Molecular breeding for virus resistance,
an applied approach in vegetable crops

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Stellingen

1. Een genetische bron van ziekteresistentie, ook indien transgeen, verdient altijd de voorkeur boven chemische bronnen van gewasbescherming.

2. Lowe *et al.* veronderstellen ten onrechte dat de directe transformatie van kiemlijncellen in het mais meristeem de basis vormt van hun transformatieprocedure.
   Lowe, K., B. Bowen, G. Hoerster, M. Ross, D. Bond, D. Pierce & B. Gordon-Kamm, 1995. Germline transformation of maize following manipulation of chimeric shoot meristems. Bio/Technology 13: 677-682.

3. De resultaten van Presting *et al.*, waarbij ook de 'vector' controle significante niveaus van resistentie vertonen, bewijzen dat eerder werk aangaande de ontwikkeling van transgene resistentie tegen het aardappelbladrolvirus nader onderzoek behoeft teneinde de mogelijkheid van somaclonale variatie te kunnen uitsluiten.
   Presting, G.G., O.P. Smith & C.R. Brown, 1995. Resistance to potato leafroll virus in potato plants transformed with the coat protein gene or with vector control constructs. Phytopathology 85: 436-442.

4. Alvorens een uitspraak te doen omtrent het mechanisme van transgene virusresistenties, dient allereerst te worden vastgesteld of het vermeende eiwitprodukt, dan wel het gen-transcript aan de resistentie ten grondslag ligt.

5. Wanneer statistiek moet worden aangewend om het effect van een monogeen transgene eigenschap aan te tonen, dient het praktische belang daarvan in twijfel getrokken te worden.

6. Het feit dat een 'Maniatis' met transformatieprotocollen voor planten nog niet bestaat, illustreert het empirisch karakter van cel- en weefselkweektechnieken.

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7. De toepassing van het groene fluorescentie-eiwit afkomstig van de kwal *Aequorea victoria* in transgene planten maakt hun verspreiding eenvoudig traceerbaar door middel van 'remote sensing'.
Niedz, R.P., M.R. Sussman & J.S. Satterlee, 1995. Green fluorescent protein: an *in vivo* reporter of plant gene expression. Plant Cell Reports 14: 403-406.

8. De produktie van antigenen in tabak maakt pruimtabak tot een oraal vaccin.
Haq, T.A., H.S. Mason, J.D. Clements & C.J. Arntzen, 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268: 714-716.

9. Uit het oogpunt van milieubescherming dient iedere vorm van financiële vergoeding voor woon-werkverkeer verboden te worden.

10. Het papierverbruik per werknemer is omgekeerd evenredig met het communicatieniveau van de organisatie waarvan de werknemer deel uitmaakt.

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Contents

1 General introduction

Virus infections cause considerable losses of yield and quality in many crops grown in modern agriculture, and can even be limiting to the production of certain crops in specific areas. The impact of viral infections is exacerbated by the intensive agriculture of monocultures of genetically identical plants in close proximity and under optimal conditions, thereby creating a conducive environment for severe disease outbreaks. Strategies for control of virus diseases are therefore widely applied, focusing on methods to prevent the occurrence of infection, or on natural sources of virus resistance within crop plants (Tomlinson, 1987; Fraser, 1989). The use of virus-free seeds and planting material can be guaranteed by appropriate indexing and certification methods. Timing of sowing or planting so as not to coincide with an influx of virus vectors, eradication of host weeds and other virus sources, and the use of pesticides to control virus vectors represent additional culture practices to limit the incidence of virus diseases. Such culture measures, however, can fail if there are changes which affect virus epidemiology, like climatic fluctuations, the emergence of novel virus strains, or changes in cropping practices. Moreover, the application of pesticides can lead to the excessive use of ecologically unacceptable chemicals.

Breeding for virus resistance is generally regarded as being the best strategy in the long term. The term resistance is used to describe the general response of the plant in which the effect of infection is reduced or eliminated, but is imprecise when the interactions between the plant and the challenging virus are considered. These interactions range from *tolerance*, whereby the plant develops mild symptoms on systemic infection with the virus, through *hypersensitivity*, whereby the spread of the virus is restricted to a few cells at the site of infection, to true *immunity* or non-host resistance, whereby the plant does not support replication of the invading virus (Zaitlin & Hull, 1987; Hull & Davies, 1992). In spite of the indiscriminate use of terms like resistance, tolerance and immunity, the precise nature of the interaction between plant, vector and virus underlying the resistant phenotype is of prime importance in breeding for virus resistance. In this perspective, the term *protection* seems to be more appropriate to refer to the general response of the plant in which the effect of virus infection is reduced or eliminated, regardless of the plant-virus interaction underlying the reduced susceptibility. *Resistance*

then could be defined in a more restricted sense as the specific plant property preventing the virus from spread and systemic symptom development, generally associated with the hypersensitive response.

Virus resistance can operate directly, by preventing virus multiplication or its deleterious effects within the plant, or indirectly, affecting the virus vector. Host factors influencing plant attractiveness to vectors, and thus the efficiency of virus transmission include physical barriers such as leaf hairs or robust surfaces, non-preferred foliage colour, secretion of alarm pheromones and the presence of anti-feeding compounds in the plant sap (reviewed by Jones, 1990). The genetic basis of these factors is mostly polygenic and not well understood, which renders them to a limited extent accessible to the plant breeder. In contrast, the genetics of resistance genes operating against the virus within the host have been studied extensively, since Holmes (1938) first demonstrated the Mendelian inheritance of resistance to tobacco mosaic virus (TMV) in tobacco. To date, this type of resistance which is usually conferred by single loci, is deployed widely against many viruses in many different crops with varying degrees of success (Fraser, 1990).

The heritable resistance of cultivars to a particular virus to which the species as a whole is normally susceptible, implies an interaction between the virus and the product of the host resistance gene, with consequent inhibition of virus multiplication or spread (Fraser, 1992; Keen, 1992). The underlying mechanism can either be constitutive if the product of the host resistance gene is itself the inhibitor, or induced if an initial recognition event triggers a host resistance response involving activation of several host genes. This initial recognition involves the interaction between the product of the host resistance gene and that of a matching viral avirulence gene (Dawson & Hilf, 1992; Fraser, 1992), as is explained by the concept of gene-for-gene incompatibility, stating that the success or failure of resistance is determined by the presence or absence of complementary genes that are present within the pathogen and the host plant, the avirulence and resistance genes respectively (Flor, 1956; Keen, 1990; de Wit, 1992).

For both modes of resistance, constitutive or induced, virus virulence is restored by modification of the avirulence factor, such that it retains its function in pathogenesis, but that inhibition or induction of the resistance mechanism is less effective or totally ineffective. It may take only a few nucleotide substitutions to change a virus from an avirulent to a virulent isolate which overcomes a cultivar resistance gene (Meshi *et al.*, 1988; Meshi *et al.*, 1989; Culver & Dawson, 1989; Calder & Palukaitis, 1992). In practice, comparatively low numbers of dominant resistant alleles proved highly durable as their long term application in resistant cultivars did not yet result in the selection of virus isolates with matching virulence. Examples of sofar durable genes include *Ry*

against potato virus Y in potato (Barker & Harrison, 1984) and *Tm-2*^{2} against tobacco mosaic virus in tomato (Pelham, 1972). But still, the co-evolution of plant resistance and viral virulence obviously poses a serious problem for the plant breeder, as the emergence of virulent virus isolates overwhelming resistance genes restricts their continued incorporation into new crop cultivars.

Outline of this thesis

As mentioned herein above, breeding for virus resistance provides the best long-term prospects of virus control, but is hampered by the paucity of useful sources of resistance. For most crops, there are only few, if any, sources of resistance in sexually compatible species available, while polygenic sources of resistance are yet less amenable in plant breeding. In contrast to the scarcity of resistance genes available to the plant breeder, the high frequency of virulent virus isolates overwhelming natural resistance genes urges the need for 'pyramiding' of resistance genes within crop cultivars, for oligogenic resistances are reasoned to be more difficult for the virus to overcome than monogenic types of resistance. Whenever the virus overcomes one resistance gene, it will be faced by yet other genes that prevent the virus from systemic infection and subsequent disease development.

Major scientific advances in stable gene transfer techniques and the molecular characterisation of viral genomes permitted the onset of molecular breeding for virus resistance employing plant genetic engineering. Over the past few years, the transgenic expression of virus-derived nucleotide sequences proved a versatile and broadly applicable strategy for achieving virus resistance, illustrated by the numerous reports of genetically engineered virus resistance for an ever-growing number of viruses and crops (reviewed by Fitchen & Beachy, 1993; Wilson, 1993; Scholthof *et al.*, 1993; Hull, 1994). Once such transgenic plants carrying novel resistance genes have been evaluated for their performance, they can be carried forward into crop breeding programs in order to extend the battery of resistance genes available to the plant breeder.

With this applied aim in mind, this thesis describes the development of transgenic progenitors of pathogen-derived resistance to a number of different viruses representing different virus genera, and for a number of different vegetable crops. Major aspects related to this research involve the construction of pathogen-derived resistance genes for beet western yellows luteovirus, zucchini yellow mosaic potyvirus, cucumber mosaic cucumovirus and tomato spotted wilt tospovirus, the development of *Agrobacterium*-mediated transformation protocols for elite genotypes of lettuce, melon and tomato, and

the subsequent characterisation of the observed resistance. The performance of the transgenic plants and their potential to function as progenitors of virus resistance in crop breeding programs is critically assessed for the various virus-crop combinations.

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2 Genetic modification of crop plants

Introduction

Molecular breeding is based on the introduction and expression of foreign genes in crop plants. The development of procedures to regenerate plants from single cells or organised tissues, and the discovery of novel techniques to deliver DNA into plant cells enabled the practical use of molecular breeding in crop improvement. From the large number of strategies for plant transformation that have been developed in the past, only a few are routinely in use. These strategies include electroporation, microprojectile bombardment and *Agrobacterium*-mediated transformation, which differ in the way the genes are transferred to the plant cell. A basic prerequisite for these transformation methods is the selection and regeneration of the initially transformed plant cells, which implies the need of a tissue culture approach. The regeneration of normal and fertile transgenic plants from the rare transformed plant cells, however, proved to be rather difficult for the majority of crop species. The main difficulty in this appears to be directing the gene transfer towards totipotent cells that are amenable to regeneration, and the subsequent selection and proliferation of the initially transformed cells. This constraint leads to the development of a variety of selectable markers that encode resistance to antibiotics or herbicides. On the contrary, the direct transformation of germ line cells in organised tissue explants circumvents the need for the selective regeneration from single transformed cells, and thus presents an attractive approach towards the genotype independent transformation of crop species. The basic principles and characteristics of different transformation techniques and selectable marker genes with practical application are outlined hereinafter. Additionally, the influence of the state of the plant genome on the expression of the transgene and the phenotype resulting therefrom is discussed, in relation to phenomena such as position effects, gene silencing and chromatin compaction.

Direct DNA transformation: electroporation and particle bombardment

In the search for species and genotype independent transformation methods, several techniques based upon the direct delivery of naked DNA into plant cells have been developed (reviewed by Potrykus, 1991; Songstad *et al.*, 1995), ranging from the use of laser technology (Guo *et al.*, 1995) to the direct injection of DNA into reproductive tissues (de la Pena *et al.*, 1987; Luo & Wu, 1988). Today only two strategies of naked DNA transformation are of practical importance: electroporation and microprojectile bombardment. The physical nature of these delivery techniques can potentially overcome biological barriers associated with other transformation procedures such as host range specificity or the regeneration of transgenic plants from protoplasts. Polyethylene glycol (PEG) has successfully been used as a chemical agent mediating direct DNA uptake in the transformation of a number of plants species including monocots (Horn *et al.*, 1988; Zhang & Wu, 1988; Wang *et al.*, 1992; Omirulleh *et al.*, 1993). However, due to the fact that PEG-mediated transformation requires an efficient protoplast system, its application range remained rather limited compared to both physical strategies of DNA delivery.

Electroporation involves short electrical discharges to cause reversible permeabilisation of the plasmalemma membrane which enable the passage of nucleic acids through the otherwise impermeable plasmalemma membrane (reviewed by Lindsey & Jones, 1990). The rigid plant cell wall, however, does not allow the efficient diffusion of macromolecules such as nucleic acids. As such, the plants cell wall constitutes a physical barrier in electroporation, which implies the preparation of protoplasts and their subsequent regeneration into fertile transformants. In order to eliminate the need for totipotent protoplasts and to avoid long periods of time in tissue culture associated with protoplast regeneration procedures, a new method of electroporation was developed to deliver DNA into morphogenic plant tissues such as immature embryos or embryonic callus tissue. This simple and inexpensive technique has been successfully applied to obtain transgenic plants starting from enzymatically or mechanically wounded immature embryos of maize (D'Halluin *et al.*, 1992a) or rice (Xu & Li, 1994).

Microprojectile bombardment employs metal particles coated with DNA that are accelerated to high velocities into plant tissues (reviewed by Christou, 1992; Sanford *et al.*, 1993; Klein & Fitzpatrick-McElligott, 1993). The particles penetrate the plant cell wall and enter the plant cell where the DNA is released and occasionally incorporated into the plant genome. This biolistic method has permitted the genetic transformation of several important but hitherto recalcitrant crop species, including maize (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990), rice (Christou *et al.*, 1991) and soybean (McCabe *et al.*, 1988). One of the advantages of the biolistic approach is that organised structures

such as meristems or embryos can be used as explant material, aiming at the transformation of the germ line cells that give rise to the future reproductive organs (Christou & McCabe, 1992; McCabe & Martinell, 1993). Due to the nature of this particular procedure, which does not involve the selective regeneration of single transgenic cells, the recovered transformants will be chimaeric. Only transformants that carry stably transformed germ line cells will yield homogeneously transformed offspring plants, that can easily be identified by means of a selectable marker such as resistance to a herbicide. The deviation from the need for the regeneration of transgenic plants from single transformed cells reduces tissue culture demands to the minimum, and thus makes this approach a promising alternative for the genotype-independent transformation of recalcitrant crop species. Its reduction to practice, however, still requires further improvement and refinement of the bombardment technology, so that germ line cells can be transformed more efficiently by modulating particle size, particle velocity and the depth of particle penetration (Sautter & Potrykus, 1991; Iglesias *et al.*, 1994; Leduc *et al.*, 1994).

In contrast to the technically demanding methods of electroporation and microprojectile bombardment, the silicon carbide fiber- or whisker-mediated transformation as recently described for the production of fertile transgenic maize plants (Frame *et al.*, 1994), is astonishing in its simplicity. The method involves the vortexing of cells from embryogenic suspension cultures in liquid medium with silicon carbide whiskers and plasmid DNA. The collisions between cells and whiskers apparently lead to cell penetration and subsequent DNA delivery (Kaeppler *et al.*, 1990). As such, whisker-mediated transformation represents a simple and inexpensive system that does not require any sophisticated and expensive equipment. However, it appears that routinely achieving high frequency DNA delivery in tissue cultures that retain their regeneration capacity will remain challenging.

Direct DNA transformation methods in general make use of plasmid DNA carrying the genes of interest, that is physically introduced into the plant cells. Since integration of the delivered DNA is not a controlled and precise event, transformed plants not only contain the genes of interest, but also fragments of redundant vector sequences. Additionally, multiple integration events, linked at only a few loci, are rather common upon direct DNA transformation. Transgene loci containing repetitive transgene copies arranged in inverted or direct repeats, however, are considered to be more prone to homology-dependent gene silencing (Flavell, 1994; Matzke *et al.*, 1994; Matzke & Matzke, 1995). Support for this concern also comes from a study in *Arabidopsis*, in which an allelic series comprising different copy numbers of the transgene was generated by recombination at a single locus, derived from one and the same primary transformant.

Alleles containing repeats were silenced and methylated, whereas alleles lacking repeats remained active and unmethylated (Assaad *et al.*, 1993). Consequently, transgenic loci containing repetitive elements are less favourable when considering their stability of gene expression over successive generations (Finnegan & McElroy, 1994).

Agrobacterium tumefaciens-mediated transformation

The first ever transgenic plants were obtained exploiting the natural gene transfer system of *Agrobacterium tumefaciens* (Fraley *et al.*, 1983), which is now widely applied for the routine transformation of dicotyledonous plants (reviewed by Hooykaas & Schilperoort, 1992; Lindsey, 1992). Despite of its simplicity in practice, the underlying processes mediating DNA transfer and stable integration comprise several complex events that are only partially understood (reviewed by Zambryski, 1992; Hooykaas & Beijersbergen, 1994; Zupan & Zambryski, 1995).

Agrobacterium tumefaciens is a plant pathogenic soil bacterium that infects wounded tissues, resulting in the formation of a crown gall at the site of infection. A large plasmid contained within the *Agrobacterium* cells and referred to as the tumour inducing or Ti plasmid, carries most of the functions for crown gall formation. The shift to tumorous cell growth is effectuated by the transfer of a piece of DNA, the T-DNA, from the Ti-plasmid into the plant cell genome. The T-DNA itself is delimited by two direct repeats of approximately 25 bp, the T-DNA borders. Any DNA between these borders is transferred to the plant cell, without any effect on the efficiency of T-DNA transfer. In wild type *Agrobacterium* strains the T-DNA contains oncogenic genes which when expressed in plant cells, direct the formation of the crown gall. The machinery for T-DNA transfer is encoded by the *vir* region, that is also located on the Ti-plasmid. The *vir* region consists of several loci that contain all information needed to duplicate and to transfer the T-DNA as a single-stranded DNA molecule from the bacterium into the plant cell (Zambryski, 1992) and into the plant nucleus (Citovsky *et al.*, 1992).

The functional separation of the Ti-plasmid into the T-DNA and the *vir* region led to the development of the binary vector system (de Framond *et al.*, 1983; Hoekema *et al.*, 1983), in which both entities are physically separated on two different plasmids. One is a deleted Ti plasmid, lacking the T-DNA region, but providing the virulence functions of the *vir* region. The binary vector itself is a much smaller plasmid that carries a 'disarmed' T-DNA from which the oncogenic sequences have been deleted, but leaving both border regions. Binary vectors can be propagated both in *Agrobacterium* and in *Escherichia coli* to permit the cloning of foreign genes onto the T-DNA. Upon introduction of the binary

vector into *Agrobacterium* both plasmids replicate independently, resulting in the reconstitution of the T-DNA transfer system through *trans*-complementation.

Agrobacterium-mediated transformation in practice

The successful recovery of transgenic plants by means of *Agrobacterium*-mediated transformation is dependent on the susceptibility of the explant material to the *Agrobacterium* strain, the ability to select for newly grown transgenic tissues derived from initially transformed cells, and the potential to regenerate fertile plants from the selected tissue. A well-known procedure is that of leaf disk transformation, in which transformed shoots regenerate directly from the wounded edges of leaf explants upon *Agrobacterium*-mediated transformation (Horsch *et al.*, 1985). The main difficulty in this approach is to direct T-DNA transfer towards totipotent cells that are amenable to regeneration, but that do not necessarily correspond with those amenable to DNA uptake and subsequent stable integration (Colby *et al.*, 1991; Sangwan *et al.*, 1992). This discrepancy can partially be overcome by the use of immature plant tissues such as seedling or embryo explants, that harbour actively dividing cells or cells that can easily be dedifferentiated. Actively dividing, dedifferentiated plants cells generally are increased amenable to transformation and subsequent regeneration (van Wordragen & Dons, 1992).

