the C-terminal three quarters of the CP (Shukla and Ward 1988). Strains of individual viruses exhibit very high sequence homology (90-99%) throughout the full length CP (Shukla et al. 1988).

Epitopes thought to be group specific were located in the trypsin-resistant Core protein region (Dougherty et al. 1985; Shukla et al. 1988). The N-terminus contains the major virus-specific epitopes and constitutes the most immunodominant region in the virus particle (Dougherty et al. 1985; Shukla et al. 1988), although the C-termini also can include immunodominant sites (Vuento et al. 1993). In johnsongrass mosaic virus (JGMV) (Shukla et al. 1989) the epitopes recognized by virus-specific monoclonal antibodies and polyclonal antibodies were shown to be linear sequences located in the N-terminal region of the coat protein.

The N and C termini of the potyviruses coat proteins are surface located and can be removed from virions by mild proteolysis (Allison et al. 1985; Dougherty et al. 1985). Mild trypsin treatment removes the N-terminal region (30-67 amino acids long, depending on the virus) and 18-20 amino acids from the C terminus of the coat proteins, leaving a fully assembled virus particle composed of coat protein Cores consisting of 216 or 218 amino acid residues (Allison et al. 1985; Dougherty et al. 1985; review, Shukla et al. 1991). The enzyme Lysyl endopeptidase selectively removes the surface exposed N terminus (Shukla et al. 1988, 1989). These Core particles were indistinguishable from untreated native particles in an electron microscope and were still infectious (Shukla et al. 1988; Allison et al. 1985; Dougherty et al . 1985), suggesting that the N- and C- termini are not required for particle assembly or for the infectivity during mechanical inoculation. It has been shown that the Core proteins can be dissociated and reassociated into potyvirus-like particles (Jagadish et al. 1993b) which indicates that all the necessary information required for polymerization of coat protein is located within its Core region.

Recently it has been shown that a TEV mutant with a deletion in the N-terminal sequence, is capable of forming virions with normal appearance at the electron microscope, supporting previous biochemical analysis that amino terminal portion of CP plays no essential role in maintaining proper virion architecture or infectivity (Dolja et al. 1994). However single and dual mutations in the Core region inhibited virion formation even in the presence of wild-type CP supplied *in trans* (Dolja et al. 1994). Using a microbial expression system (Jagadish et al. 1991) for potyvirus coat protein, in which the CP's of JGMV can be expressed and assembled into virus particles, the two charged residues R^{194} and D^{238} within the Core region of the protein were shown to be crucial for the assembly processes (Jagadish et al. 1993a).

Apart from virion assembly, potyvirus CP possesses distinct, separable activities required for cell to cell movement and long distance transport (Dolja et al. 1994). It was suggested that the Core domain of TEV CP provides a function essential during cell-to-cell movement and the variable N-and C-terminal regions are necessary for long distance movement (Dolja et al. 1995). N-terminal domain of the coat protein has also been shown to mediate aphid transmission. Successful transmission of potyviruses by their aphid vectors depends upon the interaction of two viral-encoded proteins, the coat protein and the helper component (Pirone, 1991). Substitutions in the conserved DAG triplet in the N-terminus of aphid trasmissible potyviruses results in the loss of the aphid transmissibility in tobacco vein mottling virus (TVMV) (Atreya et al. 1990, 1991) and loss of aphid transmissibility and antibody binding capacity in turnip mosaic virus (TuMV) (Kantrong et al. 1995). In the non aphid transmissible strain of ZYMV, aphid transmissibility was

restored by a mutation from threonine to alanine, confirming the previous findings (Gal-On et al. 1992). Based on a mutational analysis Atreya et al. (1995) suggested that conserved DAG triplet and the other residues near N-terminus function in some phase of the TVMV life cycle, in addition to aphid transmission.

2.4 Genetic engineering of virus resistance for ZYMV in melons

The cucurbit family includes some important vegetable crops (e.g. melons, cucumbers, squashes). Cucurbits are subjected to severe losses due to infection by at least three potyviruses: the watermelon strain of papaya ring spot virus (PRV-W), watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV) (Provvidenti et al. 1984). Among these, ZYMV is a relatively new but very aggressive member of the potyvirus group. Severe outbreaks of this virus have been reported in many countries in the world (Lisa et al. 1981; Davis and Mizuki, 1987). Muskmelon (*Cucumis melo* L.) is a high value and important crop throughout the world. It is subjected to severe losses by several viruses (Nameth et al. 1985) and so genetic engineering was a primary goal for this crop (Grumet and Fang 1990; Gonsalves et al. 1991).

Genetically engineered coat protein mediated protection against ZYMV in melons has been demonstrated by Fang and Grumet (1993). The ZYMV coat protein gene has been cloned, and the nucleic acid sequence has been determined (Grumet and Fang, 1990; Gal-On et al. 1990; Quemada et al. 1990). The gene encodes a coat protein with 279 amino acids and a calculated molecular mass of 31,214 Da. Sequence comparisons show that ZYMV shares an average of 50-60% amino acid homology in the CP with other potyviral

coat proteins. The majority of the conserved amino acids are located in the central-and carboxyl-terminal region of the protein, known as trypsin resistant Core portion of potyviral CP's (Shukla et al. 1988).

In an effort to genetically engineer potyvirus resistance, to test for protection against both homologous and heterologous viruses, and to gain insight into possible mechanisms of protection, Fang and Grumet (1993) utilized three versions of the ZYMV CP gene: The full-length CP gene, a truncated Core portion of the CP gene, and an antisense version of the CP gene. All the necessary information required for polymerization of coat protein is located within Core region (Shukla et al. 1988; Dougherty et al. 1985; Jagadish et al. 1993b; Dolja et al. 1994). It has been hypothesized that for several systems CP-mediated protection involves CP-RNA or CP-CP interaction (Beachy et al. 1990; Grumet, 1990; Nelson et al. 1990). Based on this Fang and Grumet (1993) hypothesized that, if these processes are critical for protection against potyvirus infection, then the Core portion of the protein would be expected to confer resistance, since domains reponsible for CP-RNA interaction and CP-CP interaction are located within Core. They further predicted that it might be possible that plants expressing the conserved CP gene fragment could be protected from infection by more than one potyvirus. Therefore the truncated version of the CP gene including the highly conserved central- and carboxy-terminal region (the Core portion) was used to test the above said hypothesis.

The three ZYMV CP-derived constructs were engineered and introduced into melon and tobacco plants and the effect of those constructs on increasing resistance to infection by ZYMV and two heterologous potyviruses, TEV and PVY was studied. Most of the

plants expressing full-length CP did not show any disease symptoms for at least 90dpi. Melon plants expressing ZYMV Core protein showed a 3- to 10-day delay in symptom appearance. Eventually, however all core plants became infected although the symptoms on the Core-protein expressing plants were milder than for control plants. The FL-CP expressing plants that did not develop symptoms also did not accumulate measurable virus levels. The virus titer in transgenic plants expressing the Core construct was intermediate between the inoculated controls and FL-CP plants. Protection against ZYMV was not correlated with the levels of protein expression.

When the transgenic tobacco plants expressing FL-CP and Core constructs were challenged with heterologous potyviruses PVY and TEV, there was a few days delay in symptom development. However there was no obvious difference among the different constructs. Transgenic plants expressing any of three forms of the ZYMV CP gene performed similarly in time to symptom appearance and symptom severity in systemic leaves. The disease symptoms in most plants were milder, and younger leaves often had no symptoms. Virus accumulation was correlated with the degree of visual symptoms. The results with TEV were similar to those with PVY.

2.5 Objective of this study

Although the Core protein was expressed at levels comparable to the FL-CP and did confer some protection, the Core construct was not as effective as the FL-CP construct that resulted in apparent immunity to ZYMV infection. 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 the Core. In the case of protection against 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 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, the sequence of which is virus specific, may be limited to the virus from which the CP gene was derived (Fang and Grumet, 1993).

A key feature in elucidating the mechanism involves identifying the molecules responsible for protection. The role of amino terminal portion, if any, is not known. It is not understood whether the protection conferred by FL-CP is RNA mediated or protein mediated, the mechanism underlying is not understood. In order to address these questions three things are necessary: 1. Altered forms of CP clones 2. Transgenic plants expressing these clones. 3. A model system where studies can be conducted, both with homologous and heterologous viruses.

As an initial step towards the study of mechanism of protection, my project was to make clones of altered ZYMV CP genes, (amino terminal portion alone and sense-defective version of FL-CP) and produce transgenic *N.benthamiana* plants expressing the modified CP genes and conduct preliminary analysis of transgenic plants. Virus protection studies can be conducted on these transgenic plants and alternatively the cloned CP genes can also be used to generate transgenic melon plants so that the mechanism of CP mediated protection for ZYMV can be studied in melons.

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CHAPTER TWO

CLONING OF ALTERED ZYMV COAT PROTEIN GENES FOR EXPRESSION IN PLANTS AND ANALYSIS OF TRANSGENIC PLANTS

INTRODUCTION

Coat protein mediated protection is one of the most widely used genetic engineering approaches to viral resistance. In this approach, the gene encoding the coat protein is isolated from the virus in question and inserted into the chromosomes of the susceptible plant. Coat protein mediated resistance has been demonstrated in various virus groups successfully, but so far there is no unifying hypothesis to explain the mechanism of such protection.

Zucchini yellow mosaic virus (ZYMV) is a very aggressive potyvirus, it causes major losses in cucurbit crops. In an effort to genetically engineer potyvirus resistance, to test for protection against both homologous and heterologous viruses, and to gain insight into possible mechanisms of protection, Fang and Grumet (1993) utilized three versions of the ZYMV CP gene: The full-length CP gene, a truncated Core portion of the CP gene, and an antisense version of the CP gene. All the necessary information required for polymerization of coat protein is located within Core region (Shukla et al. 1988; Dougherty et al. 1985; Jagadish et al. 1993b; Dolja et al. 1994). It has been hypothesized that for several systems CP-mediated protection involves CP-RNA or CP-CP interaction (Beachy et al. 1990; Grumet, 1990; Nelson et al. 1990). Based on this Fang and Grumet

(1993) hypothesized that, if these processes are critical for protection against potyvirus infection, then the Core portion of the protein would be expected to confer resistance, since domains responsible for CP-RNA interaction and CP-CP interaction are located within Core. They further predicted that it might be possible that plants expressing the conserved CP gene fragment would be protected from infection by more than one potyvirus. Therefore, the truncated version of the CP gene including the highly conserved central- and carboxy-terminal region (the Core portion) was used to test the above said hypothesis.

The three ZYMV CP-derived constructs were engineered and introduced into melon and tobacco plants and the effect of those constructs was studied on increasing resistance to infection by ZYMV and two heterologous potyviruses, TEV and PVY. Most of the plants expressing full-length CP did not show any disease symptoms for at least 90dpi. Melon plants expressing ZYMV Core protein showed a 3- to 10-day delay in symptom appearance. The symptoms on the Core-protein expressing plants were milder than for control plants. The FL-CP expressing plants that did not develop symptoms also did not accumulate measurable virus levels. The virus titer in transgenic plants expressing the Core construct was intermediate between the inoculated controls and FL-CP plants. Protection against ZYMV did not correlate with the levels of protein expression.