The susceptibility of the explant material to *Agrobacterium* is determined by several factors of which plant genotype and virulence of the *Agrobacterium* strain are of key importance. Among *Agrobacterium* strains that are commonly used in dicot transformation protocols, including the octopine type strain LBA4404 (Ooms *et al.*, 1981) and the nopaline type strain C58C1(pMP90) (Koncz & Schell, 1986), exist large differences in virulence that are primarily related to the nature of the *vir* region. The *vir* region of Ti plasmid pTiBo542 as present in supervirulent strain A281 is known to be more virulent than other strains to many plant species. Strain A281 carrying pTiBo542 incites large, fast-growing tumours on several solanaceous species as well as on a number of important legumes, including alfalfa, soybean and pea (Hood *et al.*, 1987; Chabaud *et al.*, 1988; Hobbs *et al.*, 1989; Puonti-Kaerlas *et al.*, 1989). This hyper-virulence and broad host range property were shown to be encoded outside of the T-DNA (Hood *et al.*, 1986; Jin *et al.*, 1987), and thus are of particular interest to be exploited for *trans*-complementation of binary vectors in *Agrobacterium*-mediated transformation.

The explants must release phenolic compounds that are recognised by *Agrobacterium* as signal molecules, resulting in the specific induction of the *vir* genes (Stachel *et al.*, 1985

and 1986). In this way, the combination of plant genotype and bacterial strain is known to affect the efficacy of transformation for many crop species. Methods for enhancing transformation efficiency are often based on the addition of phenolic compounds, such as acetosyringone (Stachel *et al.*, 1985), or on the use of feeder layers, aiming at the activation of *vir* genes during co-cultivation. Feeder layers generally consist of cell suspensions from readily transformable plants species, such as tobacco or *Petunia*, that are separated from the explants by a filter paper. The beneficial effect of feeder layers probably is not only due to the secretion of phenolic compounds, but also to other secreted compounds influencing dedifferentiation and subsequent regeneration of the explant cells.

In the perspective of obviating the need for the selective regeneration of single transgenic cells, two novel methods were described for the *in planta* transformation of *Arabidopsis*. Both non-tissue culture approaches are based on the inoculation of adult plants with a concentrated suspension of *Agrobacterium* cells, aiming at the direct transformation of germ line cells (Bechtold *et al.*, 1993; *Chang et al.*, 1994). The *in planta* transformation of *Arabidopsis* was first achieved by inoculating germinating seeds at the stage of imbibition. Inoculated plants were grown to maturity to produce seeds that were subsequently sown on selective medium to recover transformed plants (Feldmann & Marks, 1987). However, despite numerous attempts, this method proved poorly reproducible and thus remained rather inefficient. The vacuum infiltration of flowering plants, on the other hand, was shown to yield high frequencies of transformation (Bechtold *et al.*, 1993). On average, up to five independent transformants could be recovered from the progeny of each plant infiltrated. Likewise, the severing of apical shoots, the subsequent inoculation and the *in planta* generation of newly formed shoots from the severed sites, also yielded high frequencies of transformation (Chang *et al.*, 1994). Evidently, such simple and efficient non-tissue culture approaches present a tempting alternative for the transformation of crop species recalcitrant to regeneration and to *in vitro* tissue culture techniques. However, the successful *in planta* transformation of any other plant species than *Arabidopsis* has not yet been disclosed.

Selectable markers

As outlined herein above, one of the most difficult aspects in plant transformation is the preferential selection and regeneration of the rare cells that have been transformed. Most of the strategies for selection are based on the selective inhibition of non-transformed plant cells without significantly affecting the transformed cells, which is generally

Table I. Summary of selectable marker genes that are routinely used in the transformation of crop species, their mode of action and the corresponding selective agents.

Selective Agent	Marker Gene[1]	Mode of Action
Kanamycin neomycin paromomycin butirosin G418 (geneticin)	NPTII	detoxification
Hygromycin	HPT	detoxification
Glyphosate (Roundup™)	EPSPS GOX	complementation detoxification
Phosphinothricin (Basta™)	PAT	detoxification
Sulfonylurea chlorsulfuron	ALS	complementation

[1]NPTII: neomycin phosphotransferase II; HPT: hygromycin phosphotransferase; EPSPS: 5-enolpyruvylshikimate 3-phosphate synthase; GOX: glyphosate oxidoreductase; PAT: phosphinothricin-N-acetyltransferase; ALS: acetolactate synthase.

achieved by making use of a selectable marker that confers resistance to a herbicide or to an antibiotic (Wilmink & Dons, 1993) (Table I).

Kanamycin, belonging to the aminoglycoside type antibiotics, proved to be very efficient as selective agent in the transformation of dicotyledonous crop species. Resistance to kanamycin is obtained by the transgenic expression of the *aphA2* gene from the Tn5 transposon of *Escherichia coli* (Bevan *et al.*, 1983; Fraley *et al.*, 1983), encoding the enzyme aminoglycoside 3'-phosphotransferase (APH(3')II), better known as neomycin phosphotransferase II (NPTII). Upon phosphorylation of a specific hydroxyl group, the antibiotic is detoxified and its binding to the ribosome prohibited. Hygromycin is another aminoglycoside type antibiotic, for which plant tissues generally show a higher sensitivity compared to kanamycin. The *aphIV* gene from *Escherichia coli* confers resistance to this antibiotic and proved suitable to function as selectable marker in plant transformation (van den Elzen *et al.*, 1985; Waldron *et al.*, 1985).

In addition to antibiotics, herbicides are widely applied as selective agents in plant transformation, of which phosphinothricin and glyphosate are the most important. Both herbicides have been successfully employed for a large number of crop species, including recalcitrant monocots such as maize (Gordon-Kamm *et al.*, 1990), wheat (Vasil *et al.*, 1992) and rice (Christou *et al.*, 1991). Moreover, the transformation of crop

species with selectable marker genes conferring herbicide resistance may be a goal on its own as herbicide resistance constitutes a trait of commercial interest in field crops.

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), which is involved in the synthesis of aromatic amino acids through the shikimate pathway (Comai *et al.*, 1985). Because of its mobility in vascular tissues, glyphosate tends to accumulate in the apices and meristematic tissues, which renders it a very powerful herbicide, commercially known under the brand name Roundup™. Resistance to glyphosate is achieved by the expression of mutant EPSPS enzymes with reduced affinity to the herbicide (Comai *et al.*, 1985; Shah *et al.*, 1986), but leaving the herbicide itself unaffected. Its accumulation in meristematic tissues, however, forced the development of a detoxifying mechanism in order to fully protect crop plants upon treatment with a commercial application of Roundup™. For this purpose, the transgenic expression of a glyphosate oxidoreductase supplementing the mutant EPSPS enzyme, resulted in sufficiently high protection levels for crop plants to resist the commercial application of Roundup™ at levels several fold the routine dose (Barry *et al.*, 1992).

Phosphinothricin, also known as glufosinate, is an analogue of glutamate and acts as a competitive inhibitor of glutamine synthase, a key enzyme in the metabolism of nitrogen (de Block *et al.*, 1987). Inhibition of glutamine synthase leads to the accumulation of ammonia, which rather than the glutamine deficiency causes subsequent cell death. Resistance to the herbicide is conferred by phosphinothricin-N-acetyltransferase, that inactivates phosphinothricin through acetylation and that is encoded by two similar genes isolated from *Streptomyces* species (Thompson *et al.*, 1987; Wohlleben *et al.*, 1988).

A third herbicide that has occasionally been used as selective agent in plant transformation is chlorsulfuron, belonging to the group of the sulfonylureas. This type of herbicides inhibits the enzyme acetolactate synthase, which is involved in the synthesis of branched-chain amino acids. Mutant acetolactate synthases with reduced sensitivity to chlorsulfuron have been isolated from a number of sources including plant species (Haughn *et al.*, 1988; Lee *et al.*, 1988), that have been applied as selectable markers in the transformation of field crops like sugarbeet (D'Halluin *et al.*, 1992b), maize (Fromm *et al.*, 1990) and rice (Li *et al.*, 1992).

The success of a selectable marker in plant transformation is partly based on the mechanism by which resistance is conferred to the plant cells. In general, the detoxification of the selective agent will tend to decrease the concentration of the selective agent in the vicinity of transformed plant tissue, which may lead to the regeneration of escapes (Christou *et al.*, 1991; Escandón & Hahne, 1991). If the selection is based on the expression of a modified target enzyme tolerant to the selective agent involved, the regeneration of escapes is less likely to occur as the concentration of the

selective agent remains unaffected. But still, the effective concentration of any selective agent needs to be determined empirically for each crop, for each type of explant and for each phase of the transformation protocol, regardless of the mode of selection. In this respect, a concentration which prevents the growth of non-transformed plant cells without causing too much cell death is favourable in selecting for the proliferation and regeneration of transformed cells.

Transgene expression

Although the transformation technology in general has reached a high level of refinement, the integration of the foreign DNA into the plant genome remains a random process. The random insertion of the delivered DNA into chromosomal regions that are differentially regulated during plant development gives rise to variation in quantitative and qualitative expression of the transgene, referred to 'position effects' (Dean *et al.*, 1988; Peach & Velten, 1991). Depending on the chromosomal environment surrounding the insertion site, the expression of the transgene may be enhanced, suppressed or deregulated. In general, however, the majority of transformants express the introduced transgenes at relatively low levels. Moreover, when integration occurs during cell dedifferentiation, the potential danger exists that transformation events result in silenced transgenes upon regeneration of the differentiated plant that is accompanied by *de novo* methylation or *de novo* chromatin condensation of the surrounding plant genome. Data that endorse this concern come from a study on *Arabidopsis*, which describes the reactivation of a transgene in callus induced on explant material derived from the differentiated plant in which the transgene was suppressed (Mittelsten Scheid *et al.*, 1991). Similar observations have been made with transgenes in other crops as well, describing their progressive but reversible silencing (Linn *et al.*, 1990; Kilby *et al.*, 1992; Meyer *et al.*, 1992; Cherdshewasart *et al.*, 1993; personal observations in transgenic lettuce and tomato). Such observations emphasise the importance of extensive analysis on the regulation and expression of transgenes, including field trials closely reproducing the environmental conditions which the transgenic plants will be exposed to upon their commercial release.

Chromosomes of higher eukaryotes are functionally organised in constraint domains that define units of co-ordinately regulated genes by means of chromatin compaction or relaxation (reviewed by Eissenberg & Elgin, 1991; Jackson, 1991; Dillon & Grosveld, 1993). Such functional domains are delimited by nucleoprotein complexes known as boundary elements, at which the chromatin is attached to the proteinaceous nuclear

matrix. By virtue of their binding to the nuclear matrix, boundary elements control the limits of chromatin condensation and serve as a topological means of constraining interactions between regulatory elements in different domains. In this way boundary elements separate regions that are differentially regulated. Putative boundary elements or 'matrix associated regions' have been isolated from animals (Grosveld *et al.*, 1987; Phi-Van & Strätling, 1988; Kellum & Schedl, 1992) as well as plants (Hall *et al.*, 1991; Slatter *et al.*, 1991; Breyne *et al.*, 1992; van der Geest *et al.*, 1994).

Research on animal systems has demonstrated that flanking transgenes with boundary elements can insulate the transgene from the influence exerted by the chromosomal environment, thereby reducing position effects (Stief *et al.*, 1989; Bonifer *et al.*, 1990; Phi-Van *et al.*, 1990; Klehr *et al.*, 1991; McKnight *et al.*, 1992). Recently, the bracketing of a plant reporter gene with a boundary element from the chicken lysozyme gene (Mlynárová *et al.*, 1994) or from the β-phaseolin gene (van der Geest *et al.*, 1994) was also shown to reduce position effects in transgenic tobacco. Although maximum expression levels of the transgenes remained largely unchanged, the average expression level was increased as the presence of the boundary element seemed to prevent the occurrence of lower levels of transgene expression. However, other boundary elements only enhanced gene expression leaving inter-transformant variability unaffected (Allen *et al.*, 1993; Schöffl *et al.*, 1993), or normalised transgene expression at sub-maximal levels (Breyne *et al.*, 1992). These data illustrate that different boundary elements behave differently in their effect on transgene expression and that much remains to be learned about the organisation of the chromosome in higher order structures, and its effects on gene expression. The normalisation of transgenes, however, aiming at their reliable expression independent of environmental fluctuations and the physiological state of the plant, definitely will contribute to the successful introgression of transgenic traits into crop breeding programs.

Concluding remarks

The recent progress in the tissue culture and transformation technology of plant species allows the stable integration and expression of foreign genes in a still growing number of crop species. The practical application of plant genetic engineering relates to the development and introduction of novel traits, so as to enrich the crop gene pool and to expand the genetic variability that is available to the breeder. In general, the final result consists of a population of transgenic lines that carry the genes of interest at different positions in their genome, and that differ in the quantitative and qualitative expression of

the transgenes. Once the initial selection based on molecular and biochemical analyses of expression of the transgene has been made, it is the breeder who has to evaluate the behaviour and performance of the novel trait in different genomic backgrounds and under different culture conditions. Ultimately, only the breeder can decide which transgenic lines can be considered as elite lines that meet the demands for incorporation into the crop breeding program.

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3 Pathogen-derived resistance to viral infections in transgenic crops

Introduction

The transgenic expression of nucleotide sequences derived from plant viral genomes can render crops resistant to infection by the homologous virus. The concept that host resistance to a particular pathogen could be achieved by transformation of the host with nucleotide sequences derived from the pathogen was already hypothesised in the early eighties, and was theoretically formulated in a publication by Sanford & Johnston (1985). This type of genetically engineered resistance is based upon the existence of pathogen-encoded functions which are essential to the pathogen, but not to the host. Transgenic expression of such a key gene product in pathogenesis in a dysfunctional form, in excess, or at an inappropriate developmental stage could disrupt the normal equilibrium of viral components and thereby the process of infection. In the most successful instances, such disruptions would prevent the replication or subsequent movement of the virus beyond the initially infected cell, while having minimal effects on the host itself.

With the advent of stable gene transfer techniques and the molecular characterisation of viral genomes, the concept of pathogen-derived resistance has been put to practice for a still growing number of plant-virus combinations. Successful strategies include the expression of viral sequences encoding coat proteins, subunits of the viral replicase, movement proteins, but also sequences not involved in encoding proteins, including satellite sequences. Current strategies of pathogen-derived virus resistance will consecutively be described herein after, and their underlying mechanism discussed. Additionally, the various strategies will be critically assessed in the perspective of their future application in practical agriculture.

Plant virus infection cycles

Plant viruses differ considerably in their particle morphology and in their genomic organisation, comprising single-stranded as well as double-stranded DNA or RNA genomes (Matthews, 1991; Zaccomer *et al.*, 1995). Despite substantial differences in replication strategies, plant viral life cycles are characterised by broadly similar steps (Hull, 1990). Viruses generally enter a host plant cell following mechanical damage, or via insects, fungi or nematodes that penetrate the cell wall during infection or feeding. The virus particle is then thought to disassemble, which exposes the viral genome to the plant cellular environment (Verduin, 1992). Strategies underlying the expression of viral genomes are diverse, but ultimately mRNAs are translated to produce structural and non-structural viral proteins that are required to fulfil the virus life cycle. All viruses encode proteins that, in concert with host factors are involved in replication of the parental genome to produce progeny. Upon replication, most viruses spread from cell to cell via plasmodesmata as single-stranded nucleic acids that are protected from degradation and assisted in movement through the association with a movement protein. These movement proteins interact specifically with plasmodesmata, gating them to enable the nucleoprotein complex to pass to the adjacent cell (Lucas & Gilbertson, 1994). Yet other viruses employ tubuli that penetrate the plasmodesmata allowing intact virus particles to traverse the plant cell wall (Perbal *et al.*, 1993; Kasteel *et al.*, 1993). The long distance spread of viruses through the phloem in most cases requires the presence of viral coat protein, as is the case when the virus is transmitted from plant to plant. While for some viruses it is the coat protein that interacts directly with the vector and that determines specificity, others encode one of more non-capsid proteins that facilitate vector transmission (Hull, 1994a). Any other viral gene product generally adapts the viral replicon for its expression and replication within the plant host.

Conclusively, plant virus replication requires a subtle blend of host- and virus-encoded proteins, and each stage of the infection cycle, i.e. disassembly, translation, replication, movement and transmission, carries the potential of being perturbed. The approach of molecular breeding for virus resistance is to transform crop plants with a portion of the viral genome, superimposing interfering proteins or nucleic acids in order to obtain resistance. The recent advances in the understanding of the genome organisation and gene functions for many of the diverse groups of plant viruses (Goldbach *et al.*, 1990) enabled the development of such novel resistance genes that proved to be highly effective for a still growing number of plant viruses (reviewed by Wilson, 1993; Scholthof *et al.*, 1993; Hull, 1994b).

Coat protein gene-derived resistance

The concept of creating virus resistance by engineering crop plants to express part of a viral genome evolved from empirical observations on the use of mild or symptomless strains of viruses to protect crops against closely related, but severely pathogenic virus strains (Fulton, 1986; Urban *et al.*, 1990). In fact, this phenomenon referred to as cross-protection represents an example of naturally occurring pathogen-derived protection (Hamilton, 1980). Among the hypotheses put forward to explain the mechanism of cross-protection was the suggestion that the coat protein of the protecting virus inhibited the uncoating and subsequent replication of the incoming strain (Sherwood & Fulton, 1982; Sequeira, 1984). In 1986, Beachy and co-workers demonstrated that the expression of tobacco mosaic virus (TMV) coat protein in transgenic tobacco could provide a considerable level of protection against the TMV disease (Powell Abel *et al.*, 1986). Since then, coat protein gene-derived protection has been reported for a still growing number of over 20 viruses in at least 10 distinct taxonomic groups, and in a wide variety of plant species (reviewed by Beachy *et al.*, 1990; Fitchen & Beachy, 1993). Although the mechanism of coat protein-mediated protection is not fully understood, it was argued that the accumulation of coat protein in transgenic plants interferes directly with the replication and transport of the invading virus (Beachy *et al.*, 1990; Reimann-Philipp & Beachy, 1993). In cases like TMV, potato virus X (PVX) and alfalfa mosaic virus (AlMV) the degree of protection indeed correlated directly with the expression level of the coat protein gene when protection was scored across several transformant lines accumulating different levels of transgene-derived coat protein. Moreover, transgenic plants expressing translationally defective coat protein genes of TMV (Powell *et al.*, 1990) or AlMV (van Dun *et al.*, 1988a) were not protected against the corresponding virus, which proves that it is the coat protein rather than the mRNA transcript that confers protection to these viruses. However, the protection was never absolute. Within homogeneous populations only a proportion of the transgenic plants resisted infection, while others exhibited merely reduced numbers of infection sites on inoculated leaves, a delay in systemic symptom development, and a reduction in virus accumulation. The proportion of plants that remained without symptoms and the duration of the delay in symptom development in those plants that did become infected were reduced when the concentration of virus in the challenge inoculum was increased.

In addition to the expression level of the coat protein gene, the strength of the protection depends on the relationship between the transgenically expressed coat protein and that of the challenging virus. In most instances, coat protein-mediated protection extends only to the homologous virus and related strains with a substantially similar coat protein, but

there are a few instances where the expression of the viral coat protein from one virus provides at least some limited protection against heterologous viruses (Nejidat & Beachy, 1990).

In contrast to TMV, PVX and AlMV, a positive correlation between levels of protection and levels of accumulation of the transgene-derived coat protein does not exist for potato leafroll luteovirus (Kawchuk *et al.*, 1991; van der Wilk *et al.*, 1991) and for potyviruses in general (Lindbo *et al.*, 1993a). The absence of this correlation was further demonstrated by the analysis of transgenic plants expressing potyviral coat protein genes lacking an initiating methionine codon. Transgenic plants carrying such translationally defective coat protein genes were as resistant to infection as were plants carrying intact reading frames derived from the coat protein genes of potato virus Y (PVY) (van der Vlugt *et al.*, 1992), tobacco etch virus (TEV) (Lindbo & Dougherty, 1992a and 1992b) or zucchini yellow mosaic virus (Fang & Grumet, 1993; Chapter 6 of this thesis). Despite the origin of the sequence encoding the viral coat protein, the mechanism of resistance to potyviruses appears to be mediated at the transcript level and is independent of the accumulation of the coat protein per se. As such, the general term 'coat protein-mediated protection' (CPMP) is heavily misleading in these instances, and should better be replaced by 'coat protein gene-derived protection'. Many individual plants accumulating the coat protein transcript, remained entirely asymptomatic and plants actually showed virtual immunity as accumulation of virus did not occur. Moreover, levels of resistance were independent of the inoculum concentration over the range used, but were confined to the homologous potyvirus only. Similar results were obtained for tomato spotted wilt virus (TSWV), an enveloped plant virus with a negative-strand RNA genome (German *et al.*, 1992). Transformation of tobacco with a translationally defective gene cassette of the TSWV nucleoprotein, which like coat proteins is involved in wrapping of the viral genome, equally generated virtual immunity to TSWV infection (de Haan *et al.*, 1992; Chapter 7 of this thesis). Such extreme levels of resistance illustrate the potential of RNA interference in pathogen-derived resistance to plant viruses, but also suggest a mechanism other than antisense or RNA-RNA interactions.

From their observations on transcript-mediated resistance to TEV infection, Dougherty and co-workers (Lindbo *et al.*, 1993b) postulated the induction of a highly specific 'antiviral state', triggered by the accumulation of the transgene transcript and the replicating viral genome. In plant tissues immune to TEV infection, the level of accumulation of the transgenic mRNA was shown to be markedly reduced, when compared to mRNA levels in unchallenged plant tissues. Transcription rates, however, as determined by nuclear run-off assays appeared to be unchanged. Collectively, these observations suggest that the decrease in steady state transcript levels results from a post-

transcriptional activity that targets specific RNA sequences for accelerated degradation. This down-regulation of the steady state level of the transgenic transcript then coincides with the elimination of viral sequences from which the transgenic transcript was derived, thereby yielding the TEV resistant phenotype. In support of this novel concept that plants can somehow sense intolerably high levels of an RNA transcript and target that RNA for accelerated degradation is another study on transcript-mediated resistance resulting from the transgenic expression of a translationally deficient gene cassette for the coat protein of PVY (Smith *et al.*, 1994). Plants that transcribed the transgene at high levels, yet accumulating only low levels of the translationally deficient coat protein transcript were resistant to PVY infection, again implying the action of a cytoplasmic and sequence specific RNA turnover mechanism induced by elevated transcript levels.

The examples of coat protein gene-derived protection described herein above, illustrate the existence of multiple mechanisms for different virus genera. Moreover, the resistance derived from a single coat protein gene in a single crop species may act by more than one mechanism and may inhibit several different stages in the infection process such as initiation of infection, replication, spread of the infection throughout the plant, and symptom development (Reimann-Philipp & Beachy, 1993). The difference in both basic mechanism of protection, protein- versus transcript-mediated, is generally reflected by the frequency of resistant lines within a series of transgenic lines carrying the same coat protein gene construct. When protein-mediated, any transformed line accumulating coat protein to a substantial level will exhibit some degree of protection. When transcript-mediated, the degree of protection does not correlate with steady state levels of gene expression and as such, the accumulation of transcripts presents no guarantee for protection. Only a proportion of the recovered transgenic lines will exhibit the resistant phenotype, but generally at higher levels compared to protein-mediated protection. Although the spectrum of transcript-mediated resistance against related viruses may be rather limited, the virtual immunity as associated with the 'antiviral state' illustrates the potential of RNA interference in coat protein gene-derived resistance to plant viruses. As more control experiments are completed to unravel mechanisms underlying genetically engineered resistance to viral infections, more examples of transcript-mediated resistance are likely to be discovered. However, in order to be ever sure on the mechanism of resistance, the transformation of the acceptor host with a translationally defective coat protein gene and the analysis of a sufficiently large number of independent transformants for virus resistance is inevitable.

Replicase gene-derived resistance

A key event in virus infection cycles is the replication of the parental genome to produce new generations of the virus. Thus, the various virus- and host-derived components that constitute the replication complex present attractive targets to inhibit the multiplication and spread of the invading virus (reviewed by Carr & Zaitlin, 1993). The first instance of engineered virus resistance conferred by the transgenic expression of a replicase-related protein was demonstrated by Zaitlin and co-workers (Golemboski *et al.*, 1990). The TMV replicase is expressed from a large open reading frame at the 5' end of the viral genome, which encodes two proteins: a 126 kD protein and a second read-through protein of 183 kD, harbouring the glycine-aspartic acid-aspartic acid (GDD) motif characteristic of RNA-dependent RNA polymerases (Goldbach, 1987; Koonin & Dolja, 1993). The read-through portion of the replicase gene potentially encodes a third protein of 54 kD that also includes the GDD motif, but that has never been observed *in vivo*. Transgenic expression of the 54 kD read-through portion of the replicase gene from TMV rendered tobacco plants highly resistant to TMV infection, out competing coat protein-mediated levels of TMV resistance. Although not halted completely, TMV replication at the site of infection appeared so severely inhibited that subsequent systemic spread was strongly impeded (Carr & Zaitlin, 1991). In fact, transgenic tobacco plants showed virtual immunity as they remained free of systemic symptoms through maturity and did not accumulate virus, even when challenged with very high inoculum concentrations. Although accumulation of the 54 kD polypeptide could not be detected, mutagenesis rendering the 54 kD open reading frame translationally defective strongly suggested that it is the protein rather than the transcript that presents the active entity (Carr *et al.*, 1992). In contrast to the 54 kD protein which confers high levels of resistance to the homologous virus only, the transgenic expression of the full-length TMV replicase gene carrying engineered stop codons to yield truncated proteins, appeared to confer broad spectrum resistance to a range viruses related to TMV (Donson *et al.*, 1993).

Similar experiments with transgenic plants expressing full length or truncated replicase proteins have been performed with pea early browning virus (MacFarlane & Davies, 1992), cucumber mosaic virus (CMV) (Anderson *et al.*, 1992) and PVX (Braun & Hemenway, 1992, Longstaff *et al.*, 1993). As was the case with TMV, plants expressing replicase-derived transgenes were generally more effectively protected to virus challenge than plants expressing the coat protein genes from these viruses. A correlation between expression levels of the replicase-derived transgene and resistance levels, however, was never established, which does not exclude a resistance mechanism mediated at the RNA

level. Indeed, translationally defective deletion mutants of brome mosaic virus (BMV) RNA2 effectively interfered with viral replication of BMV in barley protoplasts, thereby demonstrating that a defective replicase protein was not responsible for the decreased replication (Marsh *et al.*, 1991a). Likewise, translationally defective replicase genes derived from PVX and from CMV RNA2 yielded high levels of resistance to the homologous virus, but not to related virus strains (Mueller *et al.*, 1995; de Haan, personal communication). For the PVX-derived replicase gene, the strain-specific virus resistance was shown to be correlated with low-level accumulation of the transgene transcript, but yet high transcription rates, characteristic for the 'antiviral state' postulated by Dougherty and co-workers (Lindbo *et al.*, 1993b; Smith *et al.*, 1994). Additionally, resistance conferring transgenes were shown to be able to *trans*-inactivate homologous transgenes, a phenomenon referred to as homology-dependent gene silencing (Kooter & Mol, 1993; Matzke & Matzke, 1993 and 1995). Based on these observations it was proposed that the 'antiviral state' underlying transcript-mediated virus resistance, and homology-dependent gene silencing may be due to the same cytoplasmic mechanism that degrades RNA with sequence homology to the silencing transgene (Mueller *et al.*, 1995).
Simply transforming plants with viral replicase sequences, however, does not necessarily confer virus resistance. On the contrary, the transformation of tobacco with native replicase genes from AlMV (van Dun *et al.*, 1988b; Taschner *et al.*, 1991) or from brome mosaic virus (Mori *et al.*, 1992) resulted in the successful complementation of deficient virus inocula. The design of defective or mutant replicase proteins, however, still presents a plausible approach to achieve replicase-mediated resistance to these viruses, which recently proved successful for AlMV. Transformation of tobacco with a number of replicase genes carrying various mutations in the GDD motif was shown to afford protection to AlMV infection, due to the accumulation of mutant replicase proteins rather than to a transcript-mediated gene silencing mechanism (Brederode *et al.*, 1995).

Antisense-mediated protection

The antisense expression of RNA sequences derived from the viral genome is another potential strategy to obtain virus resistance. Most examples of antisense resistance to virus infection resulted from studies on coat protein-mediated resistance. Transgenic plants expressing antisense transcripts to the coat protein genes from CMV (Cuozzo *et al.*, 1988), PVX (Hemenway *et al.*, 1988) or TMV (Powell *et al.*, 1989) showed only limited protection at levels lower than those observed for protein-mediated protection. Similarly, transgenic plants expressing antisense RNA to other regions of the CMV

genome than the coat protein gene were generally not resistant to CMV infection (Rezaian *et al.*, 1988). However, the high copy number of viral genomes and their association with proteins at all stages of replication suggest that a simple antisense strategy is unlikely to be successful. Having sufficient blocker molecules to control a replicating virus and knowing which are the best regions to target are important questions related to this strategy. In this perspective it is early replication or transcription signals that represent tempting targets (Morch *et al.*, 1987). Antisense transcripts to the intercistronic control sequence from brome mosaic virus RNA3 for instance, were shown to interfere with replication of the virus in barley protoplasts (Huntley & Hall, 1993a), as did small transcripts targeted to the minus strand RNA promoter (Huntley & Hall, 1993b). Transgenic expression of an RNA transcript complementary to a replication-associated region from the genome of tomato golden mosaic geminivirus, a single-stranded DNA virus that replicates in the nucleus, likewise resulted in a positive correlation between the accumulation of antisense mRNA and the reduction in symptom development upon virus challenge (Day *et al.*, 1991; Bejarano & Lichtenstein, 1994).

Satellite and defective interfering RNA-mediated protection

Virus satellites are small RNAs that rely on a helper virus for their replication and encapsidation, but that are not related to the helper virus by sequence homology (reviewed by Collmer & Howell, 1992). Satellite RNAs usually attenuate symptom expression associated with helper virus infection, but in some cases disease symptoms may aggravate. The ability of satellite RNAs to act as molecular parasites of their helper virus, thereby attenuating symptom expression, led to their use in the vaccination of commercial crops (Tien & Wu, 1991). In analogy to this approach, the transgenic expression of cloned copies of symptom-ameliorating satellites from CMV and tobacco ringspot virus was also shown to provide protection from the severe effects of their helper viruses, but without satisfactory explanation (Harrison *et al.*, 1987; Gerlach *et al.*, 1987; Saito *et al.*, 1992). A clear correlation between the suppression in symptom development and a reduction in virus replication could not be established (reviewed by Yie & Tien, 1993).

Several concerns exist which limit the widespread application of the satellite-mediated approach. As satellite-mediated protection is to be classified as tolerance rather than resistance, the transgenic crop acts as a virus reservoir that might endanger the culture of adjacent crops. Moreover, one satellite that alleviates symptoms in the target crop may aggravate symptoms in another, depending on the helper strain and the crop cultivar

involved. Additionally, the mutation of the transgenically expressed satellite RNA from a benign form into a virulent form during its amplification in conjunction with virus infection, constitutes a constant risk inherent to satellite-mediated protection, for the difference between attenuating and virulent satellites may be as little as a few nucleotides (Palukaitis, 1988; Masuta & Takanami, 1989). Finally, superinfection of crop plants immunised with a satellite-attenuated virus strain by a second non-related virus may lead to synergistic effects causing severe losses.

Defective interfering (DI) RNAs differ from satellites in that they do share extensive sequence homology with their helper virus, as they arise from rearrangements of the viral genome (Hillman *et al.*, 1987). Generally, DI RNAs result from internal deletions of essential sequences from the viral genome, which render them smaller in length and helper-dependent on the replication of the parent virus. Although rather common for animal viruses, native DI RNAs have been described for only a limited number of plant viruses, including cymbidium ringspot virus, tomato bushy stunt virus and tomato spotted wilt virus (Burgyàn *et al.*, 1991; Knorr *et al.*, 1991; Resende *et al.*, 1991). Like satellite RNAs, they can intensify (Li *et al.*, 1989), or attenuate symptom expression through interference with the replication of the parent virus (Jones *et al.*, 1990). Expression of cloned forms of DI RNAs similarly conferred tolerance to the cognate virus. Transformation of *Nicotiana benthamiana* with a native DI RNA from cymbidium ringspot tombusvirus prevented the occurrence of apical necrosis and plant death normally caused by infection with the parent virus (Kollàr *et al.*, 1993). This amelioration of symptoms was associated with the replication of the parasitic DI RNA, inhibiting the replication of the parent virus. Likewise, transgenic expression of a defective, subgenomic DNA fragment from African cassava mosaic virus conferred protection to this DNA virus (Stanley *et al.*, 1990). Inoculation of transgenic plants resulted in the episomal replication of the artificial parasitic molecule interfering with the replication of the parent virus. Systemic infection took longer to become established and symptoms were less severe.

An alternative to seeking natural occurring DIs is the construction of artificial DIs from replicase binding sites. For most plant viruses, these are likely, at least in part, to be located at the 5' and 3' ends of the viral genome. Internal deletions of brome mosaic virus RNA2 were shown to yield artificial DIs, reducing or even eliminating the replication of the parent virus RNAs in barley protoplasts (Marsh *et al.*, 1991b). Mutations that prevented translation of the DI RNA did not affect interference, but those involving sequences controlling replication reduced interference, thus demonstrating the need for active replication of the parasitic DI RNA. This example of an artificial DI RNA illustrates the principle of the DI approach and confirms its feasibility. When furnished

with latent suicide genes encoding phytotoxic proteins that are controlled from subgenomic RNA promoters, the DI-mediated tolerance can even be converted into a resistance type of protection. Upon replication of the artificial DI, the latent suicide gene will be expressed from the subgenomic RNA promoter, causing localised cell death and prohibiting the cognate virus from systemic infection. As such, this particular strategy of pathogen-derived resistance would mimic the hypersensitive response of host resistance genes. One type of phytotoxic proteins that can be envisioned to function as lethal determinant are non-specific ribonucleases, that have likewise been employed to engineer male sterility in plants (Mariani *et al.*, 1990). The plant cell suicide concept, however, requires the extreme and strict transcriptional control of the phytotoxic genes, in order to prevent their uncontrolled or leaky expression jeopardising crop yield and product quality.

Other strategies of engineered virus resistance

Another target for engineering virus resistance is the process of spread of viruses, involving movement proteins (Hull, 1991; Deom *et al.*, 1992; McLean *et al.*, 1993). The understanding how movement proteins function enables the design of decoy or crippled derivatives that block the spread of the virus. Transgenic tobacco plants accumulating dysfunctional movement proteins from TMV mutants were delayed in the development of systemic symptoms, presumably through interference with cell-to-cell movement of the challenging TMV particles (Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993; Cooper *et al.*, 1995). Transgenic expression of a mutated movement protein derived from the white clover mosaic potexvirus, was likewise shown to act as a dominant negative mutation, generating general potexvirus protection (Beck *et al.*, 1994). Expression of wild type TMV movement protein genes did not yield protection, but complemented movement deficient mutants of TMV (Holt & Beachy, 1991), and even increased susceptibility to virus infection (Cooper *et al.*, 1995). On the contrary, transgenic expression of the native movement protein gene from tomato golden mosaic geminivirus was shown to reduce the systemic infectivity of the related African cassava mosaic geminivirus in *Nicotiana benthamiana* (von Arnim & Stanley, 1992). The implication of movement proteins in systemic symptom development, however, makes the use of movement protein genes to engineer virus resistance a rather ambiguous approach.

Since the first demonstration that functional mouse monoclonal antibodies can successfully be expressed and assembled in plant cells (Hiatt *et al.*, 1989), the transformation of crop plants with engineered 'plantibodies' directed to essential viral

proteins is thought to provide an alternative to pathogen-derived resistance strategies (Conrad & Fiedler, 1994). Only recently, the constitutive expression of a single chain monoclonal antibody engineered against the coat protein of artichoke mottled crinkle tombusvirus, was shown to confer protection to virus infection in *Nicotiana benthamiana* (Tavladoraki *et al.*, 1993). Transgenic plants showed reduced incidence of infection, a delay in symptom development and lower virus accumulation levels. Likewise, the expression of a TMV-specific antibody imparted reduced levels of susceptibility to TMV infection in tobacco (Voss *et al.*, 1995). Although the precise mechanism of protection remains obscure, the approach of engineered immunisation seems to be of great potential to protect crops from viral infections, because of the virtually unlimited repertoire of 'plantibody' specificity.

Within the area of plant immunisation mimicking the mammalian immune system, the transformation of potato with the 2',5'-oligoadenylate synthase gene from rat was claimed to yield protection against PVX infection (Truve *et al.*, 1993). In mammals, this enzyme takes part in the antiviral response induced by interferon (Samuel, 1991). Once activated by the presence of double-stranded RNA, the typical replication intermediate of RNA viruses, the enzyme polymerises ATP to a series of 2'-5' oligoadenylates, which on their turn activate a latent endoribonuclease thought to degrade viral as well as cellular RNAs. Upon inoculation with PVX, virus concentrations in transgenic potato plants carrying the 2',5'-oligoadenylate synthase gene were reduced and in some lines were lower than in transgenic potato expressing the PVX coat protein gene. In theory, the realisation of a functional 2'-5' oligoadenylate synthesis pathway in plants should lead to a generalised protection against RNA viruses and thus potentially allows the creation of plants with a broad spectrum of protection to viral infections. However, this speculation has never been sustained by scientific data.

Concluding remarks

The number of examples of pathogen-derived resistance exploiting a variety of viral sequences is still growing rapidly. The implementation of such non-conventional resistance genes in crop breeding programs will increase the genetic sources that plant breeders can use to combat plant virus diseases. Thus far, coat protein gene-derived protection is more widely applied than any other approach, because it was the first one described. The transgenic expression of other viral sequences, however, especially those involving resistance mechanisms mediated at the transcript level, can provide higher levels of protection, albeit against a more narrow range of viruses. With the increasing

knowledge on virus function and pathogenesis, other strategies are likely to become apparent. The development of such alternative strategies and the combination of multiple strategies within one crop species may provide broad protection at sufficiently high levels to protect crops from virus infections in the field, thereby opening a new era in controlling plant viral diseases.

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4 Transgenic expression of the coat protein gene from beet western yellows virus in lettuce

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Abstract

The commercial culture of lettuce suffers appreciable losses from beet western yellows virus (BWYV) infections. As reliable sources of natural resistance are lacking, the transformation of lettuce with the BWYV coat protein gene presents a potential alternative to obtain genetic resistance. In that perspective, this chapter describes the construction of plant gene cassettes of the BWYV coat protein gene, their subsequent transformation to an elite lettuce cultivar, and the evaluation of their potential to yield engineered resistance to BWYV infections in transgenic lettuce. In spite of the numerous number of individual lettuce transformants analysed, substantial levels of protection against BWYV infection were not obtained. At best, a delay in symptom development of only a few days could be observed, but eventually all plants turned diseased. Apparently, the coat protein-mediated approach is inadequate to protect transgenic lettuce from BWYV infections.

Introduction

Lettuce (*Lactuca sativa* L.) is an almost world-wide grown vegetable crop, that is predominantly used in salads. The prevalent virus disease in field lettuce crops arises from beet western yellows virus (BWYV) infections. Lettuce plants infected with this luteovirus show a typical interveinal chlorosis of the older leaves, especially under high light intensities, thereby reducing their marketability. Due to its broad host range and its world-wide distribution, BWYV represents the most important member of the luteovirus group (Casper, 1988). Over 100 plant species from 21 dicotyledonous plant families, including vegetable crops such as lettuce, spinach, radish and several *Brassica* species, are readily infected (Duffus, 1972). Field infections of crop plants commonly originate from weed species like *Senecio vulgaris* and *Capsella bursa-pastoris*, which function as natural overwintering hosts of the virus. The occurrence of the virus within the plant is confined to the phloem and neighbouring cells, and the virus is obligatory transmitted by aphids in a persistent, circulative manner. *Myzus persicae* (Sulz.) probably is the most efficient and most important aphid vector for BWYV under natural conditions.