When the transgenic tobacco plants expressing FL-CP and Core constructs were challenged with heterologous viruses potyviruses PVY and TEV, there was no obvious difference between transgenic plants expressing any of three forms of the ZYMV CP gene

in time to symptom appearance, or symptom severity in systemic leaves. However, there was a few days delay in symptom development and the disease symptoms in most plants were milder, and younger leaves often had no symptoms. Virus accumulation was correlated with the degree of visual symptoms. The results with TEV were similar to those with PVY.

Although the Core protein was expressed at levels comparable to the FL-CP and did confer some protection, the Core construct was not as effective as the FL-CP construct that resulted in apparent immunity to ZYMV infection. 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 the Core. In case of protection against 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 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, the sequence of which is virus specific, may be limited to the virus from which the CP gene was derived (Fang and Grumet, 1993).

A key feature in elucidating the mechanism involves identifying the molecules responsible for protection. The role of amino terminal portion, if any, is not known. It is not understood whether the protection conferred by FL-CP is RNA mediated or protein mediated, the mechanism underlying is not understood.

As an initial step towards the study of the mechanism of protection, my project was to make clones of altered ZYMV CP genes, (amino terminal portion alone and sense-

defective version of FL-CP) and produce transgenic *N.benthamiana* plants expressing the modified CP genes and conduct preliminary analysis of transgenic plants. Virus protection studies can be conducted on these transgenic plants and alternatively the cloned CP genes can also be used to generate transgenic melon plants so that the mechanism of CP mediated protection for ZYMV can be studied in melons. In this chapter, the construction of two versions of ZYMV CP genes and expression of these genes in transgenic *N.benthamiana*, and analysis of transgenic plants are described.

MATERIALS AND METHODS

Plasmids and recombinant DNA manipulations:

The plasmid pTL37 which contains the TEV 5' nontranslated lead sequence (NTR) was obtained from Dr. W. Dougherty (Oregon State University). The plasmid pGA643, which contains the CaMV 35S promoter, T-DNA borders, neomycin phosphotransferase gene (NPTII gene) and tetracycline resistance gene, was used as the T-DNA vector (An et al. 1988). The full-length coat protein sequence (CP) (Fig. 1) of ZYMV was cloned into plasmid pTL37 by Fang and Grumet (1993). All recombinant DNA and bacterial manipulations were carried out using standard methods (Sambrook et al. 1989), unless otherwise indicated. Restriction enzymes were purchased from Boeringher Manheim and were used according to supplier's instructions. *In vitro* transcription and translation were performed using the Promega riboprobe and rabbit reticulocyte lysate systems respectively. The polymerase chain reaction (PCR) was carried out using the Gene Amp PCR Reagent kit from Perkin Elmer Cetus following the protocol provided by the manufacturer. The random primed labeling kit was purchased from United States Biochem Inc. and was used according to suppliers instructions.

Cloning of the amino-terminal portion of the coat protein gene (CP-NT):

The cloning of the amino terminal portion of ZYMV-CP gene is summarized in Figure 2. The full length coat protein sequence (CP) of ZYMV in plasmid pTL37 (Fang and Grumet 1993) was used as a template for PCR amplification of the 279 base pair

fragment which includes 150 bases of the TEV 5'NTR and 129 bases of the amino terminal portion of the full-length coat protein (FLCP); just upstream of KDVKD motif (see Fig. 1). Primers were designed to match the sequences at the 5'end of TEV 5'NTR (RG 6 :5' AGATC TAAAT AACAA ATCTC AACAC AACA 3') and the 3' end of the amino terminal portion of the full length coat protein sequence (RG22: 5'CTTGA GCTCC GTGAC AGCTG CTAG 3') and to introduce a Sac1 site at the 3' end of the amino terminus of the FLCP. The amplified fragment of TEV 5' NTR+N-term was digested with NcoI and SacI to release the amino terminal portion of the FLCP gene (129 bases), corresponding to positions 498 to 627 of the cDNA sequence of the terminal 3' 1546 nucleotides of ZYMV (numbering as published by Grumet and Fang, 1990; See Fig.1). which was ligated into NcoI-SacI digested pTL37 to form pTL37+NT.

The 3' nontranslated region of ZYMV was amplified from pTL37+FLCP using primers designed to match the 3'end of the coat protein (RG7: 5'AGATCTCTGCA GCCCTTTTTTTTT 3') and to introduce a SacI site upstream of the 3'NTR (RG23:5' GGTGAGCTCACAATGCAGTAAAGG 3') via PCR (see fig. 1). The 3'NTR clone (250 bases) includes 15 base pairs from the 3'end of the coat protein corresponding to positions 1318-1332 (See fig.1, Grumet and Fang 1990). The 3'NTR fragment was digested with SacI and PstI and ligated into SacI, PstI- cut pTL37+NT to form pTL37+NT+3'NTR (Fig 2). As a result of addition of some sequence from the 3' end of the FLCP 5 amino acids, namely valine→glutamic acid, aspargine→leucine, threonine, methionine, glutamine and a stop codon are added to the 3'NTR. Introduction of the

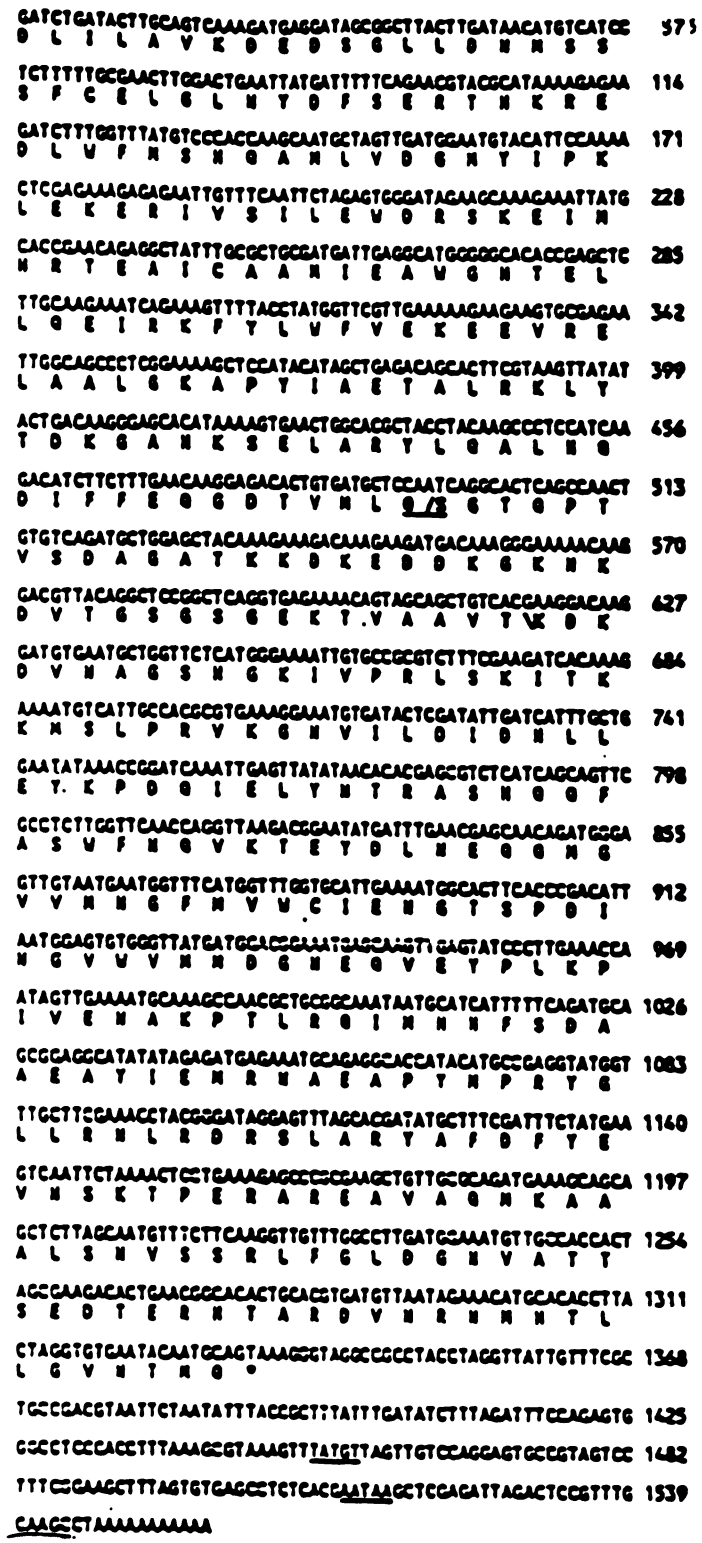

Fig. 1. The cDNA sequence and predicted amino acid sequence of the terminal 3' 1546 nucleotides of ZYMV. The proposed polymerase-coat protein protease cut site is underlined and marked with a slash. The protein protease cut site is underlined and marked with a slash. The beginning of the conserved trypsin-resistant core protein is marked with a blacklash. Possible polyadenylation signals are underlined. Figure from: Grumet and Fang (1990).

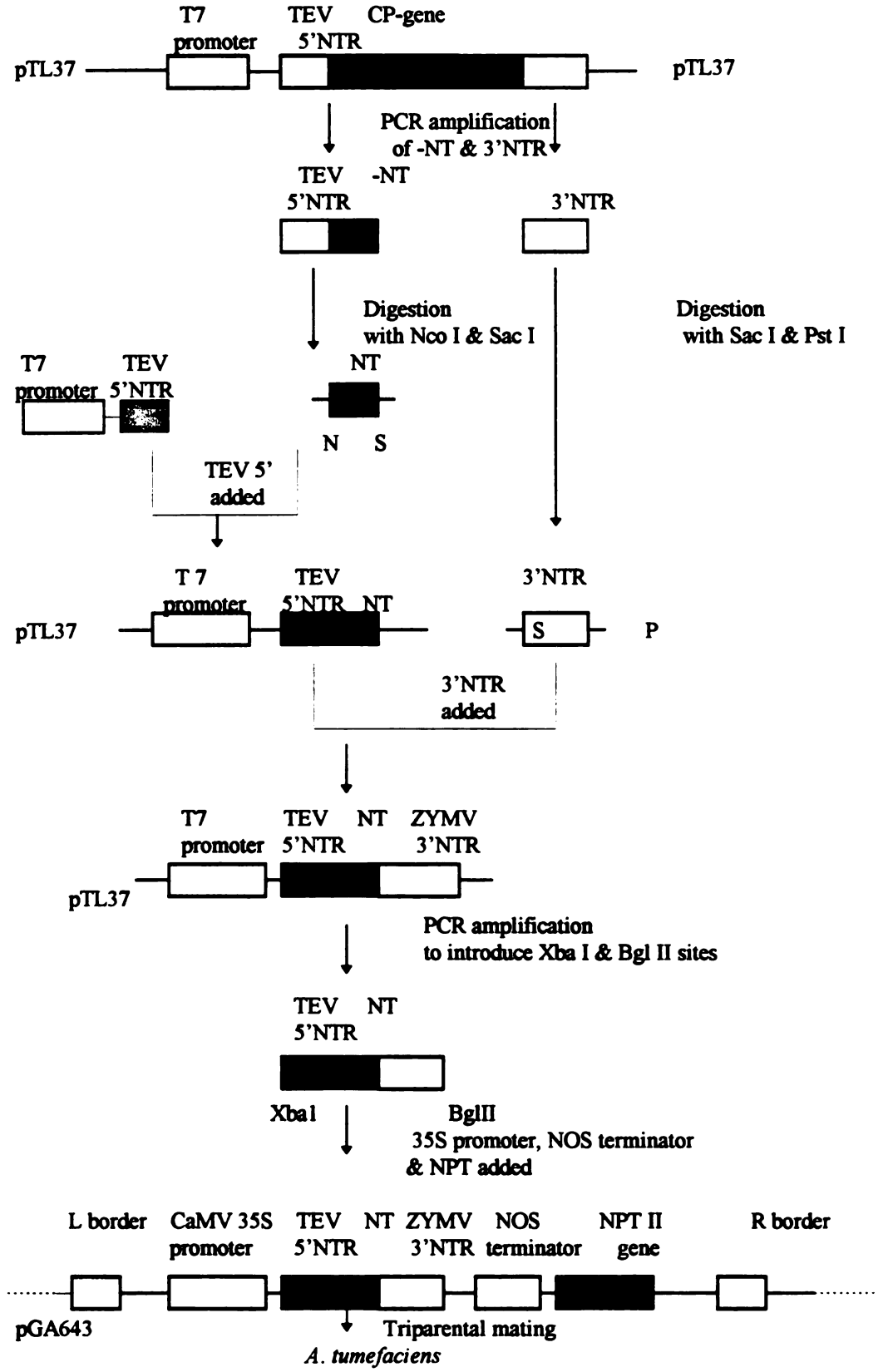

Figure 2. The genetic engineering of ZYMV CP-NT gene for expression in plants.