The genome of luteoviruses comprises one molecule of single-stranded RNA of positive polarity that is devoid of a substantial polyadenylate sequence (Martin *et al.*, 1990). At its 5' end the viral RNA is covalently bound to a genome-linked protein. The complete nucleotide sequence of the BWYV genome has been determined, revealing its genomic organisation (Veidt *et al.*, 1988). The open reading frame encoding the coat protein of 23 kD was positively identified by *in vitro* translation and immunoprecipitation experiments. The coat protein (CP) gene is located near the middle of the genome and is expressed from a subgenomic mRNA. A striking feature of the CP gene is the presence of a second open reading frame (ORF5) embedded within the CP gene, encoding a protein of 20 kD that is presumed to correspond to the viral movement protein.

Thus far, growers have relied on chemical control of the aphid vector to limit the incidence of the yellowing disease as natural sources for tolerance to BWYV infections have been inadequate (Watts, 1975). The current opinion, however, to reduce the use of pesticides in vegetable crops urges the need for genetic sources of protection. Newly identified sources of natural resistance that are currently being backcrossed from wild relatives (Pink *et al.*, 1991; Maisonneuve *et al.*, 1991), need further evaluation in order to assess their potential in protecting lettuce cultivars. Therefore, the transformation of lettuce with the viral CP gene in order to obtain engineered resistance to BWYV infections presents an attractive alternative.

In the past decade, virus resistance conferred by the expression of viral CP genes in transgenic plants, referred to as coat protein-mediated protection, has been described for

a considerable number of plant viruses from several different virus groups (reviewed by Beachy *et al.*, 1990; Gonsalves & Slightom, 1993). To date, the rapidly growing number of open field trials illustrates the success of coat protein-mediated protection to protect transgenic crops like squash, tomato, cucumber and potato from viral infections (Nelson *et al.*, 1988; Kaniewski *et al.*, 1990; Gonsalves *et al.*, 1992; Jongedijk *et al.*, 1993; Sanders *et al.*, 1992; Kaniewski & Thomas, 1993). Here, we describe the transformation of lettuce with the BWYV CP gene and its potential to engineer resistance to BWYV infections in transgenic lettuce.

Materials and methods

Virus and plant material. BWYV strain FL1 was originally isolated from lettuce in 1982 in southern France and has since been maintained and propagated in *Physalis floridana* by means of serial passages using *Myzus persicae* (Sulz.) as transmitting vector. *Physalis* host plants and apterous aphids were grown at 22 °C in a small phytotron with a 16 hr photoperiod. A collection of overlapping cDNA clones covering the complete genome of BWYV strain FL1 was purchased from the CNRS, 'Institut de Biologie Moléculaire des Plantes', Strasbourg, France (Veidt *et al.*, 1988).
Lettuce genotype L4607 was used as recipient in transformation experiments. This elite inbred line typifies the South-European market of butterhead lettuce types. Transgenic lettuce plants were grown under certified greenhouse conditions according to the legislation imposed by the Dutch and French authorities, the 'Voorlopige Commissie Genetische Modificatie' (VCOGEM) and the 'Commission du Génie Biomoléculaire' (CGB), respectively.

Construction of plant transformation vectors. All manipulations involving DNA were essentially performed according to standard procedures (Ausubel *et al.*, 1987). The BWYV CP gene was cloned either directly from cDNA clone pBW17 (Veidt *et al.*, 1988) or upon amplification by means of the polymerase chain reaction (PCR) using an appropriate pair of oligonucleotide primers. The latter approach was employed to create modified versions of the BWYV CP gene by means of site-directed mutagenesis. Upon assembly of the BWYV CP genes into plant gene cassettes controlled by the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) terminator, the chimaeric BWYV CP gene cassettes were cloned into binary plant transformation vector pBIN19 (Bevan, 1984). The resulting transformation vectors were subsequently mobilised into the non-oncogenic *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et*

al., 1981) by means of triparental mating using pRK2013 as helper plasmid (Ditta *et al.*, 1980). Before plant transformation, recombinant *A. tumefaciens* strains were checked for the integrity of their binary transformation vectors through Southern blot analysis.

In vitro *transcription-translation of the BWYV CP gene.* In order to target the BWYV CP gene for *in vitro* transcription-translation, the BWYV CP gene was positioned downstream of the RNA polymerase promoters as present at the pBluescript cloning vectors (Stratagene). To this purpose, redundant CaMV 35S promoter sequences were deleted from the various gene cassettes using appropriate restriction enzymes. Upon digestion with *Bgl* II at the 3' end of the *nos* terminator, linearised plasmid templates were transcribed using T3 or T7 RNA polymerase to produce capped *in vitro* run-off transcripts according to the supplier's instruction (TransProbe T Kit, Pharmacia). Capped transcripts were subsequently translated in a rabbit reticulocyte lysate system (*In vitro* Express^{TM} Translation Kit, Stratagene), following the supplier's prescription. Upon denaturation, ^{35}S-labelled protein samples were fractionated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and visualised through fluorography of dried gels impregnated with En^{3}hance (New England Nuclear).

Agrobacterium-*mediated transformation of lettuce.* Lettuce seeds were surface-sterilised for 20 minutes in a commercial bleach solution diluted to a final concentration of 1g/l NaOCl. Following several washes with sterile water, seeds were placed onto MS10 medium: Murashige and Skoog (MS) plant salt mixture (Murashige & Skoog, 1962) (Flow Laboratories Inc.) supplemented with 10 g/l sucrose and solidified with 7.5 g/l plant agar (Duchefa, Haarlem, the Netherlands). Seeds were incubated at 22 °C with a 16 hr photoperiod. After four days of germination, fully expanded cotyledons were excised from the seedlings. While immersed into the *Agrobacterium* suspension, cotyledons were cut transversally to provide two explants with two cut edges. *Agrobacterium* suspensions were prepared from overnight cultures that were washed and diluted 25 times in MMS30 medium: MS plant salt mixture, 1 g/l 2-[N-morpholino]ethanesulfonic acid (MES), 30 g/l sucrose, Gamborg B5 vitamins (Gamborg *et al.*, 1968) and 2.0 µM folic acid. Cotyledon explants were floated for 15 to 30 minutes onto the *Agrobacterium* suspension and subsequently transferred to co-cultivation plates consisting of MMS30 medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 mg/l 6-(g,g-dimethylallylamino)-purine (2-IP) with their abaxial side up. At 2-days-intervals, the explants were transferred to regeneration medium: MMS30 medium, 2.0 mg/l 2-IP and 0.5 g/l carbenicillin, and subsequently to selective regeneration medium containing 100 mg/l kanamycin, now with their axial side up. Explants were incubated at 22 °C and

subcultured every two weeks. The light regime with a 16 hr photoperiod and an intensity of 6 to 8 10^3 Lux was supplied by Sylvania GRO-LUX/36W and Philips 33/58W fluorescent tubes. Plant tissue culture media were solidified with 7.5 g/l plant agar (Duchefa, Haarlem, the Netherlands). Between 4 to 8 weeks after infection, shoot primordia were cut from the explants and rooted on MS10 medium supplemented with 0.5 g/l carbenicillin and 50 mg/l kanamycin. Rooted shoots were potted in soil, transferred to the greenhouse and were allowed to self-pollinate to produce offspring.

Northern blot analysis. Total RNA was extracted from transgenic lettuce plantlets according to Logemann *et al.* (1987). RNA samples of approximately 10 µg were fractionated on 1.2% agarose gels under formaldehyde denatured conditions and transferred to Hybond-N membranes (Amersham) by capillary blotting (Ausubel *et al.*, 1987). The resulting blots were subsequently hybridised with a ^{32}P-labelled DNA fragment, containing the BWYV CP gene in a SSC based hybridisation buffer.

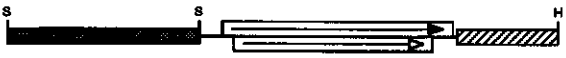
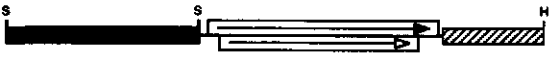
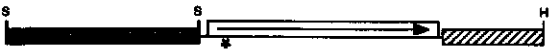
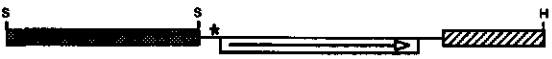
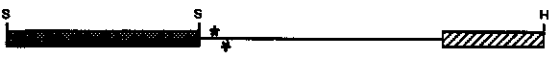
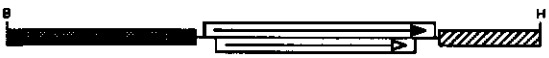
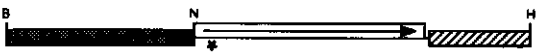
Analysis of protection to BWYV infection upon aphid-mediated inoculation. When lettuce seedlings reached their two- to three-leaves stage, approximately 3 to 4 weeks after sowing, progeny populations were exposed to viruliferous aphids that had been feeding on *Physalis floridana* plants infected with BWYV. To this purpose, a small piece of an infested *Physalis* leaf with approximately ten to thirty viruliferous aphids (larvae and adults) was placed on each lettuce seedling. For each transformant line twelve S1 progeny plants, segregating for the BWYV CP, were analysed. After two to three days of feeding, aphids were killed by spraying with 0.75 g/l pyrimicarb. This inoculation procedure was repeated a few days later, and lettuce plants were subsequently monitored for the development of yellowing symptoms at the older, most exterior leaves.

Results

Construction of plant gene cassettes for the BWYV CP gene.

Originating from cDNA clone pBW17 (Veidt *et al.*, 1988), the BWYV CP gene was assembled into a series of plant gene cassettes differing in the length and type of their 5'-untranslated leader, and in the gene arrangement of the BWYV CP gene itself (Table I). Promoter sequences were derived from the cauliflower mosaic virus (CaMV) 35S promoter, that in case of pZU030 and pZU046 was fused to the 5'-untranslated leader sequence from tobacco mosaic virus with the objective to enhance translation (Gallie *et al.*, 1987). The terminator sequence was derived from the nopaline synthase (*nos*) gene.

Table I. Schematic representation of the plant gene cassettes for the BWYV CP gene and their number of independent lettuce transformants analysed for protection to BWYV infection. Chimaeric gene cassettes comprised the CaMV 35S promoter, the BWYV CP gene, and the *nos* terminator. In case of pZU030 and pZU046, the CaMV 35S promoter was enhanced with the 5'-untranslated leader from tobacco mosaic virus. Full and open arrows refer to functional open reading frames encoding the BWYV coat protein and the ORF5 gene product respectively. B: *Bam*H I; Bc: *Bcl* I; H: *Hind* III; N: *Nco* I; R: *Rsa* I; S: *Sac* I.

Schematic Representation	Plant Gene Cassette	Number of Transformants
	pZU014	24
	pZU015	16
	pZU016	35
	pZU080	6
	pZU081	12
	pZU087	5
	pZU030	15
	pZU046	38

The rationale behind this series of gene cassettes was not only to engineer resistance to BWYV infections in lettuce, but also to unravel the mechanism underlying the anticipated resistance. To this purpose the BWYV CP gene encompassing the ORF5 gene was mutated at either or both ATG translation initiation codons to yield plant gene cassettes in which the translation of the CP gene itself (pZU81), the ORF5 gene (pZU080), or both genes (pZU087) was prevented. Both mutations were embedded within the recognition sequences of appropriate restriction sequences that enabled to check their presence.

The success of the site-directed mutagenesis of the BWYV CP gene was confirmed by *in vitro* transcription and subsequent translation in a rabbit reticulocyte lysate system. Modified CP genes were shown to be expressed as anticipated, resulting in the accumulation of both or only one of both proteins (Fig. 1). In spite of its downstream position, the ORF5 protein appeared to accumulate in about equal quantities compared to

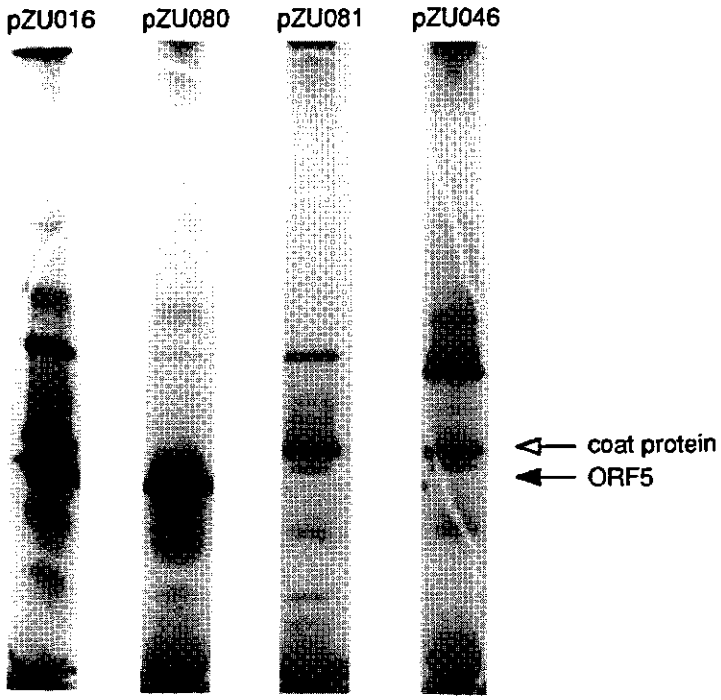


Fig. 1. In vitro transcription-translation of modified BWYV CP genes. Upon removal of spurious CaMV 35S promoter sequences, the BWYV CP genes were transcribed *in vitro* to produce capped transcripts that were subsequently used to program a cell-free rabbit reticulocyte lysate system. Denatured ^{35}S-labelled protein samples were fractionated by SDS-polyacrylamide gel electrophoresis and subsequently visualised through fluorography. The full and open arrow at the left point at the protein products translated from the open reading frames encoding the coat protein and the ORF5 protein respectively.

the coat protein. This result confirms earlier results obtained for potato leafroll luteovirus (Tacke *et al.*, 1990) and barley yellow dwarf luteovirus (Dinesh-Kumar & Miller, 1993), demonstrating the efficient translation of the nested ORF5 gene in transient expression assays. Apparently, the suboptimal AUG context of the CP gene causes a significant portion of the ribosomes to bypass the most proximal AUG and to initiate translation at the second AUG of the downstream ORF5 gene, which is situated in a more preferred context (Lütcke *et al.*, 1987; Kozak, 1992).

Using appropriate restriction enzymes the various gene cassettes of the BWYV CP gene were cloned onto the T-DNA of pBIN19, adjacent to the neomycin phosphotransferase II (NPTII) selectable marker gene already present (Bevan, 1984). The resulting transformation vectors were subsequently mobilised into *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.*, 1981).

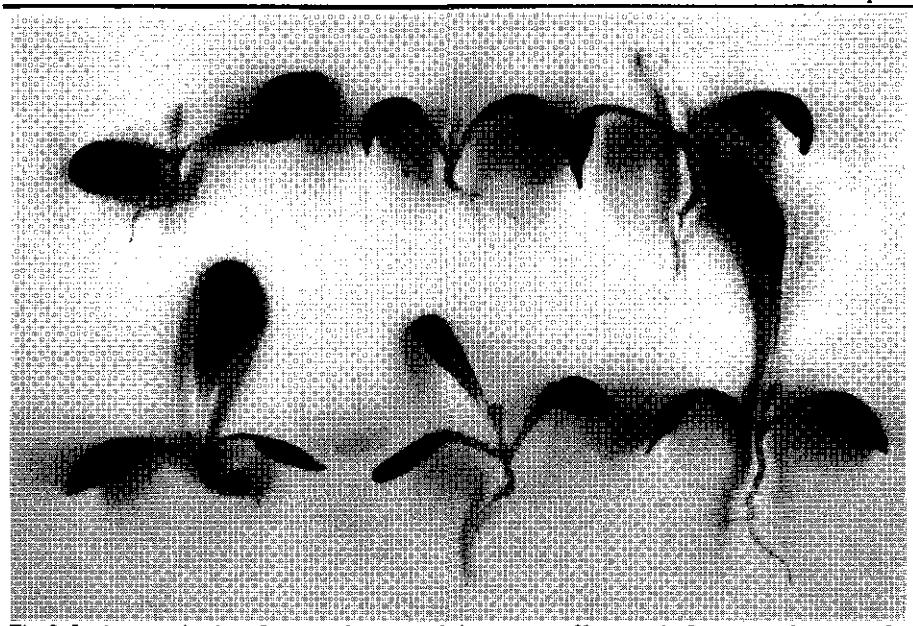


Fig. 2. In vitro germination of progeny lettuce seeds in presence of kanamycin. Lettuce seeds were surface-sterilised and subsequently germinated on MS10 medium supplemented with 100 mg/l kanamycin. On top, segregant seedlings susceptible to kanamycin; below, transgenic seedlings resistant to kanamycin. Seedlings were photographed at approximately 1 week after germination.

Agrobacterium-*mediated transformation of lettuce.*

Plant gene cassettes of the BWYV CP gene were transformed to lettuce by means of *Agrobacterium*-mediated transformation. Cotyledon explants were prepared from *in vitro*-grown seedlings of lettuce genotype L4607, which typifies the South-European market of butterhead lettuce types. After two days of co-cultivation, the cotyledon explants were transferred to regeneration medium containing the plant cytokinin 6-(g,g-dimethylallylamino)-purine (2-IP) to induce shoot regeneration. As from four days after infection, the regeneration medium was supplemented with kanamycin at a concentration of 100 mg/l to select for the regeneration of transformed cells. Regenerated shoots generally emerged between four to six weeks after infection. Putative transformants were rooted at half-strength kanamycin selection (50 mg/l) to eliminate non-transformed shoots that tended to bleach and more importantly, that failed to develop roots. The success of the protocol was confirmed by the transformation of lettuce with the β-glucuronidase (GUS) reporter gene (Jefferson, 1987) that was shown to be highly expressed (data not shown).

Upon rooting, transformants were potted in soil, transferred to the greenhouse and self-pollinated to produce offspring. The number of independent transformant lines generated for each gene cassette of the BWYV CP gene is shown in Table I. *In vitro* germination of

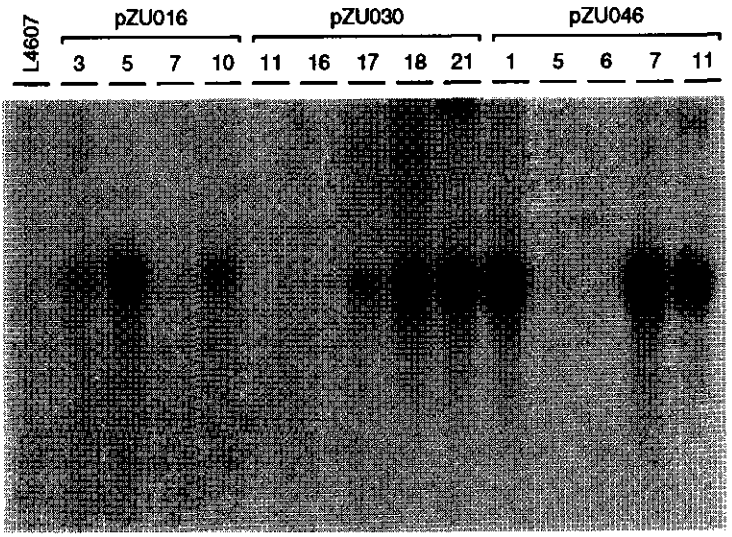


Fig. 3. Northern blot analysis of transgenic lettuce lines carrying the BWYV CP gene. Total RNA was fractionated on formaldehyde denatured agarose gels, blotted and hybridised with a ^{32}P-labelled DNA fragment derived from the BWYV CP gene. Numbers refer to the primary lettuce transformants. L4607: non-transformed lettuce used as recipient genotype in transformation experiments.

the S1 progeny lines on medium supplemented with kanamycin (100 mg/l) confirmed their transgenic nature. Upon germination, kanamycin-resistant seedlings could easily be discriminated from non-resistant segregants by virtue of the colour and shape of their first true leaf. While resistant seedlings did not differ from control seedlings germinated in the absence of kanamycin, segregant seedlings exhibited a bleached appearance and remained retarded in development (Fig. 2). Although the expression of the CP gene cassettes at the RNA level could readily be demonstrated by Northern blot analysis (Fig. 3), the accumulation of transgenically expressed coat protein could never be detected, neither by ELISA nor by Western blot analysis.