SacI site resulted in a change of two of the amino acids in the CP sequence that was added to the 3'NTR, 1. valine to glutamic acid 2. asparagine to leucine.

Cloning of the sense-defective CP construct (CP-SD):

A frameshift mutation was introduced into the CP sequence to make the CP gene untranslatable. The CP sequence in pTL37 was modified to introduce an **AvaI** site after the 10th base pair by introducing a single guanine residue using the primer RG25 (5' GCTCCATGGCAGGCACTCGAGCCAACTGTG 3'). RG25 and RG7 were used to amplify the sense-defective version of the FLCP. Sequence analyses were performed using the DNASIS program (Hitachi software Engineering) to check the consequences of the introduced frame shift mutation. The initial stop codon at position 10 was followed by several other stop codons throughout the CP sequence. The amplified fragment was digested with Nco I and PstI and ligated into the plasmid pTL37 which had been digested with NcoI and PstI (Fig 3).

In vitro transcription and translation:

Functionality of the TEV 5' NTR- ZYMV sense-defective CP gene construct and the amino- terminal portion of the CP gene was verified by *in vitro* transcription from the T7 promoter followed by *in vitro* translation. Tritium labeled leucine was used to label the sense defective CP. The translational product derived from approximately 0.5-1.0 micrograms of transcript was run on a SDS polyacrylamide gel and visualized by

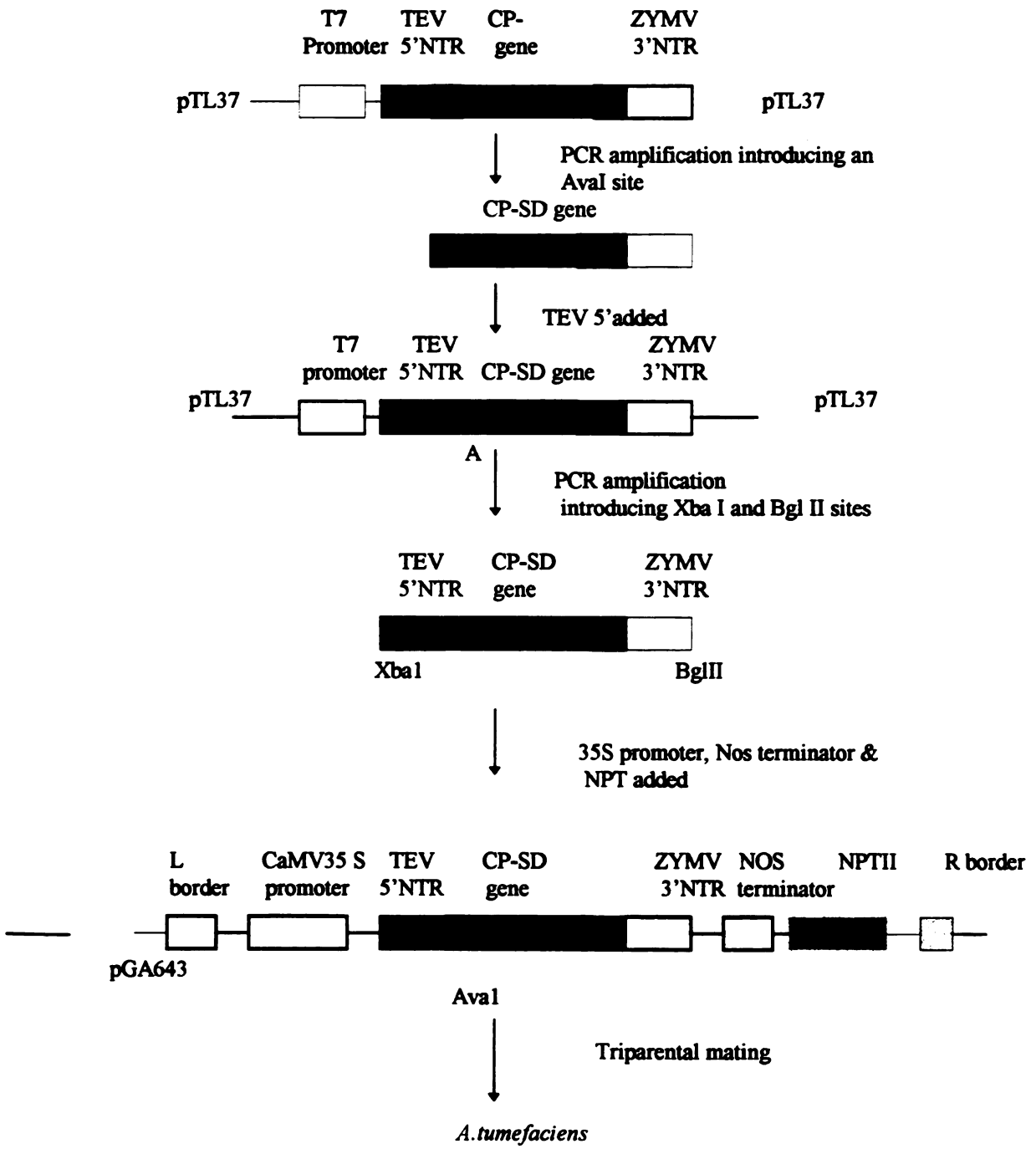

Figure 3. The genetic engineering of ZYMV CP-sense defective gene for expression in plants

autoradiography. Due to the small size and absence of leucines, the pTL37+NT product was labeled with tritium-labeled lysine. There are thirteen lysines in FL-CP, seven of which are located within the amino-terminal portion. The expected relative sensitivity for CP-NT was 50%, when compared to lysine labelled FL-CP . An amino acid mix consisting of all amino acids except lysine was prepared at a concentration of 1mM of each amino acid. The entire translation product was run on a 10% tricine SDS-PAGE gel according to the protocol of Schagger and Von Jagow (1987). In another experiment, the translation product was run on the gel and was transferred to 0.1micrometer nitrocellulose. After western blotting the nitrocellulose membrane was exposed to X-ray film.

Cloning the genes into a T-DNA vector:

The FLCP, FLCP sense-defective, and CP-NT fragments were amplified through 20 cycles of the PCR using the primers (RG26 & RG27) designed to introduce a XbaI site at the 5' end of the TEV 5'NTR and a BglII site at the 3' end of the 3' NTR. Amplified fragments were digested with XbaI and BglII and ligated into XbaI and BglII-cut pGA643, adjacent to the plant-selectable marker for kanamycin resistance, the NPTII gene. Resulting clones were analyzed by restriction enzyme digestions to verify the orientation of the inserts with respect to the CaMV 35S promoter and terminator. Three constructs were generated: ZYMV-FLCP, which contains the FL-CP gene sequence in the sense orientation; ZYMV-SD, which contains sense-defective version of FL-CP gene; and ZYMV-NT, which contains the amino-terminal portion of CP. All three constructs

contain the CaMV 35S promoter, TEV 5' NTR, ZYMV 3' NTR, and NOS terminator. The ZYMV gene constructs and NPTII gene were located within left and right *A. tumefaciens* T-DNA borders.

Agrobacterium transformation:

pGA643-derived binary vectors containing the ZYMV CP gene constructs namely, CP-SD and CP-NT, 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. pGA643 was maintained on plates with tetracyline at a concentration of 10mg/l. Antibiotics in YEB plates used for screening colonies after triparental mating were as listed: tetracycline 6mg/ml, kanamycin 10mg/ml, and rifampicin 35mg/l. *Agrobacterium* transformants were verified by restriction enzyme digestions. Plasmid was extracted from putative *Agrobacterium* transformants according to the alkaline lysis procedure (Birnboim and Doly 1979). About 100 nanograms of the extracted plasmid was used to back-transform competent cells of DH5α. DH5α transformants were grown and plasmid was extracted by the alkaline lysis procedure (Birnboim and Doly 1979). Restriction analysis was performed on the extracted plasmid to verify the transformants.

Plant transformation:

Nicotiana benthamiana plants were transformed individually with pGA643+CP-SD, pGA643+CP-NT, pCIB10+FLCP, pCIB10+Core, and vector pCIB10 using the protocol of An et al. (1988). Shoots were regenerated on medium consisting of Murashige and

Skoog (1962) salts, sucrose (30 g/l), benzyladenine (1mg/l) napthalene acetic acid (0.1mg/l), kanamycin (100mg/l) and carbenicillin (300 mg/l). Transformed shoots were subsequently rooted on phytohormone-free medium containing 100 mg/l of kanamycin prior to transferring to soil. The tissue culture growth room conditions were 25-26° C with a 16hr photoperiod provided by cool white fluorescent lamps (ca. 2500 lux). *Agrobacterium* cultures were grown and maintained on YEB medium with 50mg/l kanamycin. Regenerated plantlets were transplanted to sterile soil mix as soon as roots appeared and transferred to the growth chamber. The pots were covered with plastic bags and plants were hardened by gradually exposing them to full light and heat. The interval from explant to rooting stage was about 4-5 weeks and from explant to seed set was 14-15 weeks.

Enzyme-linked immunosorbent assay (ELISA):

Samples of leaf tissue from kanamycin-resistant regenerated plants were initially screened for expression of NPT II protein using double-antibody sandwich ELISA. The NPT11 assay kit was purchased from 5 prime>3 prime, Inc.(Boulder, CO), 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 according to a modified protocol developed from the procedure of Dellaporta et al. (1985). Instead of grinding the leaf samples in liquid nitrogen, the leaf sap was extracted by pressing the leaves in a pasta maker. About 0.5-1gm of fresh leaf tissue was added to

750 microliters of extraction buffer in a plastic bag and the bag was rolled in a pasta maker. The extracted leaf sap was placed into an eppendorf tube, 50 µl of 20% SDS was added and the mixture was incubated for 10 minutes at 65° C. 250 µl of 5M potassium acetate was added and the mixture was chilled on ice for 5 minutes, followed by centrifugation for 10 minutes. The supernatant was precipitated with 0.7 vol. isoproponal and 0.1 vol. 3M sodium acetate. The precipitate was resuspended in 500 µ1 of 50 mM Tris, 10 mM EDTA (pH 8.0) and purified by a phenol chloroform extraction. The extracted DNA was treated with RNase A. The presence of the inserted ZYMV CP fragments was verified by PCR using the primers specific for the 5' end of the TEV non-translated lead sequence and the and 3' end of ZYMV 3' NTR i.e., RG26 and RG27 for CP-SD, CP-NT., RG6 and RG7 for FLCP and core. About 50ng of total plant DNA was used with 40 cycles of PCR. Cycle conditions were as follows, 94°C 1 min., 58°C 1min., 72°C 2 min. Concentrations were : magnesium sulphate ($MgSo_4$)-1.5mM, dNTP's-200mM, primers-150 nanograms, Taq polymerase-2 units. Samples were preheated for 5 minutes at 94^0 C before starting the amplification.

Transcriptional analysis of transformants:

The transcripts of ZYMV FLCP, FLCP-SD, CP-NT were examined by northern analysis. Total RNA was isolated from NPT-positive R_1 transgenic plants essentially as described by Nagy et al. (1988). The RNA was separated by electrophoresis in a 1.8% agarose gel containing formaldehyde and MOPS buffer (Sambrook et al. 1989) and transferred to MSI magna nylon transfer membrane (Fisher Scientific) for northern analysis. The RNA on the

nylon membrane was fixed by UV-cross linking (Sambrook et al. 1989). To prepare the probe, the ZYMV-CP fragment was isolated from the plasmid pTL37+CP by digesting with NcoI and PstI and electroeluting the CP fragment from the agarose gel. The CP fragment was ^{32}P-labelled using a random primer DNA labelling kit (United States Biochemical., Corp., Cleveland, OH) and unincorporated nucleotides were removed using a spun column (Sambrook et al. 1989) of 1ml G-50 sephadex in TES was used (20mM Tris, 20mM NaCl, 2mM EDTA, pH 8). The column was washed twice with 100 microliters of TES. After adding the sample it was spun in a clinical centrifuge (#4) for 5 minutes and the sample was collected in an eppendorf tube.

Protein analysis of transgenic plants:

Total soluble protein was extracted from 0.1gm leaf tissue of R_0 transgenic plants according to the protocol of Lin and Thomashow (1991), and the concentration was determined by the method of Bradford (1976). For FL-CP and Core protein, analysis was performed on R_1 plants, whereas in CP-NT and CP-SD, analysis was performed on R_0 plants. FL-CP, Core, and CP-SD, protein was separated using a 10% SDS-PAGE gel. CP-NT protein was separated on a 10% tricine SDS gel . Electrophoresed proteins were transferred to 0.1micrometer pore size nitrocellulose using an electroblotter. The ZYMV CP gene fragment was detected by rabbit anti-ZYMV CP polyclonal antibodies (Hammar and Grumet, unpublished) and alkaline phosphatase-conjugated goat-anti rabbit secondary antibodies (Sigma, St. Louis, MO).

Genetic analysis:

To test for the inheritance of the introduced NPT II gene, transformed *N.benthamiana* plants were allowed to self-pollinate in the growth chamber. The progeny were examined for the expression of NPT gene by ELISA as described above.

RESULTS

Construction of two versions of ZYMV CP gene.

Two versions of ZYMV CP, a sense defective construct (CP-SD) and a construct comprised of the amino- terminal portion of the CP (CP-NT) have been constructed (see Fig. 4). The sense defective construct has the full length coat protein sequence of ZYMV but it was rendered untranslatable by introducing a frame shift mutation early in the sequence. As a result of the frame shift mutation, several stop codons have been introduced. The initial stop codon at amino acid position 10 was followed by several other stop codons throughout the CP sequence. The amino-terminal construct has the 100 amino-terminal bases of ZYMV CP generated by PCR using the ZYMV FL-CP construct as the template and cloned in such a way that it has 150 base pairs of 5' NTR (of TEV) on the 5' end and 250 base pairs of 3'NTR (of ZYMV) on the 3' end. This process led to the addition of five amino acids and a stop codon, from the 3' end of the coat protein to the 5' end of the 3' NTR, namely valine→glutamic acid, aspargine→leucine, threonine, methionine and glutamine. The FL-CP, CP-SD and CP-NT genes were ligated individually into the binary vector pGA643 adjacent to the transferable plant selectable marker for kanamycin resistance, the NOS/NPTII chimeric gene. Both the ZYMV gene construct and NPT II gene were located within left and right *A.tumefaciens* T-DNA borders.

FL-CP and Core (Fang and Grumet, 1993) which had been cloned previously were used for comparisions. All the constructs have 5' NTR (of TEV) at the 5' end and 3' NTR (of ZYMV) at the 3' end. The constructs CP-SD and CP-NT, which were cloned

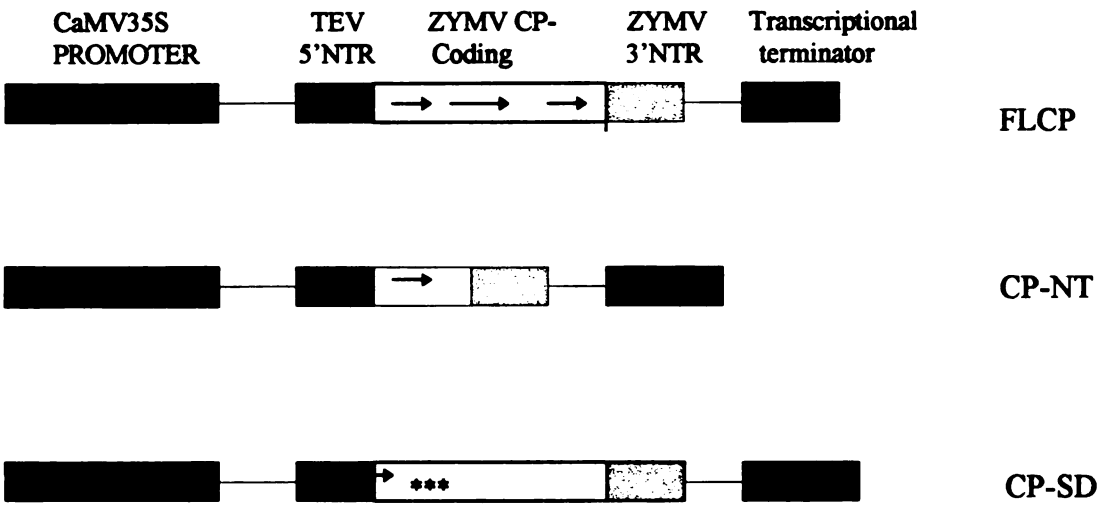

Figure 4. Zucchini yellow mosaic virus coat protein (ZYMV-CP) constructs. Each CP-derived construct contains the *Agrobacterium tumefaciens* T-DNA left and right border sequences, the cauliflower mosaic virus 35S promoter and either 35S or NOS terminator, the tobacco etch virus (TEV) 5'nontranslated region (NTR), all or a portion of the ZYMV-CP coding sequence, the ZYMV 3'NTR, and the selectable marker gene for kanamycin resistance, neomycin phosphotransferase (NPT II). The full length-coat protein (FL-CP) construct includes the full length ZYMV-CP coding sequences. The CP-NT construct contains only the 100 amino-terminal amino acids of the ZYMV CP gene. The sense defective (CP-SD) construct includes same the components as the FL-CP construct, except that the ZYMV-CP coding sequence is an untranslatable version.

into the T-DNA vector pGA643, have the CaMV 35S promoter and NOS terminator, whereas FL-CP and Core, which were cloned into a different T-DNA vector, pCIB10, have the CaMV 35S promoter and terminator (Fang and Grumet, 1993). The CP-SD construct was generated to identify whether the coat protein or its mRNA is required to confer protection against homologous or heterologous viruses. The CP-NT construct was generated to study the role of the amino-terminal portion of the ZYMV coat protein in conferring coat protein mediated protection.

To determine whether the CP-SD and CP-NT constructs were translationally functional, the protein products of in vitro transcription and translation were examined for presence and correct size by SDS-PAGE and visualized by autoradiography. The FL-CP construct and the template from the kit were used as positive controls. The expected 60 kDa protein with kit positive control and 30 kDa CP protein was visible within 3 days of exposure (Fig. 5). Nothing was detected in the negative control, which had all the reagents except the template. No protein was detected in the sense defective construct even after 4 weeks of exposure (Fig. 6), however the expected size transcript was detected after *in vitro* transcription, indicating that the construct was transcriptionally functional but translationally defective. In similar assays the CP-NT peptide also was not detected (results not shown), but the transcript levels were comparable to controls. Western blotting on in vitro translation products of CP and CP-NT was unsuccessful. Possible explanations for the result with NT construct are the NT peptide is not stable or the amount produced was at a level below the detection limits of the assay used.

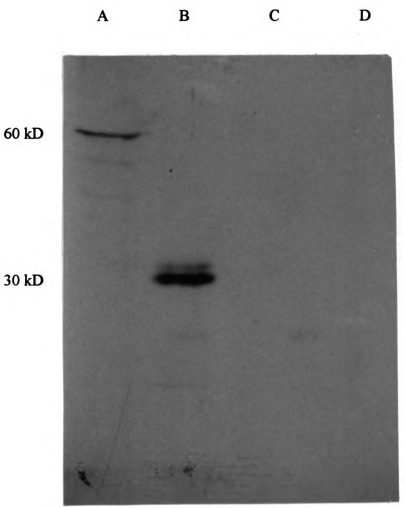

Figure 5. *In vitro* transcription and translation of the ZYMV CP and CP-SD genes after 3 days of exposure. ZYMV CP and ZYMV CP-SD cDNA in plasmid pTL37 were used as the templates for *in vitro* transcription, and then translated in vitro using rabbit reticulocyte lysate (Promega). The protein products were labelled with ^{3}H-leucine. Lane A, translation product using the positive control template provided in the kit, expected size is 60kD; Lane B, translational product when ZYMV FL-CP RNA was used as template, expected product is 30kD; lane C, the protein product using ZYMV CP-SD RNA as template; lane D, negative control has reagents but no template.

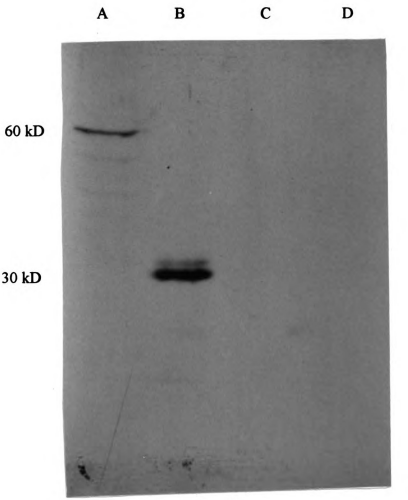

Figure 6. *In vitro* transcription and translation of the ZYMV CP and CP-SD genes after 4 weeks of exposure. ZYMV CP and ZYMV CP-SD cDNA in plasmid pTL37 were used as the templates for *in vitro* transcription, and then translated in vitro using rabbit reticulocyte lysate (Promega). The protein products were labelled with ^{3}H-leucine. Lane A, translation product using the positive control template provided in the kit, expected size is 60kD; Lane B, translational product when ZYMV FL-CP RNA was used as template, expected product is 30kD; lane C, the protein product using ZYMV CP-SD as template; lane D, negative control, reagents but no template.