Analysis of protection to BWYV infection upon aphid-mediated inoculation.

As luteoviruses are not sap-transmissible but obligatory transmitted by aphids, segregating S1 progeny populations were challenged with BWYV by means of viruliferous aphids that were propagated on *Physalis floridana* plants infected with the virus. Aphids were allowed to feed for a few days on the lettuce seedlings before being killed by the application of an insecticide. After inoculation, lettuce plants were monitored individually for the appearance of characteristic yellowing symptoms associated with BWYV infections. The development of chlorosis, however, is strongly

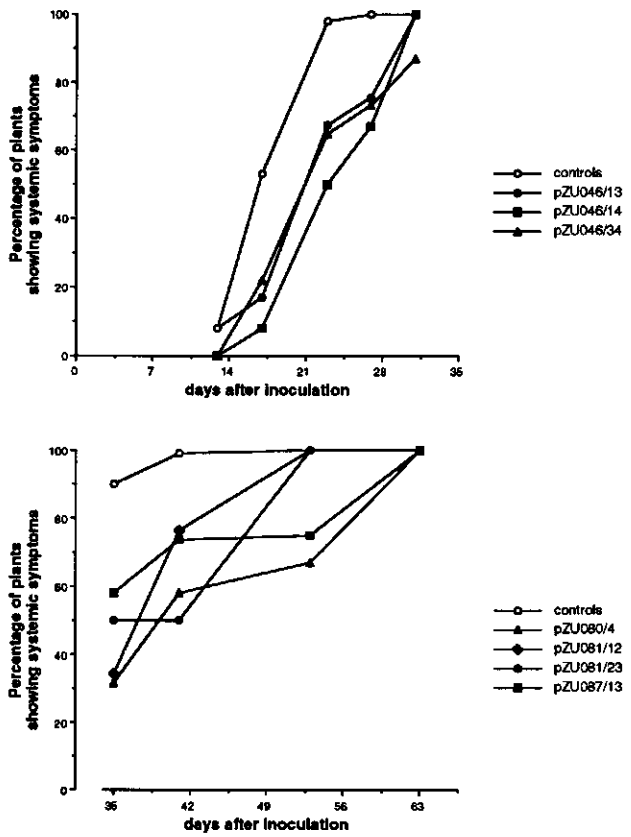


Fig. 4. Protection of segregating S1 progeny populations to aphid-mediated BWYV infection. Lettuce seedlings were challenged with viruliferous aphids that were nourished on *Physalis floridana* plants systemically infected with BWYV. Upon inoculation, lettuce plants were monitored individually for the development of chlorosis on the outermost leaves. Diagram A and B shows the results obtained from two separate experiments. Control plants (open circles) consisted of non-transformed L4607 acceptor plants.

dependent on the light intensity during the monitoring period. At high light intensities lettuce plants develop chlorosis within three weeks after infection, at lower intensities the appearance of chlorosis may be delayed to more than five weeks, and thus is highly variable between separate experiments. Results obtained for two series of transformants evaluated in two consecutive experiments are shown in Fig. 4. In spite of the large number of independent transformants analysed (Table I), any transformant line truly resisting BWYV infection was not identified. A number of lines, however, showed a clear delay in symptom development, but finally all plants turned diseased and accumulated high levels of virus as determined by ELISA. The variation in disease

development between separate experiments excludes the precise quantification and the comparison of the delay in symptom development between transformant lines from different series. Nevertheless, delayed disease development was not only observed for gene cassettes pZU046 and pZU080 that potentially express the CP at the protein level, but also for gene cassettes pZU081 and pZU087 (Fig. 4) that express the CP gene only at the mRNA level. These results suggest the underlying mechanism to be mediated at the transcript level rather than at the protein level by the accumulation of the BWYV coat protein itself.

Discussion

In an attempt to obtain engineered resistance to BWYV infections in transgenic lettuce, nucleotide sequences encoding the BWYV CP gene were transformed to an elite butterhead lettuce genotype. However, in spite of the numerous independent lettuce transformants analysed, a prominent level of resistance to BWYV infections was never observed. At best, a delay in symptom development of only a few days was observed, a level of protection too poor to be of commercial interest. This minor protection could not be assigned to a specific gene construct, and was also observed for gene constructs like pZU081 and pZU087, in which the coat protein gene was translationally inactivated through site-directed mutagenesis of the ATG initiation codon. As such, this observation suggests the observed protection, albeit minimal, to be transcript-mediated rather than protein-mediated.

Over the past few years, a number of reports on engineered resistance to luteoviruses appeared in literature, all of them dealing with resistance to potato leafroll virus (PLRV) in potato (Kawchuk *et al.*, 1991; van der Wilk *et al.*, 1991; Barker *et al.*, 1992). Although engineered resistance to PLRV infections through the expression of the PLRV CP gene was claimed, the significance of the observed protection is questionable. Potato plants never resisted infection, but merely showed reduced levels of virus accumulation, and the virus was readily transmitted to progeny plants emerging from transgenic tubers (van der Wilk *et al.*, 1991). In tobacco, the transgenic expression of the PLRV CP gene likewise afforded only marginal levels of protection (Barker *et al.*, 1993). Finally, the protection observed in potato might not even arise from the transgenic expression of the PLRV CP gene, but from somaclonal variation induced by the tissue culture process (Evans *et al.*, 1986; Potter *et al.*, 1991). In support of this explanation are data demonstrating the occurrence of reduced susceptibility to PLRV infections in control transformant lines carrying vector sequences only (Presting *et al.*, 1995).

The limited success in engineering resistance to luteovirus infections may possibly be explained by their restricted occurrence to the phloem tissue. In phloem sieve tube elements plant gene expression is drastically reduced as nuclei are degenerated. Consequently, the transgenic expression of CP gene-derived sequences at the primary site of infection may just be too limited to combat luteovirus infections.

In summary, it may be concluded that the transgenic expression of the BWYV CP gene in lettuce did not yield levels of protection of commercial value and is not expected to do so upon minor modifications such as the use of other promoters. Only by developing alternative strategies for engineered resistance, protection levels of commercial interest may be obtained for luteoviruses in general, and for BWYV in particular. In this respect, the transformation of replicase-derived sequences seems to be a promising option as replicase-mediated resistance generally outcompetes coat protein-mediated protection (Carr & Zaitlin, 1993, Baulcombe, 1994).

There is accumulating evidence that engineered resistance to plant viral infections through the transgenic expression of viral sequences is mediated at the transcript-level, rather than at the protein-level, although the precise mechanism remains unknown for the time being (Dougherty *et al.*, 1994; Smith, 1994; Chapter 6 and 7 of this thesis). In this light, the transgenic expression of viral sequences involved in early functions such as replication and transcription of the viral genome might confer higher levels of protection than viral sequences encoding structural proteins. In case of BWYV these sequences are located at both ends of the viral RNA and at the intergenic region upstream of the coat protein gene. The subgenomic promoter, located in this intergenic region, directs the transcription of a subgenomic mRNA from which the coat protein and ORF5 are translated (Veidt *et al.*, 1988). The transgenic expression of such early replication signals, in both the sense as well as the antisense orientation, presents an attractive alternative to the transgenic expression of sequences encoding viral proteins in order to obtain high levels of resistance to BWYV infection. The potential of RNA-interference targeted to regulatory control sequences has already been demonstrated for bromo mosaic virus (Huntley & Hall, 1993a and 1993b). Antisense transcripts to the minus strand promoter or to the intercistronic control sequence harbouring the subgenomic promoter from bromo mosaic virus RNA3 were shown to interfere with *in vitro* replication of the virus in barley protoplasts.

The transgenic expression of an artificial defective interfering (DI) RNA consisting of all regulatory sequences involved in replication and transcription of the viral genome presents yet another approach to obtain engineered resistance to BWYV infections. Internal deletion mutants of bromo mosaic virus RNA2 have already been shown to act as parasitic DIs, reducing or even eliminating genomic RNA replication of the parent

virus RNAs in barley protoplasts (Marsh *et al.*, 1991). Likewise, Veidt and co-workers reported on the successful assembly of a full-length cDNA clone of the BWYV genome (Veidt *et al.*, 1992). Agroinoculation of host plants with a plant gene cassette of this full-length cDNA clone controlled by the CaMV 35S promoter, resulted in a systemic infection with BWYV (Leiser *et al.*, 1992), thereby demonstrating the successful replication of the virus from a transiently expressed mRNA transcript and the feasibility of the DI approach. When equipped with a latent suicide gene encoding a phytotoxic protein that is controlled from the subgenomic or the minus strand RNA promoter, the engineered tolerance mechanism might even be converted into an active resistance mechanism. Upon BWYV infection and subsequent replication of the artificial DI, the accumulation of the phytotoxic protein will evoke localised cell death, that might prohibit the invading virus particles from systemic infection. In this way the engineered resistance mechanism would mimic the hypersensitive response associated with cultivar resistance genes.

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5 Coat protein-mediated protection to cucumber mosaic virus infections in cultivated tomato

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Abstract

Cucumber mosaic virus (CMV) infections rank among the most devastating diseases in the commercial culture of tomato (*Lycopersicon esculentum* Mill.), for which suitable sources of natural resistance are not available. The concept of pathogen-derived resistance, however, offers an alternate approach to combat plant viral diseases by transformation of crops with nucleotide sequences derived from the viral genome. This report demonstrates the successful application of such a pathogen-derived resistance gene comprising the CMV coat protein (CP) gene, to generate protection to CMV infections in cultivated tomato. Transformation of an inbred tomato line with the CMV CP gene isolated from a subgroup I strain, engendered high levels of protection to various CMV strains, including a virulent strain causing lethal necrosis and a typical subgroup II strain. Moreover, when challenged by natural infection through aphid vectors in open field, levels of protection were largely maintained in hemizygous hybrids. In all, these results demonstrate that synthetic resistance genes based on the CMV CP gene make excellent sources of broad spectrum resistance to CMV infections for introgression into tomato breeding programs.

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Introduction

Cucumber mosaic virus (CMV) is known as one of the economically most important plant viruses, because of its wide host range and the large number of different strains identified (Douine *et al.*, 1979; Kaper & Waterworth, 1981). Outbreaks of diseases incited by CMV infections have caused significant yield losses in many important vegetable crops, including tomato (Tomlinson, 1987). Breeding for CMV resistant tomato cultivars is severely hampered by the lack of suitable resistance sources in *Lycopersicon* species, which is illustrated by the fact that sofar CMV resistant tomato cultivars did not reach the market. The absence of resistant cultivars only leaves culture practices, such as control of the aphid vector population by the application of insecticides, to limit the incidence of the CMV disease. Proposed reductions in the application of pesticides for reasons of environment protection, however, urge the need for the development of novel sources of genetic resistance to CMV infections for introgression into the breeding programs of tomato.

The CMV genome consists of three positive-sense RNA species and a subgenomic mRNA encoding the viral coat protein (CP) gene. Some naturally occurring CMV strains include a fifth RNA species, which depends on the CMV helper virus for its replication and its encapsidation. This satellite, designated CARNA5 for CMV-associated RNA5, modulates disease symptoms induced by its helper virus. Most satellites associated with CMV attenuate symptom expression, but in exceptional cases specific satellite RNAs may intensify symptom expression (Waterworth *et al.*, 1979).

According to their nucleotide sequence homologies and serological properties, CMV strains are divided into subgroups I and II (Devergne & Cardin, 1973; Piazolla *et al.*, 1979; Owen *et al.*, 1990). Within subgroups, isolates share more than 95% homology in the amino acid sequence of their coat proteins. Between subgroups, the degree of homology is only approximately 80%. Nevertheless, genomic RNAs of subgroup I and II are fully compatible in pseudo-recombinants formed between both subgroups (Palukaitis *et al.*, 1992), and the emergence of novel CMV strains through (pseudo)recombination constitutes a continuous threat to the culture of vegetable crops.

The pathology of CMV infections in tomato is quite divers, ranging from asymptomatic to severe stunting with leaf curl, referred to as the fern leaf syndrome. Moreover, when associated with certain satellite RNAs, CMV infections can induce lethal necrosis in tomato (Kaper & Waterworth, 1977; Kaper *et al.*, 1990). Because of the lack of CMV resistant cultivars, CMV strains carrying attenuating satellite RNAs have been exploited to control the CMV disease in commercial tomato crops in the field. Pre-inoculation or vaccination with such attenuated CMV strains resulted in the effective cross-protection

of tomato plants against more virulent strains of the virus (Tien *et al.*, 1987; Gallitelli *et al.*, 1991; Sayama *et al.*, 1993). However, such high risk and laborious culture practices are generally regarded inferior to stable sources of genetic resistance to CMV.

Since the first report that transgenic plants expressing a plant viral CP gene showed reduced susceptibility to the homologous virus (Powell Abel *et al.*, 1986), this strategy, referred to as CP-mediated protection, has been widely adopted for protecting plants to viruses (reviewed by Beachy *et al.*, 1990). CMV is one of the still growing number of viruses for which CP-mediated protection has been demonstrated, not only in tobacco (Cuozzo *et al.*, 1988; Quemada *et al.*, 1991; Namba *et al.*, 1991; Nakajima *et al.*, 1993), but also in vegetable crops like cucumber (Gonsalves *et al.*, 1992) and melon (Yoshioka *et al.*, 1993; Gonsalves *et al.*, 1994). To study the applicability of this technology in tomato, we transformed an inbred tomato genotype with a chimaeric gene cassette comprising the CMV CP gene. Transgenic progeny plants that resisted mechanical inoculation with CMV were used as parents in the production of experimental hybrids. When exposed to natural inoculation by viruliferous aphids in open field, hemizygous hybrids showed high levels of protection, thereby demonstrating the potential of this transgenic source of resistance for protecting commercial tomato crops against CMV infections.

Materials and methods

Virus and plant material. CMV strain ZU represents a laboratory strain that was maintained in squash by repeated mechanical inoculation over many years. The strain was originally isolated from *Stellaria media* in 1972 in southern France. Necrogenic CMV strain ARN5 causing lethal necrosis in tomato, was recently isolated from tomato in southern France. Filimorphic strain I17F, which incites the typical fern leaf syndrome (Jacquemond & Lauquin, 1988), was obtained from the 'Instituut voor Planteziekten-kundig Onderzoek' (IPO-DLO) in Wageningen, the Netherlands. Subgroup II strain A was recently isolated from infected tomato plants in Australia. Virus strains were stored as desiccated leaf material at 4 °C in presence of $CaCl_2$, or as fresh leaf material in liquid nitrogen. Before their use in inoculation experiments all strains were multiplied in *Nicotiana tabacum*.

In transformation experiments parental tomato line ATV847 was used as recipient. This inbred line is used as male parent in the production of a number of hybrids for the South European market, that represent fresh market tomatoes of the indeterminant type. Transgenic hybrids were obtained by cross-pollination of ATV847 transformants with

parental line ATX011. The resulting fresh market hybrid was tentatively named *Astrid*. Transgenic tomato plants were grown under certified greenhouse conditions according to the legislation imposed by the Dutch and French authorities, the 'Voorlopige Commissie Genetische Modificatie' (VCOGEM) and the 'Commission du Génie Biomoléculaire' (CGB), respectively.

Construction of the plant transformation vector. All manipulations involving DNA were essentially performed according to standard procedures (Ausubel *et al.*, 1987). The CMV CP gene was amplified by means of the polymerase chain reaction (PCR), following reverse transcription of total RNA samples prepared from squash systemically infected with CMV strain ZU. Deproteinised RNA samples were isolated essentially as described by Logemann *et al.* (1987) by grinding 1 g of leaf material in 5 ml extraction buffer (8 M guanidine-HCl, 20 mM 2-[N-morpholino]ethanesulfonic acid (MES) pH 7.0, 20 mM EDTA, 50 mM beta-mercaptoethanol), followed by phenol extraction and ethanol precipitation. Reverse transcription and subsequent PCR-amplification was performed using the *GeneAmp* Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin-Elmer Cetus), according to the supplier's instruction and applying 40 cycles of 1 min. denaturation at 95 °C, 1.5 min annealing at 55 °C and 2 min extension at 72 °C, each cycle prolonged with 3 sec. Oligomer primers that were used for reverse transcription and amplification were Jan049 (5' CGAGCC**ATG**GACAAATCTGAATC 3') and Jan050 (5' GAACCTGCAG**TCA**GACTGGGAGCACTCCAGATGT 3'). Primer Jan049 hybridises to the ATG region of the CP gene and introduces the recognition sequence of *Nco* I comprising the ATG initiation codon. Primer Jan050 is complementary to the 3' end of the CP gene and introduces a *Pst* I site immediately downstream of the TGA stop codon. The PCR-amplified fragment of 0.8 kb was digested with *Nco* I and *Pst* I and ligated into expression vector pZU119. The resulting gene cassette pZU120 contains the cauliflower mosaic virus (CaMV) 35S promoter fused to the 5'-untranslated leader sequence from tobacco mosaic virus, the CMV CP gene and the nopaline synthase (*nos*) polyadenylation signal. The complete gene cassette was released as an *Xba* I fragment and cloned into the binary plant transformation vector pBIN19 (Bevan, 1984), yielding transformation vector pZU123A, that was subsequently transferred to the non-oncogenic *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.*, 1981) by triparental mating using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). Before plant transformation the recombinant *A. tumefaciens* strain was checked for the integrity of the binary transformation vector by Southern blot analysis.

Preparation of a polyclonal antiserum to the CMV coat protein. To facilitate the preparative purification of CMV coat protein, the CP gene was cloned into bacterial expression vector pET11t, yielding expression vector pZU137. Bacterial expression vector pET11t is derived from pET11d (Novagen, Inc.) by the introduction of additional unique cloning sites immediately downstream of the *Nco* I recognition sequence. When cell cultures of strain BL21(DE3, pLysE) transformed with expression vector pZU137 reached an OD_{600} of 0.6, expression was induced by the addition of IPTG to a final concentration of 0.4 mM, and growth continued for an additional 5 hrs (Studier *et al.*, 1990). Bacterial cells were pelleted and subsequently resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) beta-mercaptoethanol, 10% (v/v) glycerol, 0.001% bromophenol blue). After boiling, denatured protein samples were analysed by electrophoresis in 12.5% SDS-polyacrylamide gels (Laemmli, 1970). For large scale preparations of the CMV coat protein, total proteins from 50 ml IPTG-induced cultures of strain BL21 (DE3, pLysE, pZU137) were resolved on 1.5 mm thick 12.5 % preparative SDS-polyacrylamide gels (Protean™ II, Bio-Rad). Gels were stained in an ice cold solution of 0.1 M KCl and the coat protein band was excised from the gel. Purified CMV coat protein was recovered from the gel slices through electro-elution in 20 mM Tris, 150 mM glycine, 0.01% SDS for 5 hours at 100 V and 4 °C, applying an ISCO electrophoretic concentrator (ISCO, Inc.). Eluted proteins were dialysed against phosphate buffered saline (PBS), analysed on a SDS-polyacrylamide gel and quantified using the Bio-Rad Protein Assay according to the manufacturer's procedure. Portions of 100 to 200 µg of purified coat protein were emulsified with Freund's incomplete adjuvant and injected twice into the hind legs of rabbits at an interval of two weeks. Starting two weeks after the second injection, rabbits were bled several times and the gamma-globulin fractions were isolated according to Clark & Adams (1977). The titer and specificity of antisera were tested by Western blotting and ELISA using serial dilutions of the *E. coli* expressed coat protein.