Plant transformation

The *A. tumefaciens* binary transformation system was used to introduce ZYMV CP constructs into *N. benthamiana*. Kanamycin resistant plants were initially screened for expression of NPT II protein by ELISA. Of the *N. benthamiana* regenerants, about one third of the Core (43 out of 113 samples tested), pCIB10 (29 out of 88) and CP-SD (26 out of 75), and approximately one quarter of the FL-CP (19 out of 88 samples), and CP-NT (17 out of 64) were NPT II positive (see Table. 1)

The presence of the inserted ZYMV CP gene sequences in the regenerated, NPT-positive R_o *N. benthamiana* plants was tested by PCR amplification (see Table. 1) from plant genomic DNA. The expected size of fragment in FL-CP, CP-SD, Core, CP-NT were 1200, 1200, 1000, 500 base pairs respectively. At least three individual regenerants in each construct, amplified the expected size fragments (see Fig. 7). The full length coat protein construct in *E.coli* was used as a positive control. DNA from a non-transgenic plant was used as a negative control. The positive control generated the expected 1200 base pair fragment, and no amplification was seen in the negative control. Summaries of the data collected for each of the putatively transgenic lines is presented in Tables 2 to 5. Most of the regenerated plants were healthy, morphologically normal, and produced typical flowers. All plants transformed with CP, Core, pCIB10 produced normal seed. In plants transformed with CP-SD, one out of 20 lines one was sterile (SD 9A). There was no seed set in spite of flowering. For plants transformed with CP-NT, only five plants out of 13 plants produced seed, although all the plants flowered normally. In one line (NT 4B), in addition to sterility, some morphological abnormalities were noticed on leaves (undulations,

Table 1. Characterization of the putatively transgenic R_0 individuals of *N.benthamiana*.

Construct	Number of regenerants	Number of ELISA positive regenerants / Number of plants tested.		Number of PCR positive / Number of ELISA positive plants tested.	
		No.	%	No.	%
pCIB10+FLCP	170	19 / 88	22	3 / 3	100
pCIB10+Core	113	43 / 113	38	3 / 6	50
pGA643+SD	75	26 / 75	35	6 / 8	75
pGA643 + NT	64	17 / 64	27	8 / 13	62
pCIB10	88	29 / 88	33	-	

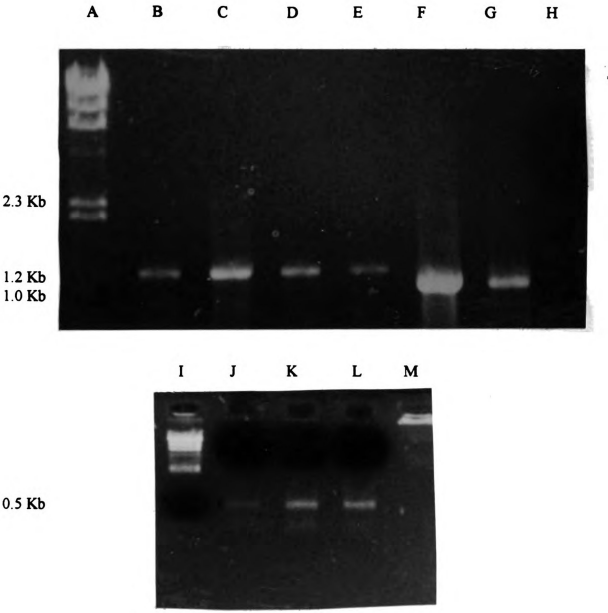

Figure 7. PCR amplified ZYMV CP DNA fragments from transgenic *N.benthamiana* plants. The samples from left to right lane A, lamba standard; lane B and C, FL-CP transformed *N.benthamiana* plants (CP 6A and CP 10F respectively); lanes D and E, CP-SD transformed *N.benthamiana* plants (SD 3D and SD 8H repectively); lanes F and G, Core transformed *N.benthamiana* plants (Core 3F and Core 3G respectively); lane I, lambda standard (Hind III cut); lanes J K and L, CP-NT transformed *N.benthamiana* plants (NT 5C, NT 5E and NT 1B respectively) ; lanes H and M are the negative control lanes. The primers used in the PCR were RG-26 and RG-27. The ZYMV FL CP and CP-SD fragments were approximately 1200 base pairs, the ZYMV Core was about 1kb and CP-NT was about 0.5 kb

mosaic like symptoms and increase in the size of the leaf), even though this particular line tested positive by PCR. Of the eight sterile individuals of the NT construct, four of them amplified the expected 500 base pair fragment when they were tested by PCR. In the second batch of plants transformed with NT sterility was observed to a lesser extent and only three plants out of 33 regenerants were sterile. It is not clear whether this sterility is associated with some factor in the tissue culture process or the construct, since sterility was relatively more when compared to individuals transformed with other constructs.

Transcriptional analysis of transformants

The transcripts of ZYMV CP constructs were examined by northern analysis in R_1 transgenics. Representative results are shown in Fig. 8. Hybridization with a labelled ZYMV CP fragment revealed strongly hybridizing bands in transgenic *N.benthamiana* plants which were individually transformed with one of the four versions of the ZYMV CP gene. The control, non-transformed plants, did not give any signal. Two lines of FL-CP were tested, of which one line (CP 10F) produced detectable levels of transcript. In the other line (CP1) transcript was below detection levels. In the case of the Core transgenic, the two lines tested (Core 3G and Core 8D) produced detectable levels of transcript. Three lines of CP-SD (SD 2H, SD 3D, SD 8H) were tested, all of which produced a detectable amount of transcript, although the level of transcript produced varied between lines. In the case of the NT transgenics, four lines were tested for transcript (NT 4C, NT

Table 2. Summary of the analyses performed on NPT-positive FL-CP transformants

(+) lines	PCR (R_0)	Northern (R_1)	western (R_1)	seed produced	segregation ratio NPT(+) : (-)
CP 1	+	-	-	yes	50 : 76*
CP 10F	+	+	+	yes	29 : 10ns
CP 6A	+	n.t	-	yes	8 : 68*
CP 1D	n. t	n. t	n.t	yes	n.t.
CP 1H	n. t	n. t	n.t.	yes	n.t.
CP 2	n.t.	n.t.	n.t.	yes	n.t.
CP 2A	n.t	n. t	n.t	yes	n.t.
CP 2H	n.t.	n.t.	n.t.	yes	n.t.
CP 2F	n.t.	n.t.	n.t.	yes	n.t.
CP 3	n.t.	n.t.	n.t.	yes	n.t.
CP 4	n. t	n. t	n.t	yes	n.t.
CP 5	n. t	n. t	n.t	yes	n.t.
CP 5G	n.t.	n.t.	n.t.	yes	n.t.
CP 6F	n.t.	n.t.	n.t.	yes	n.t.
CP 7F	n.t.	n.t.	n.t.	yes	n.t.
CP 7H	n.t.	n.t.	n.t.	yes	n.t.
CP 8D	n. t	n. t	n.t	yes	n.t.
CP 8H	n. t	n.t	n.t	yes	n.t.
CP 9C	n.t.	n.t.	n.t.	yes	n.t.

n.t.- not tested,

ns-ratio not significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3:1).

* Ratios significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3 : 1).

(+) detectable message or protein respectively.

(-) no detectable message or protein respectively.

Table

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Table 3. Summary of the analyses performed on NPT-positive Core transgenics

NPT (+) lines	PCR (R_0)	Northern (R_1)	Western (R_1)	Seed produced	Segregation ratio NPT(+):NPT(-)
Core 2A	+	n.t.	-	yes	2 : 66*
Core 3F	+	n.t	+	yes	20 : 0ns$^{(15:1)}$
Core 3G	+	+	-	yes	80 : 54*
Core 4D	n.t.	n.t.	n.t.	yes (L)	84 : 18ns
Core 5F	n.t	n.t	n.t.	yes	3 : 101*
Core 8D	n.t.	-	+	yes	19 : 35*
Core 9G	n.t	n.t.	n.t.	yes (L)	0 : 20

n.t.-not tested.
ns-ratio not significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3 :1).
* Ratios significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3 : 1).
L-comparitively less seed produced.
(+) detectable message or protein respectively.
(-) no detectable message or protein respectively.

Table 4. Summary of the analyses performed on NPT-positive sense defective CP transgenics

Total NPT (+) lines	PCR (R_0)	Northern (R_1)	western (R_0)	Seed produced	Segregation ratio NPT(+):NPT(-)
SD 3D	+	+	-	yes	39 : 13ns
SD 2H	+	+	-	yes	39 : 18ns
SD 8H	+	-	n.t.	yes	118 : 6ns (15:1)
SD 3E	+	n.t.	-	yes (L)	n.t.
SD 11H	+	n.t.	-	yes	70 : 125*
SD 11C	+	n..t.	-	yes	96 : 65*
SD 12F	-	n.t.	-	yes	n.t.
SD 2C	-	n.t.	-	yes	n.t.
SD 3A	n.t.	n.t.	n.t.	yes	n.t.
SD 12 A	n.t.	n.t.	n.t.	yes	n.t.
SD 3F	n.t.	n.t.	n.t.	yes	n.t.
SD 1H	n.t.	n.t.	n.t.	yes	n.t.
SD 2D	n.t.	n.t.	n.t.	yes	n.t.
SD 11F	n.t.	n.t.	n.t.	yes	n.t.
SD 10C	n.t.	n.t.	n.t.	yes	n.t.
SD 5B	n.t.	n.t.	n.t.	yes	n.t.
SD 9A	n.t.	n.t.	n.t.	sterile	n.t.

n.t.- not tested.
ns- ratio not significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3 : 1or 15:1).
L- a relatively low amount of seed was produced.
* Ratios significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$
(+) detectable message or protein respectively.
(-) no detectable message or protein respectively

Table 5. Summary of the analyses performed on NPT-positive NT transgenics.

NPT (+) lines	PCR (R_0)	Northern (R_1)	Western (R_0)	Seed produced	segregation ratio NPT(+):NPT(-)
NT 2C	-	n.t	n.t.	no	n.t.
NT 2E	-	n.t.	n.t.	no	n.t.
NT 3C	-	n.t.	-	yes	41 : 25ns
NT 4A	+	-	-	yes	40 : 6ns
NT 7B	+	+	n.t.	yes	51 : 12ns
NT 4C	+	+	-	yes	129: 18*
NT 3G	-	+	-	yes	143: 23*
NT 5C	+	n.t.	n.t.	no	n.t.
NT 5B	+	n.t.	n.t.	no	n.t.
NT 1B	+	n.t.	n.t.	no	n.t.
NT 2B	+	n.t.	n.t.	no	n.t.
NT 4B	+	n.t.	n.t.	no	n.t.
NT 7C	+	n.t.	n.t	no	n.t.

n.t.-not tested
ns- ratio not significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3 : 1).
* Ratios significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$
(+) detectable message or protein respectively.
(-) no detectable message or protein respectively

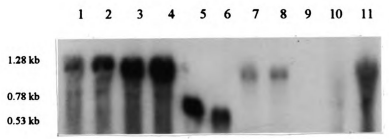

Figure 8. Accumulation of transcripts of CP gene constructs in transgenic plants. The northern blot was loaded with 10 ug of total RNA isolated from leaves of transgenic *N.benthamiana* plants. Lane 1and 2 FL- CP transformed *N.benthamiana* line CP 10F; lane 3 and 4 Core transformed *N.benthamiana* line Core 3G; lanes 5 and 6 CP-NT transformed lines NT 4C and NT 3G; Lanes 7, 8, 10 and 11 CP-SD transformed *N.benthamiana* lines SD 2H, SD 2H, SD 8H, and SD 3D respectively; lane 9- negative control. The blot was hybridized to P^{32}-labelled cDNA corresponding to ZYMV CP gene. Sizes of the bands were estimated by running RNA standards (0.155 to 1.770 Kb) (Gibco-BRL) along the side of the samples.

4A, NT 3G, NT 7B). Lines NT 4C and NT 3G produced a detectable amount of transcript of the expected size. NT 7B transcript was detected but it ran higher than expected.

The estimated size (based on markers) of the specific transcripts produced by plants transformed by the ZYMV CP gene (both sense and sense defective) was 1200 bases (Fig. 8). This compares well with the size of the RNA that was expected, which is composed of 150 bases of TEV 5' NTR, 840 bases of ZYMV FL-CP sequence and 226 bases of ZYMV 3' NTR. The ZYMV CP-NT transformed plants showed the expected bands of 500 bases (Fig. 8), which should be composed of 150 bases of TEV NTR, 100 bases of ZYMV FL-CP and 226 bases of ZYMV 3'NTR. The expected size of the Core construct is 1000 bases (Fig. 8), but it appears to be running little high. Similar observation has been made elsewhere as well. Lindbo and Dougherty (1992) observed that the transcript of their sense defective version of the coat protein was running slightly higher than expected, possibly due to termination at an alternately selected site and /or a longer poly-A tail on the transcript.

Expression of ZYMV coat protein in transformed plants

A total of 18 NPTII-positive *N.benthamiana* plants of which 16 were tested positive in PCR,were tested by western analysis using polyclonal antisera to ZYMV CP (Table 2-5). A ZYMV infected zucchini plant was used as a positive control. Vector-transformed and non-transformed *N. benthamiana* were used as negative controls. Detectable amounts of viral protein of expected sizes were found in plants transformed with full length CP and

Core fragments (Fig. 9). Three lines of FL-CP R_1 plants (CP 10F, CP 6A & CP1) were tested for the presence of ZYMV CP protein. Lines CP 10F and CP6A produced detectable amounts of protein, although the level of expression in CP 6A was less than that of CP 10F (Fig 9). Protein in line CP 1 was below detectable levels. In the case of the Core transgenics (R_1 plants), four lines (Core 8D, Core 3F, Core 2A and Core 3G) were tested for the expression of the protein. Both Core 8D and Core 3F expressed the protein at detectable levels, but the level of expression in Core 3F was much lower than Core 8D (Fig 9). The expression of protein in lines Core 2A and Core 3G was below the detectable level. In the case of SD transgenics (R_0 plants), four lines (SD 2H, SD 11H, SD 3E & SD 3D) , were tested for the expression of protein and no protein was detected in any of the SD lines (Fig 8). In the case of NT transgenics (R_0 plants), four lines (NT 4A, NT 4C, NT3C & NT 3G) were screened for expression of protein. The positive and negative controls worked, but there was no detectable amount of protein from NT transgenics. The experiment was repeated with a ten-fold increase in the concentration of antibody, but still no protein was detected in the western blot. The quantity of total plant protein from CP-NT transgenic lines loaded on the Tricine SDS-PAGE was increased by four times and the antibody concentration by ten times in another experiment and again detectable amounts of NT protein were not observed.

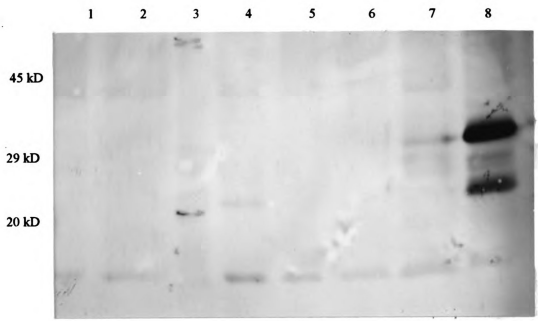

Fig.ure 9. Detection of ZYMV CP, Core, CP-SD proteins in transgenic *N.benthamiana* plants. Total soluble protein was isolated from leaf samples of transgenic plants. 50ug total protein isolated from plants transformed with CP, Core, CP-SD was separated on 10% SDS polyacrylamide gel, transferred to nitrocellulose, and treated with rabbit antibody against ZYMV CP, followed by alkaline phosphotase-conjugated goat anti-rabbit secondary antibody. Lane 1 contains protein from a non transformed plant; lane 2 contains protein from vector (pCIB10) transformed plants; lane 3 contains protein standards; lane4 contains protein from Core trasnformed plant (core 3F); lane 5 and 6 contain protein from SD (SD 2H, SD 3D) transformed plants; lane 7 contains protein from CP (CP 10F) transformed plant; lane 8 contains protein from ZYMV infected sap as a positive control. The estimated sizes of FL-CP is approximately 30kd, the Core protein is about 26kd. No protein was detected in CP-SD transformed plants and in negative control. Estimation of sizes is based on sigma wide range (6.5 - 205kDa) color markers.

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 *N. benthamiana* were analyzed for the presence of the NPT II gene by ELISA. The results of progeny analysis are shown in Table 6. Progeny from plants CP-10F, Core 4D, Core 3F, SD 3D, SD 2H, NT 7B, NT 4A and NT 3C segregated with a ratio approximating 3 : 1 (NPT + : NPT -), suggesting that the NPT genes were inserted at a single locus. The segregation ratio of progeny from plants SD 8H and Core 3F approximated 15 : 1 (NPT + : NPT -), suggesting that the NPT II gene was inserted at two loci. The reason(s) for the aberrant ratios (which did not fit the 3 : 1 expression model), in the progeny from plants CP 1, CP 6A, Core 2A, Core 3G, Core 5F, Core 8D, Core 9G, SD 11H, SD 11C, NT 4C and NT 3G are unknown.

Table 6. Segregation ratios for the expression of NPT II gene as detected by NPT-ELISA in the R_1 progeny of transgenic *N.benthamiana* plants

[1]Plant line	Total No.	NPT (+)	NT (-)	+/- ratio	X^2 [2]
CP 10F	39	29	10	3 : 1	0.034 [3] ns
CP 1	126	50	76		82.9*
CP 6A	76	8	68		167*
Core 4D	102	84	18	3 : 1	3.15ns
Core 3F	20	20	0	15 : 1	3.60ns
Core 3G	134	80	54		16.33*
Core 2A	68	2	66		186*
Core 5F	104	3	101		286*
Core 8D	54	19	35		44.6*
Core 9G	20	0	20		58.1*
SD 3D	52	39	13	3 : 1	0.03ns
SD2H	57	39	18	3 : 1	1.2ns
SD 8H	124	118	6	15 : 1	.80ns
SD 11H	195	70	125		157*
SD 11C	161	96	65		19.9*
NT 7B	63	51	12	3 : 1	1.4ns
NT 4A	46	40	6	3 : 1	3.3ns
NT 3C	66	41	25	3 : 1	5.5*
NT 4C	147	129	18		13.3*
NT 3G	168	143	23		11.2*

[1] Plants labelled as CP are transformed with the full length CP construct; Core, construct with truncated portion of the gene; SD- construct with sense defective version of the full length CP; NT-construct of amino-terminal portion of the CP.

[2] X^2 values calculated as $X^2 = \Sigma[(Io - eI- 1/2)^2 / Ie]$ using the Yate's correction factor.

[3] ns Ratios not significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$(3: 1).
Ratios significantly different from the predicted ratios by X^2 analysis at $P \geq 0.05$ (3 : 1).

DISCUSSION

Two versions of the ZYMV CP gene, a sense defective construct (CP-SD), and the amino-terminal portion of the CP (CP-NT), were constructed. The FL-CP, Core, CP-SD and CP-NT fragments were cloned into the T-DNA vector, pGA643, and successfully expressed in *N.benthamiana* plants. ZYMV CP, Core, CP-SD and CP-NT fragments were detected in more than 50% of the NPT positive plants by PCR amplification of the CP gene. The FL-CP and Core constructs were in the transformation vector pCIB710 and then transferred into the T-DNA vector pCIB10 (Fang and Grumet 1993), CP-SD and CP-NT, were carried by vector pGA643. All the constructs have the 5' NTR of TEV and the 3' NTR of ZYMV. It has been shown that the TEV 5' NTR region can function as an enhancer of translation (Carrington and Freed, 1990). The 3' NTR confers polyadenylation (PolyA) signals to the transcripts (Hari, 1995). Based on the preliminary analysis of transgenics, there was no significant difference in the percentage of regenerants that were positive for NPT II between the constructs, whether vectored in pCIB10 or pGA643 (see Table 1). In the case of constructs in pCIB10, the percentage ranged from 22% to 39%. In the case of constructs in pGA643 the number of NPT II positives in the regenerants ranged from 27% to 35%. It is not possible to compare the PCR data at this time, since only a few lines of FL-CP and Core constructs have been tested.

Northern blot analysis indicated that the ZYMV CP genes were being transcribed and maintained at detectable steady state levels. The level of mRNA produced by

different FL-CP, Core, CP-SD and CP-NT transgenic lines varied. Some plants produced a higher level of message (Fig. 7), while other plants produced either no RNA, or amounts that were below the detection level (data not shown). Overall the RNA levels in SD lines appear lower than those in FL-CP and Core (Fig. 7). This might be due to the fact that SD is untranslatable, as untranslatable sequences are usually more unstable. The plant to plant variability for the expression level may be caused by positional effects due to insertion in differently expressed chromosomal locations. Reduced levels of RNA could also be due to the cellular surveillance mechanism (Lindbo et al. 1993), according to which plant cells are able to sense elevated or aberrant RNA levels and then target and inactivate them by a cellular factor which degrades the RNA that has accumulated to a critical threshold level. In lines in which critical threshold is not reached, the response system would not be activated (Smith et al. 1995).