Transformation of parental tomato line ATV847. The CMV CP gene cassette was introduced into the genome of inbred tomato line ATV847 by means of *Agrobacterium*-mediated leaf disc transformation as described by Ultzen *et al.* (1995; Chapter 8 of this thesis). Transformants were analysed for their ploidy level by flow cytometry, and diploid transformants were subsequently analysed for accumulation of CMV coat protein by Western blotting. Selected transformants were maintained by self-pollination to produce offspring.

Western blot analysis for the accumulation of CMV coat protein. Bacterial pellets from IPTG-induced cell cultures or leaf samples from tomato transformants were homogenised in phosphate buffered saline supplemented with 0.1% Tween-20 (PBS-T). Portions of 25 µg of soluble protein were subsequently fractionated by electrophoresis in 12.5% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were blotted to Immobilon-P membranes (Millipore) by semi-dry blotting in semi-dry transfer buffer (29 mM glycine, 48 mM Tris, 0.0375% SDS and 20% methanol) for 1 hour at 0.8 mA/cm^2. Membranes were blocked for 3 hours at 37 °C in PBS-T containing 3% BSA, and subsequently incubated with polyclonal antiserum raised against purified CMV coat protein, diluted to 1 µg/ml in PBS-T supplemented with 0.3% BSA. After incubation with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Company), antigen-antibody complexes were visualised using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) as substrate according to the supplier's instruction (ImmunoSelect™, Life Technologies, Inc.). Between subsequent treatments membranes were washed with PBS-T containing 0.3% BSA.

Analysis of protection to CMV infection upon mechanical inoculation. After emergence of the first true leaf, approximately 3 to 4 weeks after sowing, seedlings were dusted with carborundum powder and wiped with cotton-wool dipped in virus inoculum. Inocula were freshly prepared by grinding 1 gram leaf material from systemically infected *Nicotiana tabacum* in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) supplemented with 1% Na_2SO_3 and kept on ice. After inoculation plants were rinsed with water. All accessions were organised in a randomised block design with at least two replications. The extent of the CMV infection was monitored by visual observation for the development of systemic symptoms. Susceptible tomato plants developed systemic symptoms within 2 to 4 weeks after mechanical inoculation with CMV. Plants were scored susceptible when any leaf younger than the inoculated leaves showed typical systemic symptoms such as wrinkling and curling (fern leaf syndrome) or necrosis. In addition, plants became stunted at later stages of infection. The absence of virus in symptomless transgenic plants was checked by ELISA using the polyclonal antiserum raised against *E. coli* expressed CMV coat protein.

Analysis of protection in transgenic hybrids upon natural inoculation in open field. In the summer of 1994, an open field trial was executed on the premises of S&G Seeds in Agadir, Morocco, according to the legislation and regulations imposed by the 'Commission du Génie Biomoléculaire' (CGB) in France. At the end of May, when plants were 10 cm in height, transgenic *Astrid* hybrids were planted in soil in open field,

organised in two replications of 14 or 15 plants each. Non-transgenic hybrids were included as susceptible control and plants were exposed to aphid-mediated inoculation throughout their complete crop cycle. The trial was managed like a normal tomato crop including the routine application of pesticides. At the end of the trial (end of August 1994), when bearing several clusters of mature fruits, plants were scored visually for the presence of disease symptoms incited by CMV infections. Only plants that were completely free of any symptoms were scored healthy. Usually potato virus Y (PVY) infections are also quite common for the Agadir region, but in summer 1994 the incidence of the PVY disease was low and in the plot of the field trial any PVY symptoms were never observed. As a consequence plants scored healthy were devoid of any symptoms incited by viral pathogens.

Results

Cloning and sequence analysis of the CMV coat protein gene.

The CMV coat protein (CP) gene was amplified by means of the polymerase chain reaction following reverse transcription (RT-PCR) of a deproteinised RNA sample prepared from squash systemically infected with CMV strain ZU. The primers used in the amplification reaction carried appropriate restriction sites to facilitate cloning and subsequent sequence analysis. The deduced amino acid sequence of the coat protein from CMV strain ZU as shown in Fig. 1A is derived from the nucleotide sequence of two PCR fragments cloned from separate RT-PCR reactions to exclude the possibility of point mutations generated by the reverse transcriptase or the *Taq* polymerase. In comparison to the amino acid sequence of other subgroup I strains the coat protein of strain ZU shows over 95% of homology. Thus, even though CMV strain ZU was maintained by repeated mechanical inoculation over many years, the amino acid sequence of the coat protein remained highly conserved. The presence of a proline residue at position 129 classifies strain ZU as non-chlorotic (Shintaku *et al.*, 1992), which is confirmed by the typical green mosaic induced in tobacco.

Prokaryotic expression and production of antiserum to CMV coat protein.

To facilitate the purification of CMV coat protein in sufficient quantities to raise a polyclonal antiserum, the CP gene was expressed *in vitro* by means of a prokaryotic expression system (Studier *et al.*, 1990). Upon IPTG-induction, the 24 kD coat protein accumulated to large quantities and could easily by recognised on coomassie brilliant blue strained gels (Fig. 2A). The coat protein was purified from polyacrylamide gels and

A

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ZU  MDKSESTSAG R-NRRRRPRR GSRSAPSSAD ANFRVLSQQL SRLNKTLAAG RPIINHPTFV  59
I17F ..........  .-........ .......... .......... .......... ..T.......  59
O   ..........  .-........ .......... .......... .......... ..T.......  59
Y   ..........  .-.....L.. .....S..S. .......... .......... ..T.......  59
A   ....G.PN.S .TS..... ..... -.G.. .GL.A.T..M L.......I. ..TL......  59
WL  ....G.PN.S .TS..... ..... -.G.. .GL.A.T..M LK..R...I. ..TL......  59

ZU  GSERCRPGYT FTSITLKPPK IDRASYYGKR LLLPDSVTEY DKKLVSRIQI RVNPLPKFDS 119
I17F ..........  .......... ...G...... .......... .......... .......... 119
O   .....K....  .......... ...G...... .......... .......... .......... 119
Y   .....K....  ......R... ...E...... .......M.. .......... .......... 119
A   ...S.K....  .........E .EKG..F.R. .S......D. .......... .I........ 119
WL  ...S.K....  .........E .EKG..F.R. .S......D. .......... .......... 119

ZU  TVWVTVRKVP ASSDLSVAAI SAMFADGASP VLVYQYAASG VQANNKLLYD LSAMRADIGD 179
I17F ..........  .......... .......... .......... .......... .......... 179
O   ..........  .......... .......... ........F. .......... .......... 179
Y   .........S  .......... .......... .......... .......... .......... 179
A   ..........  S......... ....G..N.. .......... .......... ..E....... 179
WL  ..........  S......... ....G..N.. .......... .......... ..E....... 179

ZU  MRKYAVLVYS KDDALETDEL VLHVDIEHQR IPTSGVLPV  218
I17F ..........  .......... .......... .........  218
O   ..........  .......... .......... ....R....  218
Y   ..........  ...T...... .....V.... .........  218
A   ..........  ...K..K..I .....V.... ..I.RM..T  218
WL  ..........  ...K..K..I A....V.... ..I.RM..T  218
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B

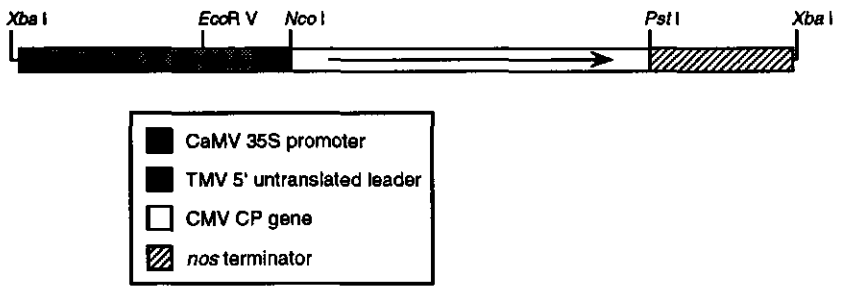

Fig. 1. A: Deduced amino acid sequence of the coat protein gene from CMV strain ZU, aligned to those of some other CMV strains. The sequences shown in boxes represent subgroup II strains. Amino acids identical to the amino acid sequence of the coat protein from strain ZU are indicated by dots, while dashes represent gaps that are introduced for optimal alignment. B: Schematic representation of the chimaeric CMV CP gene cassette comprising the CaMV 35S promoter, the tobacco mosaic virus 5'-untranslated leader, the CMV CP gene and the *nos* terminator. The complete CMV CP gene cassette was cloned into binary transformation vector pBIN19 as an *Xba* I fragment.

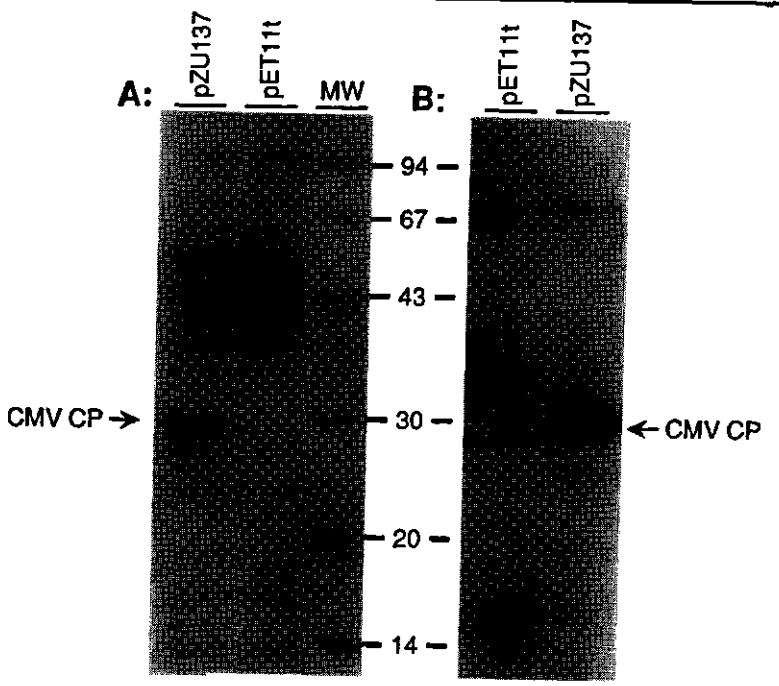

Fig. 2. Prokaryotic expression of the CMV CP gene and specificity of the polyclonal antiserum raised against purified CMV coat protein. A: The accumulation of CMV coat protein in *E. coli* cells transformed with pZU137 was induced with IPTG and total proteins analysed on a SDS-polyacrylamide gel stained with coomassie brilliant blue. Total proteins from bacterial cells transformed with the empty expression vector pET11t were included as control. B: Western blot analysis of total protein samples from IPTG-induced *E. coli* cells harbouring pET11t or pZU137. Proteins were resolved on a SDS-polyacrylamide gel, blotted to Immobilon-P membranes and the CMV coat protein detected using the polyclonal antiserum raised against purified coat protein. The protein sample in lane pZU137 was 1000-fold diluted in comparison to lane pET11t.

used to immunize rabbits. Titer and specificity of the obtained antisera were tested by immunoblot analysis (Fig. 2B). Although the antisera appeared to be slightly contaminated with immunoglobulins against bacterial proteins that were co-purified with the CMV coat protein, such contaminations are not likely to interfere with immunological analyses of plants. The detection limit on immunoblots was estimated at approximately 0.5 ng and in ELISA at approximately 2 ng of denatured *E. coli* expressed coat protein (data not shown).

Construction of the CMV CP gene cassette and transformation of tomato.

In order to assemble a plant gene cassette, the CP gene was cloned between the cauliflower mosaic virus (CaMV) 35S promoter and the polyadenylation signal derived from the 3' flanking region of the nopaline synthase (*nos*) gene, yielding plant gene cassette pZU120 (Fig. 1B). The CaMV promoter was modified by fusing the 5'-

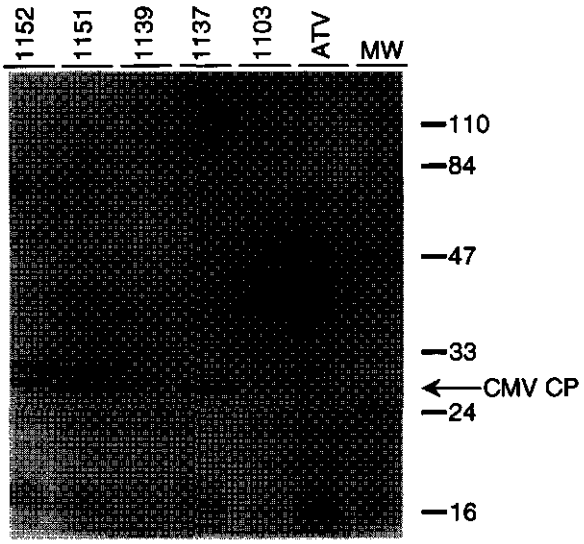

Fig. 3. Western blot analysis of putative tomato transformants carrying the CMV CP gene. Leaf protein samples were subjected to SDS-polyacrylamide gel electrophoresis, blotted to Immobilon-P membranes and CMV coat protein was detected using the polyclonal antiserum raised against purified CMV coat protein. Molecular weight markers are indicated on the right and numbers refer to independent tomato transformants; ATV: non-transformed tomato line ATV847 used as acceptor.

untranslated leader sequence from tobacco mosaic virus (TMV) immediately downstream of the transcription initiation site. The TMV leader is known to function as a translational enhancer (Gallie *et al.*, 1987). Upon cloning into binary transformation vector pBIN19, the CMV CP gene cassette was introduced into the genome of inbred tomato line ATV847 by means of *Agrobacterium*-mediated leaf disc transformation, using kanamycin resistance as selectable marker (Ultzen *et al.*, 1995; Chapter 8 of this thesis). Tomato genotype ATV847 represents an inbred line used as male parent in the production of a number of fresh market hybrids of the indeterminant type. Transformants were analysed for their ploidy level by flow cytometry and diploid transformants were subsequently subjected to Western blot analysis to identify transformants accumulating CMV coat protein (Fig. 3). Diploid transformants accumulating the CMV coat protein were maintained and self-pollinated to produce S1 offspring. None of the transformants nor their progeny populations showed any phenotypic aberrations that could be assigned to the accumulation of viral coat protein or to the insertion of the CP gene cassette into the plant genome.

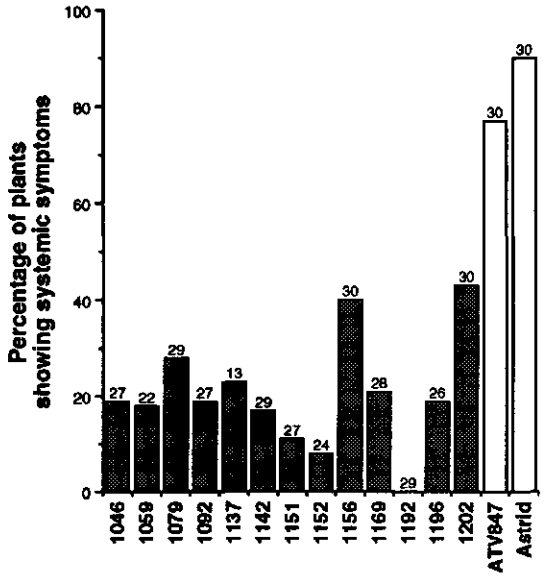

Fig. 4. Protection of segregating S1 progenies against CMV infection upon mechanical inoculation. Plants were challenged with a ten times diluted inoculum prepared from young tobacco leaves systemically infected with CMV strain I17F. Control plants (white bars) consisted of non-transformed ATV847 acceptor plants or *Astrid* hybrids derived therefrom. Figures on top of the bars refer to the number of tomato plants that have been scored for systemic symptoms in the final observation about three weeks after inoculation, including plants that did not inherit the CMV CP gene cassette through segregation.

Protection of transgenic tomato against CMV infections.

The extent of CP-mediated protection is known to be affected by the virulence and concentration of the challenge inoculum. Therefore, in order to prevent the exclusion of transformant lines with low levels of protection, tomato seedlings from S1 progeny populations were challenged by mechanical inoculation using a ten times diluted inoculum. The diluted inoculum was prepared from tobacco systemically infected with the non-necrogenic CMV strain I17F. In tomato, this strain provokes the typical fern leaf syndrome due to the wrinkling and curling of leaves (Jacquemond & Lauquin, 1988), and at later stages of infection diseased plants become stunted and reduced in height. After inoculation, plants were visually monitored for the development of systemic disease symptoms on non-inoculated leaves. The final observation was made three weeks after inoculation when susceptible controls reached infection percentages of 80% or higher (Fig. 4). From a selection of 13 S1 progeny populations, descending from primary transformants which accumulated the CMV coat protein, all transformant lines showed reduced susceptibility to CMV infection. Considering the fact that the CMV CP gene still

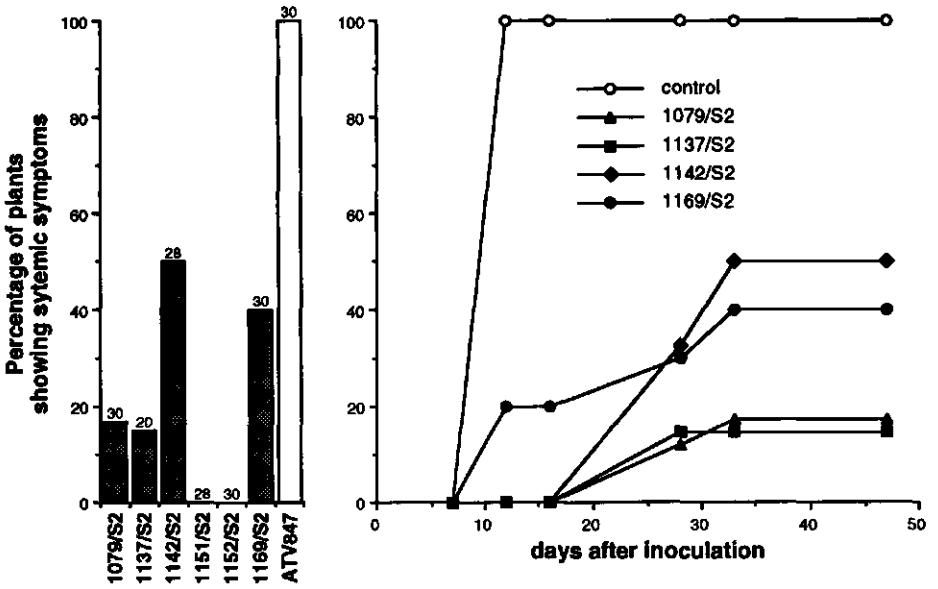

Fig. 5. Protection to CMV infection of homozygous S2 progenies upon mechanical inoculation. Plants were challenged with an inoculum prepared from young tobacco leaves systemically infected with necrogenic CMV strain ARN5. Control plants (white bars and open circles) consisted of non-transformed ATV847 acceptor plants. A: Percentage of plants showing systemic disease symptoms in the final observation about five weeks after inoculation. Figures on top of the bars refer to the number of tomato plants tested. B: Development of systemic disease symptoms in partially protected transformant lines in comparison to the susceptible control.

segregates within these S1 populations, levels of protection become even more pronounced. Protected tomato plants were free of virus as demonstrated by ELISA using the polyclonal antiserum raised against purified CMV coat protein. ELISA values of protected transformants never exceeded the mean ELISA value of non-inoculated transformants plus three times the standard deviation (threshold value = 0.13), whereas ELISA readings of infected controls were out of range. The absence of detectable amounts of virus suggests that protected plants are resistant rather than tolerant to CMV infection.