The northern and western results verified that the engineered ZYMV CP gene constructs were functional for expression in plants with the exception of the amino-terminal construct. The western blots for coat protein products revealed the presence of the 30 kD FL ZYMV CP polypeptide and the 26 kD Core protein product in transgenic plants transformed with FL-CP or Core constructs, respectively but did not detect any similar protein in SD expressing plants. In the plants expressing the CP-NT gene a 3kD protein was expected but could not be detected in western blotting despite a high level of transcription. There could be several reasons for this. The amount of protein may be below the detection limit or the polypeptide might be unstable when separated from the rest of the protein. Alternatively, the polypeptide might be present but the CP polyclonal

antibody may fail to recognize in due to a conformational change, i.e., when the CP-NT is seperated from the rest of the CP it may not be folding in the proper conformation to be recognized by the CP antibody. Though the amino-terminal portion of the FL-CP is highly antigenic (Dougherty et al. 1985; Shukla et al 1989), it is not known whether the amino-terminal portion alone retains its antigenic recognition sites. It would be interesting to know if the amino-terminus, after being removed by trypsin treatment, would retain its conformation. Doing a western blot on just the amino-terminus after has been separated from coat protein might give some information on the antigenictiy of the isolated amino-terminus. Similar to these results, Silva-Rosales et al. (1994) observed that there was no detectable level of C-terminal truncated TEV coat protein in transgenic plants. It has been proposed that perhaps truncated proteins which cannot fold and adopt native conformation are rapidly cleared from the cell (Silva-Rosales et al. 1994). However, more lines need to be screened before a conclusion can be reached in this case. In the case of NT construct, owing to the high level of sterility in the first batch of NT transgenics, only four lines have been tested for the presence of the NT protein. Even if the protein levels are below the detection levels, these lines might be still be valuable. There are many examples in the literature where no correlation exists between the protein expression and the degree of genetically engineered virus resistance (review , Grumet 1995).

The segregation studies of the progeny of transgenic *N.benthamiana* plants indicated that the introduced genes were transmitted to the next generation. In some lines, the segregation ratios of the progeny were consistent with a 3:1 (NPT positive: NPT negative) ratio suggesting the incorporation of a single gene. In some lines the ratio approximated

15 : 1 (NPT + : NPT -) suggesting insertion at two loci. Aberrant ratios in some lines are unexplained. Lines with single locus / gene insertions have been utilized extensively, but lines with multiple insertions and aberrant ratios have also been reported frequently (review, Grumet 1994; Powell -Abel et al. 1986; Tumer et al. 1987).

It is not possible to use *N.benthamiana* plants as a model system as planned to study the mechanism of CPMP. Of all the isolates tested, only ZYMV-NAT and ZYMV-Ca could systemically infect *N.benthamiana*, but these isolates also were able to overcome the CPMP in melons expressing the coat protein gene from ZYMV-Ct (Grumet et al. 1994). It is not understood whether the ability to infect *N.benthamiana* systemically is in any way related to the ability to overcome coat protein mediated protection in melons. Thus, to study the mechanism of homologous protection it would be necessary to express the CP-SD and CP-NT gene in melon plants.

However, it will be possible to study the heterologous protection against TEV and PVY, using the lines derived from the *N.benthamiana* transgenic plants expressing CP, Core, CP-SD, and CP-NT. When transgenic tobacco plants expressing the full length coat protein of ZYMV were challenge inoculated with TEV and PVY, limited protection was observed (Fang and Grumet, 1993). It would be interesting to compare the response of transgenic *N.benthamiana* plants expressing the untranslatable sense construct of ZYMV CP, to those expressing translatable sense CP in *N.benthamiana* when challenged with heterologous potyviruses. If the resistance observed between the plants expressing translatable and non translatable coat protein sequences is comparable, then it would suggest that the partial protection observed does not require the presence of coat protein

per se or that either RNA or protein could confer partial protection. If even the partial protection is not observed in plants expressing the non-translatable construct, then it would suggest that coat protein per se is required even for partial protection against heterologous potyviruses viruses. It is also possible that the plants expressing CP-SD would be better protected than those expressing FL-CP.

In earlier studies, transgenic tobacco expressing the Core construct gave limited protection against the heterologous potyviruses TEV and PVY (Fang and Grumet, 1993). The presence or absence of the amino-terminal domain did not cause any difference with regard to heterologous protection. Among potyviruses, the most variable region is the amino-terminal domain. It has been suggested that the N-terminal domain is most likely involved in classical cross protection (Shukla et al. 1991) and also that the negative cross protection between some strains is the result of different sequence motifs in the N-terminal domain (Krstic et al. 1995) (negative cross-protection is the inability of two closely related strains of a virus to cross-protect). The amino-terminal domain has already been shown to mediate aphid transmission (Atreya et al. 1990), long distance movement (Dolja et al. 1994), and possibly the host range of potyviruses (Xiao et al. 1991). It would be interesting to study the effect of the amino-terminal domain, which has been implicated in many roles, on conferring heterologous protection.

The cloned genes can also be used to transform muskmelon plants (*Cucumis melo*), and virus testing can be initiated using the homologous virus i.e., ZYMV, so that important questions about the mechanism of CP-mediated protection can be addressed. When CP-SD is expressed in transgenic melons and tested for virus resistance, there are

at least three possible outcomes. 1. It could offer no or very low protection, which would suggest that expression of coat protein is essential for conferring CPMP. 2. It could offer a higher level of protection than plants expressing translatable CP, which would suggest that the CP mRNA confers protection. 3. It could offer levels of protection similar to the lines expressing translatable coat protein. This would suggest that CP RNA is the active molecule playing a role in CPMP or that the RNA is an active molecule even when CP is being expressed and that both are playing a role. CP mRNA may exhibit an antisense activity, blocking virus replication by RNA:RNA interactions. The transcripts may alternatively, or in addition, compete for viral- and or /host-encoded replication factors (de Haan et al. 1992). There are many examples in potyviruses, where the sense-defective CP conferred protection, but at varying levels depending on the case. In TEV, untranslatable CP mRNA provided a higher level of resistance in tobacco when compared to translatable sense CP (Lindbo and Dougherty, 1992). Similarly plants highly resistant to PVY infection were generated by expressing sense-defective version of PVY CP gene (Smith et al. 1995). In another case, the non translatable RNA of the PVY CP was shown to be as efficient as the translatable construct in protection against PVY (van der Vlugt et al. 1992). Tobacco plants transformed with a sense defective version of PVY^n 605 CP gene provided only partial protection against PVY^n 605 (Farinelli and Malnoe, 1993).

Similarly, when CP-NT transgenic melon are tested for virus resistance, assuming that NT is being produced and is stable, there are several different possible outcomes. 1. They could show no significant amount of protection, which would suggest that NT by itself cannot interfere in viral life cycle and confer CPMP. 2. They could show partial

protection, which would indicate that Core and NT are interfering at different stages of viral life cycle, and the presence of both is required to obtain good protection. 3. They could show significant protection, which would indicate that the NT is playing a key role in interfering with the viral life cycle or in causing CPMP. This kind of response is possible because of various roles attributed to the amino-terminal domain of the coat protein. It has been suggested (Ward and Shukla. 1991) that the N-terminal region of potyviral CP is involved in virus-specific functions or host-vector-virus interactions due to the high variability observed in this region. It has also been suggested that variable N- and C-terminal regions are necessary for long distance movement (Dolja et al. 1995). In the case of AlMV the amino-terminus of the CP has been shown to play an important role in CPMP, since a mutation in the amino-terminal domain made the plants expressing CP susceptible to viral infection (Tumer et al. 1991). Further, in potexviruses viruses specifically PAMV, (Abouhaider and Lecrelc, 1995), removal of the N terminal domain abolished the CPMP, indicating that the amino-terminus of the PAMV CP is the active domain of the protein which is involved in CPMP. If the exposed amino-terminus interacts with some plant component in the virus life cycle, then constitutive expression of NT in plants could possibly interfere in virus infection. However, in ZYMV, removal of the amino-terminus lowered but did not abolish CPMP (Fang and Grumet , 1993), so it is possible that the NT and Core are both involved in conferring protection. It would be interesting to see if the reduction in the amount of protection offered in Core transgenic melon can be restored to the levels of FL-CP transgenics by crossing with NT transgenics. Removal or mutation of the amino-terminal domain has been shown to affect CPMP, but

the effect of expressing just the amino-terminal domain is not known. Hence the studies that would be conducted with the amino-terminal transgenics may lead to some important information about the mechanism of CPMP. Identifying the molecule (CP or CP mRNA) and/or the particular domain responsible for conferring the protection would enable us to gain some insight into the mechanism and help us to design future experiments to elucidate the exact molecular mechanism involved in coat protein mediated protection.

In summary, CP-SD and CP-NT genes of ZYMV have been successfully cloned. FL-CP, Core, SD, NT genes have been transformed into *N.benthamiana* plants. The transgenic lines have been verified for the expression of transcript and protein. Lines have been analyzed for the segregation analysis of the inserted genes in the progeny of transgenic plants. Based on the analysis, the inserted genes have been found to be expressed in at least some lines. In each construct, at least one line has been identified which expresses the transgenic transcript and protein, if expected, with the exception of CP-NT, where protein was not detectable.

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CHAPTER THREE

ZYMV SUSCEPTIBILITY STUDIES ON *N. benthamiana*

INTRODUCTION

In many cases, limitations of gene transfer and plant regeneration technologies have necessitated the use of plant hosts (often *Nicotiana* species) that are model systems. The goal of this study was to make clones encoding altered versions of the CP gene of ZYMV-Ct and produce transgenic plants so that the mechanism of coat protein mediated protection could be studied. Transformation and regeneration of muskmelon plants using *Agrobacterium tumefaciens* and a leaf disk procedure has been successfully demonstrated by Fang and Grumet (1990). However, the efficiency of transformation and regeneration was 3-7% (calculated from initial explant to transgenic plant). Time from initiation of experiment to plants in the green house was approximately six months. Due to the limitations of time and efficiency, it was decided to develop a model system to study the mechanism of CP-MP against ZYMV.

Nicotiana benthamiana Domin. was selected to study the CP-MP protection for ZYMV, because of its ease of transformation, regeneration at a much higher efficiency than melon, and its relatively short life cycle. Also *N. benthamiana* has a large virus range. More than fifty viruses (Christie and Crawford, 1978; Quacquarelli and Avgelis, 1975) belonging to a wide range of groups, can infect *N.benthamiana*, most of them are mechanically transmissible and cause systemic infection, including many potyviruses like,

SMV, WMV II, PVY, TEV, BYMV, PeaMV, CYVV, PeMV etc.(Namba et al. 1992). This species has been recommended as suitable host plant for maintaining and /or purifying several plant viruses (Quacquarelli and Avgelis, 1975) and has been widely used as a model system in many virus studies. As far as susceptibility to ZYMV is concerned, there were contradictory reports in the literature. Although Provvidenti and Gonsalves (1984) reported that *N.benthamiana* is not susceptible to ZYMV infection, Wang et al. (1992) reported symptomless infection on inoculated leaves with ZYMV-Ct on *N.benthamiana* and Lecoq et al. (1993) reported that a non aphid transmissible strain of ZYMV (ZYMV-NAT) (Antignus et al. 1989) systemically infects *N.benthamiana.* Based on this information, prospects for the use of *N.benthamiana* as a model system seemed promising and would allow a study of CPMP against homologous and heterologous viruses in a single species. It was hoped that even if ZYMV does not cause systemic infection in *N.benthamiana*, it might still be possible to work with inoculated leaves, or if it causes symptomless infection, to assay for virus infection by inoculation of indicator hosts or by ELISA. Studies were initiated to further characterize its susceptibility to viruses and strains of interest to this study and to optimize a system for studying ZYMV infection.