From each transformant line five individual S1 progeny plants that resisted CMV infection were selected for Southern blot analysis to determine the copy number of the CMV CP gene cassette (data not shown). The majority appeared to carry single copies of the transgene, except for transformant lines 1142, 1152 and 1192 which carry two copies. In case of transformant lines 1142 and 1152 both copies probably reside on the same chromosome as can be deduced from the fact that both copies did not segregate in five

individual S1 progeny plants tested. Selected plants were maintained and self-pollinated to produce S2 offspring. In addition, plants were cross-pollinated with parental line ATX011 to produce experimental hybrids, tentatively renamed *Astrid.* A subset of S2 progeny populations were again analysed for resistance to CMV infection, now using a concentrated inoculum of the highly virulent CMV strain ARN5 causing lethal necrosis in tomato. Only results obtained for S2 lines carrying homozygous transgenes as determined by Southern blot analysis or PCR, are presented in Fig. 5A. Although S2 progeny populations all showed reduced susceptibility to the necrogenic CMV strain, complete protection was only observed for transformant lines 1151 and 1152. Other transformant lines exhibited partial protection levels as exemplified by the S2 progeny derived from transformant line 1142. Within a population of 28 plants, all carrying one homozygous copy of the CMV CP gene, 14 plants resisted infection while the other 14 developed systemic symptoms, resulting in an intermediate protection level of 50%. Apparently, the intrinsic level of protection of such transformant lines is insufficient to fully withstand the relatively high infection pressure imposed by mechanical inoculation with the concentrated and highly virulent inoculum of the necrogenic CMV strain. However, when compared to the susceptible control, the development of systemic disease symptoms in such partially protected transformant lines is significantly delayed (Fig. 5B). Whereas the susceptible control reached 100% of diseased plants as soon as 12 days after inoculation, partially protected transformant lines remained completely free of systemic symptoms for at least 16 days, except for transformant line 1169 which comprised already a few infected plants at 12 days after infection.

Plants that resisted infection in their juvenile stages were maintained to produce fruits and monitored for disease development in later stages of their life cycle. Although some selected plants developed necrotic spots on their stems, typical for the necrogenic CMV strain, the vast majority of plants remained completely free of systemic disease symptoms and on the fruits symptoms were never observed. One example of resistant plants descending from transformant line 1152 is shown in Fig. 6.

Protection to CMV infection upon natural inoculation under field conditions.

In nature, CMV is transmitted by aphid vectors. To determine the level of protection upon natural infection, transgenic hybrids were planted in open field in Agadir, Morocco, and exposed to continuous inoculation by aphid vectors throughout their crop cycle. The Agadir region was chosen as a suitable location to conduct an open field trial, because of the high incidence of the CMV disease in the local culture of tomato over the past few years. The field trial comprised two replications of 14 or 15 hybrid plants that were planted in soil at their juvenile plant stage. At the end of the crop, when plants bore

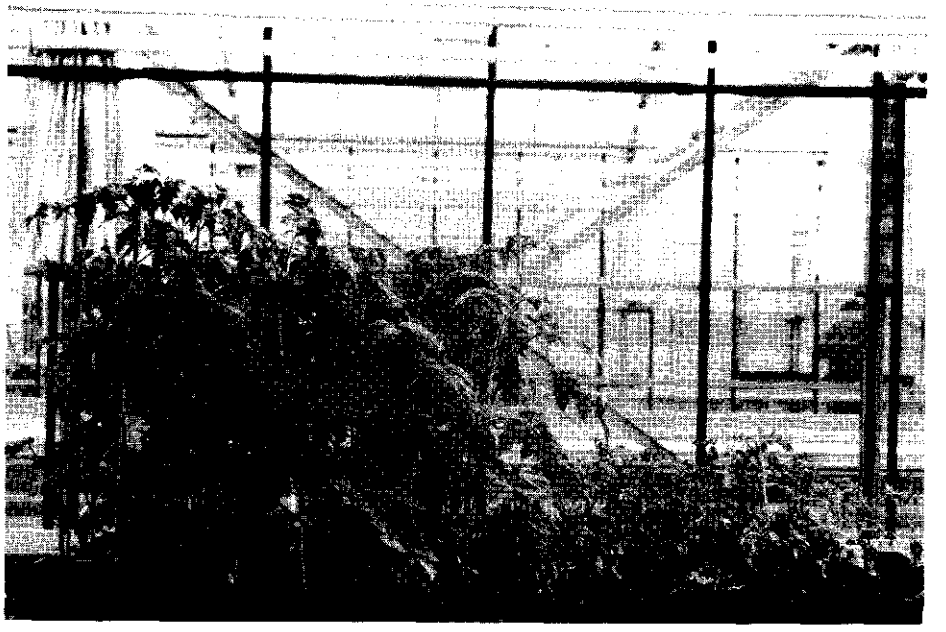

Fig. 6. Protection of the homozygous S2 progeny descending from transformant line 1151 to CMV infection upon mechanical inoculation with CMV strain I17F. Plants were photographed approximately four weeks after inoculation. At the left, transgenic tomato plants homozygous for the CMV CP gene, at the right non-transformed ATV847 acceptor plants.

several clusters of mature fruits, individual hybrid plants were carefully inspected for visual disease symptoms, not only on the vegetative plant parts, but also on the fruits. At that time, susceptible control plants consisting of non-transgenic *Astrid* hybrids, reached an average infection percentage of 73%, which illustrates the high incidence of the CMV disease in the Agadir region. Disease symptoms included the typical fern leaf syndrome, but also general leaf malformations such as wrinkling and curling, and occasional necrosis of top leaves. Affected fruits showed irregular surfaces with chlorotic and necrotic blotches that developed into soft rots at later stages of ripening. Typical symptoms incited by potato virus Y (PVY) infections, which are quite common for the Agadir region, were not observed in the transgenic field trial plot. Only plants devoid of any symptoms were scored healthy and results obtained for fixed hybrids are presented in Fig. 7. Transgenic hybrids, hemizygous for one or two linked copies of the CMV CP gene, all showed reduced susceptibility to CMV infections, while the hybrid descending from transformant line 1151 was even fully protected. This result thus demonstrates the successful protection of hemizygous tomato hybrids against natural infection by CMV.

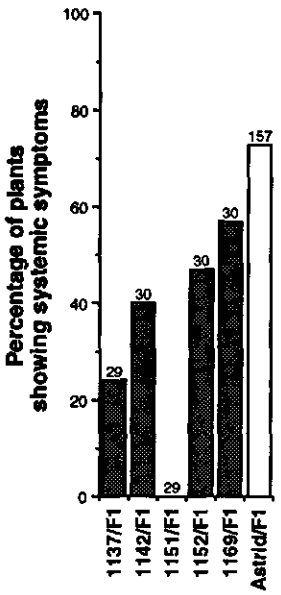

Fig. 7. Protection of hemizygous hybrids against CMV infection upon natural inoculation by aphid vectors under field conditions. Control plants (white bars) consisted of non-transformed *Astrid* hybrids. Plants were scored for systemic disease symptoms at the end of the crop cycle when plants bore several clusters of mature fruits. Only plants devoid of any disease symptoms on vegetative plants parts or on fruits were scored healthy.

Transformant line 1151 already showed full protection upon mechanical inoculation of its homozygous S2 progeny, as did transformant line 1152 (Fig. 5A). The hybrid derived from the latter transformant line, however, was not fully protected upon natural infection in open field, but was reduced in protection to an intermediate level of only 46%. The intrinsic level of protection of this transformant line appears to be too low for hemizygous hybrids to resist the challenge of continuous inoculation by the aphid vector in open field.

Protection of transformant lines to mechanical inoculation with CMV subgroup II.

The spectrum of protection conferred by expression of the CMV CP gene in transgenic tomato was further characterised by challenging transformant lines with a typical CMV subgroup II strain A. The amino acid sequence of the coat protein of this subgroup II strain shares 82% of identity with that of subgroup I strain ZU, from which the transgenically expressed CP gene was derived (Fig. 1A). Infection of tomato plants with subgroup II strain A incites rather mild disease symptoms. Top leaves of diseased plants exhibit a typical dark green mosaic and are distorted, but never as pronounced as the fern leaf syndrome typically observed for infections by subgroup I strain I17F. Upon mechanical inoculation of a subset of homozygous S2 populations, plants were monitored visually for disease symptoms. Although susceptible controls never reached infection percentages higher than 76%, the selected transformant lines showed a clear

reduction in the incidence of disease development ranging from high levels of protection for transformant lines 1137, 1142 and 1152, to an intermediate level of protection for transformant line 1169 (Fig. 8). Whether these levels of protection will hold upon more stringent inoculation conditions remains to be determined, but this result illustrates the rather broad spectrum of the engineered CMV protection conferred by expression of a subgroup I CP gene in tomato.

Discussion

In view of the common opinion to reduce the application of chemicals in modern agriculture, the identification and exploitation of genetic sources of resistance to viral pathogens is the ideal approach for controlling plant virus diseases in the long term. To this end, the introgression of naturally available resistance genes into crops by breeding, contributed largely to the development of virus resistant cultivars. Since the first report on the successful protection of transgenic plants expressing a viral CP gene against the homologous virus (Powell Abel *et al.*, 1986), the concept of CP-mediated protection has been widely adopted for protecting plants against viral infections (Beachy *et al.*, 1992). This transgenic approach thus offers a promising alternative in vegetable crops where suitable sources of natural resistance genes are lacking, as is the case for CMV resistance in *Lycopersicon* species. For cucumber and melon it was previously described that expression of the CMV CP gene confers protection to CMV infections (Yoshioka *et al.*, 1993; Gonsalves *et al.*, 1992; Gonsalves *et al.*, 1994). In this report we successfully demonstrate the use of a similar CP gene cassette to create tomato hybrids that are protected to CMV infections not only upon mechanical inoculation, but also upon natural inoculation by aphid vectors in open field.

The CMV CP gene was cloned from a typical subgroup I strain, assembled into a plant gene cassette, and subsequently introduced into the genome of a parental tomato line. When challenged by mechanical inoculation applying mild screening conditions, transformant lines accumulating CMV coat protein all showed reduced susceptibility to CMV infection. Out of six transformant lines that were pursued for further analysis, two transformant lines were identified that showed complete protection when their homozygous S2 progenies were challenged by mechanical inoculation with a virulent necrogenic CMV strain. Other transformant lines showed merely partial levels of protection, characterised by the fact that the inheritance and expression of the CMV CP does not necessarily confer resistance. However, partially protected lines still comprised

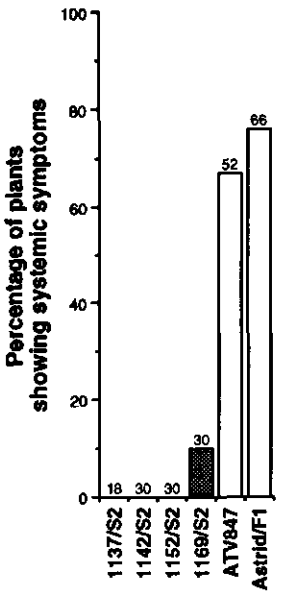

Fig. 8. Protection of homozygous S2 progenies to mechanical inoculation with subgroup II CMV strain A. Control plants (white bars) consisted of non-transformed ATV847 acceptor plants and *Astrid* hybrids derived therefrom. Figures on top of the bars refer to the number of tomato plants that have been scored for systemic symptoms in the final observation about four weeks after inoculation.

individual plants that completely resisted CMV infection and plants that did become infected were significantly delayed in disease development.

In the field, where disease pressure is exerted throughout the crop cycle by repeated inoculation from aphid vectors, levels of protection declined in hybrids carrying hemizygous copies of the CMV CP gene. The hybrid descending from transformant line 1151, however, remained fully protected, resisting infection throughout its complete crop cycle. Other hybrids showed merely partial protection levels, which is not unusual when plants heterozygous for dominant resistance genes are confronted with high disease pressures (Fraser, 1990). Apparently, the intrinsic levels of resistance in such partially protected transformant line are too low for hemizygous hybrids to fully resist the high disease pressure of repeated inoculation under field conditions. The occurrence of partial protection levels and gene dosage effects stresses the importance of screening a sufficiently large number of independent transformant lines in their hemizygous state, in order to identify suitable progenitors with high levels of CP-mediated protection to CMV. The aspect of resistance at the population level has not been taken into account in the current evaluation of the transgenic CMV protection. When resistant cultivars are planted in large numbers as commercial crops, the reduced number of infected plants will tend to lessen the disease pressure. Therefore, it is expected that levels of protection at the population level will increase, when transgenic tomato cultivars carrying the CMV CP gene are grown as commercial crops.

Recently, Anderson *et al.* (1992) reported that transformation of tobacco with a defective replicase gene derived from RNA 2 engendered resistance to CMV infection. Although this replicase-mediated resistance was claimed to be absolute and independent of the concentration of the challenge inoculum, resistance was overcome when plants were challenged with subgroup I strains that were more diverse in sequence than the strain from which the replicase gene was sequestered. Against subgroup II strains the replicase-derived transgene was totally ineffective (Zaitlin *et al.*, 1994; Hellwald & Palukaitis, 1994). Here, we demonstrate that the protection mediated by the transgenic expression of the CMV CP gene derived from a subgroup I CMV strain, does hold not only against subgroup I strains including virulent necrogenic strains, but also against a typical subgroup II strain. Consequently, it may reasonably be assumed that this synthetic CMV resistance gene will not easily be overcome by mutant CMV strains that carry point mutations in their CP gene, and thus represents a durable and reliable source of genetic resistance to CMV infections in tomato.

Other viruses that cause significant yield losses in the commercial culture of tomato are PVY and tomato spotted wilt virus (TSWV). Like in the case of CMV, suitable sources of natural resistance to limit the incidence of these viral diseases are lacking in *Lycopersicon* germplasms. However, pathogen-derived resistance genes have been described for both viruses (Gielen *et al.*, 1991; MacKenzie & Ellis, 1992; van der Vlugt *et al.*, 1992; Chapter 7 of this thesis), and in case of TSWV have already been applied in tomato (Ultzen *et al.*, 1995; Chapter 8 of this thesis). Since breeding for disease resistance is generally regarded as the best approach for sustainable crop protection, the combination of multiple pathogen-derived resistance genes within single tomato cultivars to combat plant viral diseases, represents a promising strategy towards the insecticide free culture of tomato.

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6 Transgenic resistance to zucchini yellow mosaic virus infections in melon (*Cucumis melo* L.)

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Abstract

The cultivation of melon is seriously affected by infection with a number of potyviruses, including zucchini yellow mosaic virus (ZYMV), causing significant yield losses. Since natural sources of resistance to ZYMV infections are only limitedly available, the development of engineered resistance, based on the transgenic expression of sequences derived from the viral coat protein (CP) gene, was explored. The transformation of melon with the ZYMV CP gene yielded a number of independent transformant lines that exhibited high levels of resistance when challenged with the virus. The observed resistance was shown to be largely mediated at the transcript level, as transformant lines carrying a translationally defective CP gene cassette displayed equal levels of resistance. Upon mechanical inoculation, protected melon transformants resisted viral infection and remained free of systemic symptoms. As such, transgenic melon lines carrying the ZYMV CP-derived resistance gene make excellent progenitors in breeding for ZYMV resistance.

Introduction

The Potiviridae constitutes the largest family of plant viruses and comprises a significant number of agronomically important members, including potato virus Y (PVY) and zucchini yellow mosaic virus (ZYMV). Potyviruses have flexuous rod-shaped particles that are transmitted by aphids in a non-persistent, non-circulative manner. The short acquisition and transmission period explains the limited success in controlling potyvirus infections through the application of insecticides.

The virion is composed of a single-stranded RNA of approximately 10 kb in length, encapsidated by the viral coat protein. The plus sense RNA genome possesses a 3' polyadenylate sequence and contains a single open reading frame that is expressed into one large polyprotein. The mature viral proteins are released from this initially expressed polyprotein by proteolytic processing. Any subgenomic RNAs are not produced during the replication cycle (Dougherty & Carrington, 1988; Riechmann *et al.*, 1992).

As potyviruses can have devastating effects on marketable crop yield, significant efforts have been devoted to limit the incidence of diseases incited by potyvirus infections. The use of virus resistant cultivars is the most effective way in this and traditional breeding has been successful in incorporating resistances to potyvirus infections into a number of commercially important crops (Provvidenti & Hampton, 1992). However, suitable sources of resistant germplasm are not always available and the continuous mutation of virus populations often renders resistance genes ineffective in time. The concept of pathogen-derived resistance offers the potential to expand the repertoire of resistance genes that can be deployed through breeding methods (Sanford & Johnston, 1985). To date, the still growing number of examples of pathogen-derived resistance to potyvirus infections illustrates the potential of this approach to create transgenic progenitors for potyvirus resistance (reviewed by Lindbo *et al.*, 1993a).

Cucurbit yields are often severely limited because of infection by three distinct potyviruses, i.e. the watermelon strain of papaya ringspot virus (PRSV-W), watermelon mosaic virus II (WMV-II) and ZYMV. Among these, ZYMV is a relatively new threat, but it has spread rapidly throughout the world since its first description in the late 1970s (Lisa *et al.*, 1981). The 3' terminal region of the ZYMV genome encoding the viral coat protein (CP) has been cloned and its nucleotide sequence determined for a number of different strains (Gal-On *et al.*, 1990; Grumet & Fang, 1990; Quemada *et al.*, 1990). Transgenic expression of the CP gene from ZYMV in muskmelon (*Cucumis melo*) conferred high levels of resistance to infection by the homologous virus (Fang & Grumet, 1993), while expression in transgenic tobacco (*Nicotiana tabacum* and *N. benthamiana*) conferred varying levels of protection against heterologous potyviruses

(Namba *et al.*, 1992). In this chapter we demonstrate the successful application of the ZYMV CP gene to generate resistance against ZYMV infection in transgenic melon. Transformation of melon with a translationally defective ZYMV CP gene, which is only expressed at the transcript level, generated equal levels of resistance, thereby indicating that the transgenic resistance is primarily RNA-mediated.

Materials and methods

Virus and plant material. ZYMV strain KB5, originating from Israel, was obtained from the 'Instituut voor Planteziektenkundig Onderzoek' (IPO-DLO) in Wageningen, the Netherlands. ZYMV strains E9 and E15 were isolated from infected melon plants in southern France. Strains were stored as desiccated leaf material kept at 4 °C in the presence of $CaCl_2$, or as fresh leaf material in liquid nitrogen. For preparation of virus inocula ZYMV strains were propagated in squash.
Melon genotype VIM3 was used as recipient in transformation experiments. This inbred melon line represents the market segment of the smooth 'Charentais' type melons, which have spherically shaped fruits with a slightly ribbed, but smooth surface. During maturation the colour of the fruits turns from greyish-green to yellow, while the fruit flesh turns orange. Transgenic melon plants were grown under certified greenhouse conditions according to the legislation imposed by the Dutch and French authorities, the 'Voorlopige Commissie Genetische Modificatie' (VCOGEM) and the 'Commission du Génie Biomoléculaire' (CGB), respectively.