MATERIALS AND METHODS

Infection of *N.benthamiana* with ZYMV-Ct

ZYMV-Ct was maintained on zucchini (*Cucurbita pepo* 'Black Jack'). Inoculum was prepared by grinding 1 gm of fresh leaf tissue from ZYMV-infected zucchini with 1ml of 20mM potassium phosphate buffer, pH 7.0. *N.benthamiana* plants which were at the 4-8 leaf stage were used for inoculations. 400-mesh carborundum was dusted on the top 3-4 leaves and rub inoculated. A total of 42 plants were used in 6 rows. One row of plants was not inoculated and another row was mock inoculated with just the buffer to serve as controls. The local infection was tested by ELISA. 2-3 punches were taken from each leaf and pooled in one well of an ELISA plate. Data was collected for local infection 4,7,10, 14, 21 days post inoculation. Data for systemic infection was collected 2, 3, and 6 weeks post inoculation using the two youngest leaves on each plant to take samples to conduct ELISA. The procedure was based on Romaine et al. (1981). The antibody was anti-ZYMV antibody raised against ZYMV virions (Ct strain, S.Hammar and R.Grumet, unpublished), Leaf disk samples were placed in a microtiter plates, frozen (at -80° C), thawed, and incubated in coating buffer at 4° C for 16 hr. Samples were reacted with 1:1,000 dilutions of antibodies for 2 hr at 37° C, followed by incubation with alkaline phosphotase-goat anti-rabbit conjugate (Sigma, St. Louis, MO) for 2 hr at 37° C. *p*-Nitrophenyl phosphate substrate was added and samples were read for absorbance using a Datatech plate reader at 405nm. Infection on *N.benthamiana* was further checked by back

inoculating one week old zucchini plants with leaf tissue from inoculated *N.benthamiana* plants at 4,7,10, 14, 21 days after inoculations. The experiment was repeated many times changing several factors: source of ZYMV inoculum, source of *N.benthamiana* seed, growing conditions (growth chamber vs green house), age of the plant at the time of inoculation etc.

Infection of *N.benthamiana* with ZYMV-NAT and ZYMV-CA strains

ZYMV-NAT strains were obtained from Dr.B Raccah (Volcani institute, Israel. referred to as ZYMV-NAT-1) and Dr.T. Pirone (University of Kentucky, referred to as NAT-2). They were denoted separately to keep the identity of the source. The strains were maintained on zucchini plants. Symptoms caused by these two strains were different. NAT-1 showed reduced leaf lamina and shoe string distortion, with very dark green color around veins, while NAT-2 showed mosaic symptoms initially and reduction in leaf lamina at later stages. ZYMV-CA was obtained from Dr. R. Provvidenti (Cornell University). *N.benthamiana* plants at the 4-8 leaf stage were rub inoculated with ZYMV strains. The top four leaves were dusted with 400-mesh carborundum and rub inoculated. One gram of Ca-strain infected zucchini leaf tissue tissue was ground in 1ml of 20mM potassium phosphate buffer, pH7.0. Symptoms were noted at regular intervals. Leaf punches were taken from the young leaves and ELISA was performed. Infected leaves of *N. benthamiana* were used to back inoculate one week old zucchini plants to observe the virus symptoms.

Challenging CP-transgenic melon with ZYMV-NAT strains

Transgenic CP-expressing plants (line 401 R_2 tetraploid) and control melon plants were tested for response to inoculation by ZYMV isolates NAT-1, NAT-2 and Ct at the true leaf stage in three treatments. Five transgenic and 7 control plants were inoculated with each virus. Negative controls were mock inoculated. Symptoms were observed starting 5 days post inoculation and continuing up to 8 weeks.

RESULTS

Infection of *N.benthamiana* with ZYMV-Ct There was no detectable virus infection in the ZYMV Ct- inoculated *N. benthamiana* plants. There was no local or systemic symptom development and nno measurable virus as determined by ELISA. Back inoculation onto zucchini did not show any virus infection as verified by symptoms and ELISA. There were, however, some rare high readings in the ELISA for the punches taken from inoculated leaves of *N.benthamiana*, which could not be repeated on the punches taken from other parts of the same leaves. It appears that ZYMV-Ct failed to establish infection in *N.benthamiana.* Random high readings in ELISA could be due to the presence of the virus in the inoculated cells.

Infection of *N.benthamiana* with ZYMV-NAT

Within 1-2 weeks, mosaic, puckering, and greening symptoms appeared on the new leaves of *N.benthamiana* plants inoculated with ZYMV-NAT-1 (Table 1). ELISA readings showed the presence of virus in all collected samples (data not shown). When ZYMV-NAT-1 infected *N.benthamiana* leaves were used to back inoculate zucchini, typical symptoms were observed on zucchini plants. It can be concluded that ZYMV-NAT-1 successfully infects *N.benthamiana*. Interestingly no symptoms were seen on the

plants inoculated with NAT-2 and no virus infection could be seen when the leaf tissue from NAT-2-inoculated plants were used to back-inoculate zucchini.

Challenging CP-transgenics with ZYMV-NAT

Plants inoculated with ZYMV-Ct did not show any symptoms of infection, as was observed by Fang and Grumet (1993) nor did the plants inoculated with ZYMV-NAT-2. Moreover transgenic melons expressing CP were not protected against ZYMV-NAT-1. Symptoms started appearing 7 days post

Table 7. Response of ZYMV isolates to transgenic melon expressing ZYMV-CP and their ability to infect *N.benthamiana*

Viral strain	Infection in *N.benthamiana*	Disease response in transgenic melon
ZYMV-Ct	No infection	Resistant
ZYMV-NAT-2	No infection	Resistant
ZYMV-NAT-1	Systemic infection	Susceptible
ZYMV-Ca	Systemic infection	Susceptible

inoculation. Thus the NAT-2 strain which failed to establish systemic infection in *N.benthamiana*, could not infect transgenic melon whereas the NAT-1 strain that could infect *N.benthamiana* also could overcome the ZYMV CP-mediated protection. Similar results were obtained when the experiment was repeated several times (Grumet, 1995. unpublished). Ct- and both NAT strains showed very strong symptoms on all non transgenic melon plants, which started appearing 6 days post-inoculation. No symptoms were noticed on negative controls. Both ZYMV-NAT strains caused systemic infection comparable to Ct- strain.

Infection of *N.benthamiana* by ZYMV-Ca

The results with NAT-1 prompted an examination of other ZYMV isolates. Among several tested (Grumet et al. 1994) only one other, ZYMV-Ca could overcome the CP-mediated protection. When ZYMV-Ca was tested for ability to infect *N.benthamiana* mosaic symptoms were observed 1-2 weeks post inoculation. ELISA readings showed the presence of virus infection (data not shown). Back inoculated zucchini plants showed typical symptoms of ZYMV-Ca. Based on these results it was concluded that Ca-strain causes systemic infection in *N.benthamiana*.

DISCUSSION

Initial studies were focused on developing *N. benthamiana* as a model system for studying CP-MP against ZYMV in melons. ZYMV-Ct failed to infect *N.benthamiana* systemically, or locally, as verified by symptomology, ELISA, and back inoculation on to zucchini. Even after repeated attempts, ZYMV-Ct did not establish infection in *N.benthamiana*. Wang et al. (1992) also reported negative reaction in ELISA tests for systemic leaves, but demonstrated local symptomless infection by ELISA for ZYMV-Ct. Even this did not happen in our case, since ELISA readings were very low.

When it wasn't possible to establish infection with the homologous strains, other strains of ZYMV were investigated. Different ZYMV strains have different host ranges (Grumet et al. 1994). ZYMV-NAT was reported to be causing systemic infection in *N.benthamiana* (Lecoq et al. 1993), so studies were initiated using this strain.. ZYMV-NAT-1 caused systemic infection in *N.benthamiana*, as verified by symptomology, back inoculation to zucchini, and by ELISA, which is consistent with the reports of Lecoq et al. (1993). Surprisingly, NAT-2 strain failed to establish an infection in *N.benthamiana*. In order to utilize ZYMV-NAT strains to study the coat protein mediated protection using *N.benthamiana* as model system, one important criterion to be met is that coat protein

expressing plants be protected. In addition to conferring complete protection against the Ct-strain from which the CP gene was derived, the full-length CP expressing melons were also completely protected against infection by several other North American, middle eastern, and Asian strains (Grumet et al. 1994). However, when NAT strains were tested against transgenic melon expressing the coat protein of ZYMV-Ct, ZYMV-NAT-1 overcame the coat protein mediated protection in melons. This strain seemed to be a resistance breaking strain. Interestingly, the transgenic melons expressing ZYMV-Ct coat protein were protected against NAT-2 strain, which failed to establish infection in *N.benthamiana* (Table 7). Based on these results it was not possible to use NAT strains to study the CP-MP in *N.benthamiana* using a homologous virus.

The results with NAT strains led to the question as to whether there is a relationship between the ability to systemically infect a species that is not generally a host for this virus, and the ability to overcome the CP-mediated resistance. In order to investigate this question, studies were conducted on the Ca-strain, it is a mild strain of ZYMV which also overcomes CP-MP (Grumet et al. 1994). When Ca-strain was inoculated onto *N.benthamiana*, it was able to establish systemic infection as verified by symptoms, ELISA and back inoculation to zucchini. Ca- was also able to systematically infect *Phaseolus vulgaris* cv. Black Turtle 2, unlike other strains (Grumet et al. 1994). Comparisons among predicted amino acid sequences suggest that the ZYMV-CP resistance-breaking property is not due to mutations in the CP gene itself (Grumet et al. 1995). It appears that the ability to overcome ZYMV CP mediated resistance in melons is associated with altered host range (Grumet et al. 1995).

In general ZYMV systemic infection is seen only in cucurbits and some legumes (Lisa et al. 1981), but it appears that some strains of ZYMV could cause systemic infection in *N.benthamiana*. In addition to the NAT strain, some French isolates can cause systemic infection (Lecoq, H. 1994. personel communication). ZYMV-TW (Taiwan strain) and ZYMV-Ct strains have been reported to cause symptomless local infection in *N.benthamiana*, whereas the Florida strain and French mild strain could not cause an infection (Wang et al. 1991). Namba et al. (1992) reported that with Fl- strain virus was detectable in the inoculated leaf.

Although we found a strain that could systemically infect *N.benthamiana* under our conditions it also overcame the ZYMV CP-mediated resistance. Currently it is not possible to use *N.benthamiana* to study the CP-MP against homologous virus. The cloned genes have to be transformed into melon to address the questions of mechanism of CP-MP. However, heterologous protection studies can still be conducted with the *N.benthamiana* transgenics expressing altered coat protein genes even though it is not a host for ZYMV. Stark and Beachy (1989) reported that tobacco plants expressing the SMV CP gene are resistant to two potyviruses that are not closely related to SMV, namely TEV and PVY. This resistance was conferred despite the fact that tobacco is not a host of SMV. Similarly tobacco plants expressing the CP gene of PRV, another potyvirus that does not infect tobacco, were highly tolerant of infection by TEV, PVY, and PeMV(Ling et al. 1991). Tobacco plants expressing the CP genes of ZYMV, which does not infect tobacco, conferred partial protection against other potyviruses like PVY and TEV (Fang and Grumet, 1993). The transgenic *N.benthamiana* plants expressing different

forms of coat protein genes can be used to gain insight and gather additional information regarding heterologous protection offered by ZYMV CP and altered CP genes.

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