Construction of plant transformation vectors. All manipulations involving DNA were essentially performed according to standard procedures (Ausubel *et al.*, 1987). The ZYMV coat protein (CP) gene including its 3' non-translated region was amplified by means of the polymerase chain reaction (PCR), following reverse transcription of total RNA samples prepared from squash leaves systemically infected with ZYMV strain KB5. Deproteinised RNA samples were isolated essentially as described by Logemann *et al.* (1987) by grinding 1 g of leaf material in 5 ml extraction buffer (8 M guanidine-HCl, 20 mM 2-[N-morpholino]ethanesulfonic acid (MES) pH 7.0, 20 mM EDTA, 50 mM beta-mercaptoethanol), followed by phenol extraction and ethanol precipitation. Reverse transcription and subsequent PCR amplification were performed using the *GeneAmp* RNA PCR Kit, according to the supplier's prescription (Perkin-Elmer Cetus). Oligomer primers that were used for reverse transcription and amplification were deduced from the nucleotide sequence from the Connecticut strain of ZYMV: EMBL accession number

D00692 (Grumet & Fang, 1990). Primer ZUP048 (5' ATGCTCCCCATGGGCACTCAG-CCAACTG 3') hybridised to the 5' end of the CP gene and introduced an ATG initiation codon immediately upstream of the CP gene, embedded in the recognition sequence of *Nco* I. Primer ZUP091 (5' CAGGTCGACATGGTGCACTTAGCCTAACT 3') also hybridised to the 5' end of the CP gene introducing an ATG initiation codon, but the open reading frame of the CP gene was distorted by the introduction of frame shift mutations (underlined nucleotides) causing abortive translation. Primer ZUP058 (5' TTTT-CTGCAGTTAGGCTTGCAAACGGAGTCT 3') is complementary to the ultimate 3' end of the ZYMV genome and introduces a *Pst* I site immediately downstream of the 3' untranslated region. Both PCR-amplified fragments of 1.1 kb were polished with T4 DNA polymerase and subsequently cloned into *Eco*R V linearised pBluescript. Upon their release by digestion with *Pst* I, both ZYMV CP genes were assembled into plant gene cassettes controlled by the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) polyadenylation signal. The resulting gene cassettes were released as *Bam*H I/*Kpn* I fragments and cloned into the binary plant transformation vector pBIN19 (Bevan, 1984), yielding transformation vectors pZU193 to pZU195 (the antisense orientation of the translationally defective CP gene cassette was omitted from further analysis). Upon their mobilisation into the non-oncogenic *Agrobacterium tumefaciens* strain GV3101(pMP90) (van Larebeke *et al.*, 1974; Koncz & Schell, 1986) by means of triparental mating using pRK2013 as a helper plasmid (Ditta *et al.*, 1980), recombinant *A. tumefaciens* strains were checked for the integrity of the transformation vectors by Southern blot analysis.

Transformation of inbred melon genotype VIM3. The *Agrobacterium*-mediated transformation of melon genotype VIM3 was essentially performed as described by Ben Tahar *et al.*, (1989), using cotyledons as explant material and kanamycin resistance as selectable marker, but using *Agrobacterium* strain GV3101(pMP90) in stead of strain LBA4404. Putative transgenic shoots were analysed for their ploidy level by means of flow cytometry. Intact nuclei were stained with 4',6-diamidino-2-phenylindol (DAPI) by chopping leaf tissue with a sharp razor blade in a commercial staining solution (Partec GmbH). Nuclei samples were filtered through nylon cloth (30 µm), kept on ice for at least 15 minutes and subsequently run through the flow cytometer (PAS-II, Partec GmbH) to determine their relative DNA-content (de Laat *et al.*, 1987). Diploid shoots were subjected to Southern blot analysis in order to identify true transformants, that were subsequently potted in rockwool and transferred to the greenhouse.

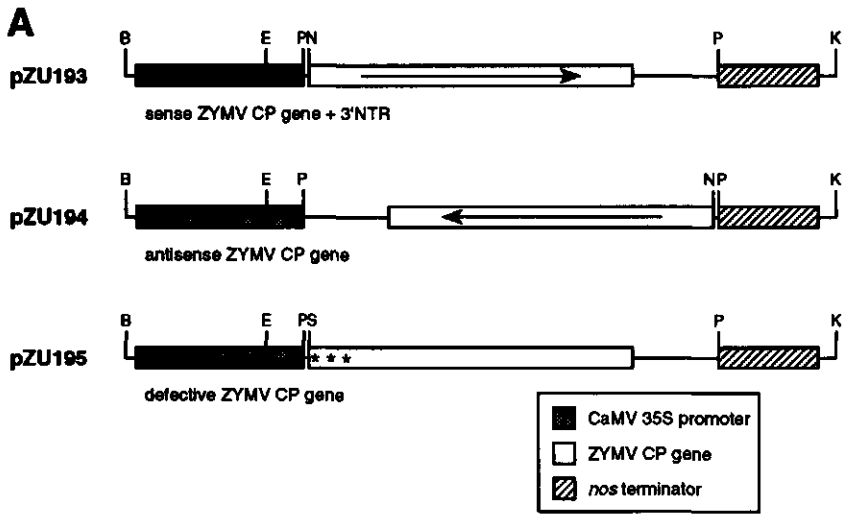
Southern blot analysis. Total DNA was extracted from melon plants essentially as described by Doyle & Doyle (1990), using an isolation buffer containing 2% hexadecyltrimethylammoniumbromide (CTAB). Portions of 10 µg DNA were digested with *Eco*R V, fractionated by electrophoresis in 0.8% agarose gels and transferred to Hybond-N membranes (Amersham) in alkaline transfer buffer employing a vacuum blotter apparatus, according to the supplier's instruction (Pharmacia). The blot membranes were subsequently hybridised to a ^{32}P-labelled DNA fragment containing the ZYMV CP gene in a SSC based hybridisation buffer containing 10% dextran sulphate (Wahl *et al.*, 1979).

Analysis of protection to ZYMV infection upon mechanical inoculation. Upon their emergence, approximately one week after sowing, seedling cotyledons were dusted with carborundum powder and wiped with cotton-wool dipped in virus inoculum. Inocula were freshly prepared by grinding 1 gram leaf material from systemically infected squash in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) supplemented with 1% Na_2SO_3 and kept on ice. After inoculation plants were rinsed with water. This inoculation procedure was repeated between one to two weeks later, now inoculating the first true leaf. The extent of the ZYMV infection was monitored by visual observation for the development of systemic symptoms. Susceptible melon plants developed systemic symptoms within 2 to 3 weeks after inoculation. Plants were scored susceptible when any leaf younger than the inoculated leaves showed typical systemic symptoms such as a yellow mosaic, leaf malformations or necrosis. At later stages of infection infected plants became stunted and reduced in height.

Results

Construction of plant gene cassettes for the ZYMV coat protein gene.

The ZYMV coat protein (CP) gene was amplified by means of the polymerase chain reaction following reverse transcription (RT-PCR) of deproteinised RNA samples prepared from squash plants systemically infected with ZYMV. The primer set used in the RT-PCR reaction hybridised to the protease cleavage site of the CP gene and to the extreme 3' end of the ZYMV genome, encompassing the entire CP gene plus the 3' non-translated region. Since potyvirus coat proteins are normally released from an initially expressed polyprotein by proteolytic processing, the primer hybridising to the 5' end of the ZYMV CP gene was designed to introduce an artificial ATG translation initiation codon immediately upstream of the open reading frame of the ZYMV CP gene (Fig. 1B).



B

```
                                      1   2   3   4   5   6
native ZYMV     ... ... CTC CAA TCA GGC ACT CAG CCA ACT GTG ... ...
                ... ... Leu Gln Ser Gly Thr Gln Pro Thr Val ... ...

pZU193          ........tgctccc ATG GGC ACT CAG CCA ACT GTG ... ...
                                Met Gly Thr Gln Pro Thr Val ... ...

                                    *       *       *
pZU195          ......caggtcgac ATG GTG CAC TTA GCC TAA CTGTG......
                                Met Val His Leu Ala OCH
```

Fig. 1. Schematic representation of the plant gene cassettes for the ZYMV CP gene. A: Chimaeric gene cassettes consist of the CaMV 35S promoter, the ZYMV CP gene along with its 3' untranslated region, and the *nos* terminator. Arrows indicate the orientation of the ZYMV CP gene, asterisks refer to the position of point mutations. The complete ZYMV CP gene cassettes were cloned into binary transformation vector pBIN19 as *Bam*H I-*Kpn* I fragments. B: *Bam*H I; E: *Eco*R V; K: *Kpn* I; N: *Nco* I; P: *Pst* I; S: *Sal* I. B: Nucleotide sequences of the ATG region of ZYMV CP gene cassettes pZU193 and pZU195, aligned to the native nucleotide sequence derived from the Connecticut strain of ZYMV (Grumet & Fang, 1990). Deduced amino acid sequences are shown underneath the nucleotide sequences and numbered starting from the first amino acid of the mature ZYMV coat protein. The open reading frame of CP gene cassette pZU195 is distorted through the introduction of three point mutations causing the abortive translation of the pZU195 transcript. Substitutions and additions of single nucleotides are indicated with an asterisk.

The ZYMV CP gene was subsequently cloned as a 1.1 kb *Pst* I fragment between the cauliflower mosaic virus (CaMV) 35S promoter and the polyadenylation signal derived from the 3' flanking region of the nopaline synthase (*nos*) gene. The sense and antisense orientation of the ZYMV CP gene yielded plant gene cassettes pZU193 and pZU194 respectively (Fig. 1A). A third gene cassette (pZU195) consisted of a derivative of the sense orientation of the CP gene, in which the open reading frame was distorted by the introduction of a frame shift mutation immediately downstream of the ATG initiation

codon (Fig. 1B). Consequently, gene cassette pZU195 is translationally defective and thus is only expressed at the mRNA-level. Upon introduction of the chimaeric gene cassettes into binary vector pBIN19, the resulting transformation vectors were transferred to *Agrobacterium tumefaciens* strain GV3101(pMP90) (Koncz &Schell, 1986).

Transformation of inbred melon genotype VIM3.

Transgenic melon plants were obtained by means of *Agrobacterium*-mediated transformation of cotyledon explants using genotype VIM3 as acceptor. This inbred melon line represents the market segment of the 'Charentais' type melons, and are characterised by their orange flesh and their smooth, slightly ribbed surface of their fruits. The transformation protocol was optimised to obtain workable frequencies of transformation for this melon genotype, using kanamycin resistance as selectable marker. About six weeks after co-cultivation, approximately 7% of the cotyledon explants gave rise to shoot primordia, that were cut from the explants for elongation and subsequent rooting. Rooted shoots were analysed for their ploidy level by means of flow cytometry. Approximately 70% of the shoots appeared to have retained the diploid ploidy level. Diploid shoots were subsequently analysed by Southern blot analysis to identify true transformants carrying the ZYMV CP gene cassettes (results not shown). In spite of the selection for kanamycin resistance during shoot regeneration, the majority of the shoots appeared to be escapes. On average, only 10% of the shoots was actually shown to be transformed. Because of this high escape rate, the effective transformation frequency, expressed as the percentage of explants yielding independent diploid transformants, dropped to 0.5%. Diploid transformants were potted in rockwool, transferred to the greenhouse and maintained to produce offspring by self-pollination. None of the transformants nor their progeny populations exhibited phenotypic aberrations that could be assigned to the insertion or the expression of the ZYMV CP gene cassette.

Protection of transgenic melon against ZYMV infections.

In order to identify protected transformant lines, melon seedlings from S1 progeny populations were challenged twice by mechanical inoculation using an inoculum prepared from squash systemically infected with ZYMV strain E15. This strain was isolated from a commercial melon crop in southern France and incites a typical yellow mosaic. At later stages of infection infected plants show general leaf malformations, become stunted and are reduced in height compared to mock inoculated plants. The final observation was made 3 weeks after inoculation when the susceptible control reached maximum infection levels (Fig. 2A). In spite of their segregating nature, four transformant lines were identified that showed reduced susceptibility to ZYMV infection,

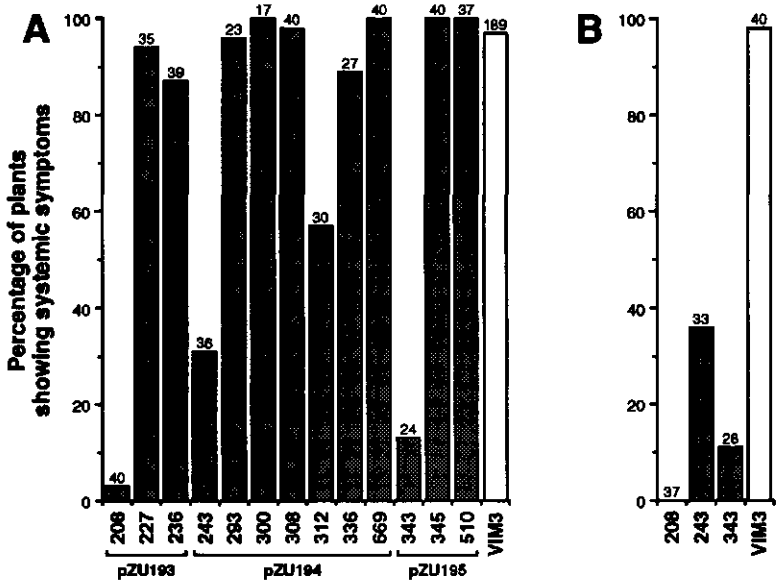


Fig. 2. Protection of segregating S1 progenies upon mechanical inoculation with ZYMV. Melon seedlings were challenged twice with an interval of approximately two weeks by mechanical inoculation with inocula prepared from young squash leaves systemically infected with ZYMV strain E15 (A) or strain E9 (B). Control plants (white bars) consisted of non-transformed VIM3 acceptor plants. Figures on top of the bars refer to the number of melon plants that have been scored for systemic symptoms in the final observation approximately 3 weeks after inoculation, including plants that did not inherit the ZYMV CP gene cassette through segregation.

amongst a total number of 13 S1 progeny populations analysed. While susceptible control plants normally developed a yellow mosaic, protected transformant lines (208, 243, 312 and 343) contained a significant percentage of plants that resisted infection and that remained free of systemic symptoms. Engineered resistance was not only observed for ZYMV CP gene cassette pZU193 (transformant lines 208 and 243), but also for gene cassettes pZU194 and pZU195 that are only expressed at the transcript level (Fig. 1). This result proves the involvement of the mRNA transcript in the mechanism underlying the engineered resistance. Protected melon plants were free of virus as demonstrated by ELISA using a commercial antiserum raised against purified ZYMV virions (data not shown). The absence of detectable amounts of virus suggests that protected plants are resistant rather than tolerant to ZYMV infection.

From each transformant line that resisted ZYMV infection, individual S1 progeny plants were subjected to Southern blot analysis to determine the copy number of the ZYMV CP gene cassettes. Total genomic DNA was digested with *Eco*R V to release border

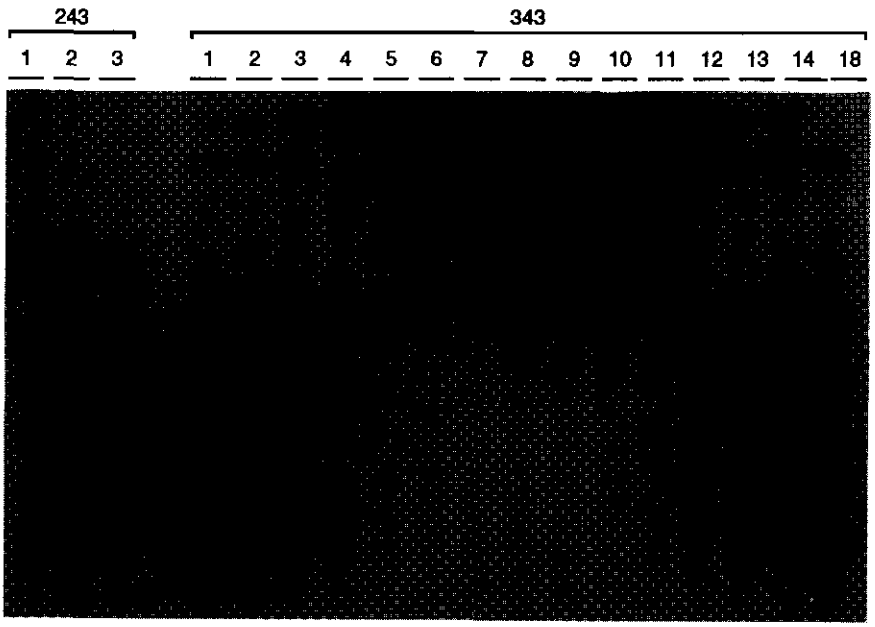


Fig. 3. Southern blot analysis of S1 progeny plants descending from transformant line 343 that resisted ZYMV infection upon mechanical inoculation with ZYMV strain E15. Total DNA was extracted from leaf tissue, digested with *Eco*R V, fractionated by agarose gel electrophoresis and blotted to Hybond-N membranes. Genome fragments comprising the ZYMV CP gene cassette were detected by hybridisation with a ^{32}P-labelled DNA fragment containing the ZYMV CP gene. The *Eco*R V digest releases a number of border fragments correlated with the copy number of the ZYMV CP gene cassette.

fragments comprising the ZYMV CP gene. The number of border fragments hybridising then correlates directly to the copy number of the transgene. In this way transformant lines 312 was shown to carry one single copy of the transgene, in contrast to transformant lines 208 that carried at least five copies distributed over at least three independent loci, as could be deduced from their segregation pattern (data not shown). Transformant line 243 appeared to carry two copies, probably linked at one chromosome. Southern blot analysis of transformant line 343 revealed the presence of at least three transgene copies, two of which residing on the same chromosome, as can be inferred from the fact that both copies did not segregate in 13 individual S1 progeny plants tested (Fig. 3). The third copy segregated independently and thus constitutes a second non-linked locus.

Except for transformant line 312, protected transformant lines were subsequently challenged with ZYMV strain E9. This highly virulent strain differs from E15 in that it generally provokes necrotic symptoms in melon cultivars carrying the *Fn* gene that

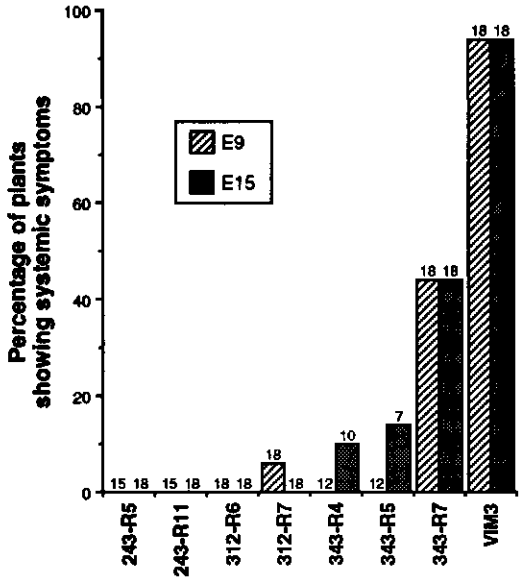


Fig. 4. Resistance to ZYMV infection of homozygous S2 progenies. Plants were challenged by mechanical inoculation with an inoculum prepared from young squash leaves systemically infected with ZYMV strain E9 or E15. After inoculation plants were inspected visually for the development of systemic disease symptoms. Control plants (white bars) consisted of non-transformed VIM3 acceptor plants. Figures on top of the bars refer to the number of melon plants that have been scored for systemic disease symptoms in the final observation about 3 weeks after inoculation.

renders melon resistant to *Fusarium* infections (Bruno Foncelle, personal observation). Acceptor genotype VIM3 also carries the *Fn* gene. Upon inoculation, plants were monitored for the development of necrosis of non-inoculated leaves (Fig. 2B). Transformant lines 208 and 343 both exhibited high levels of protection in that the majority of plants resisted infection. Transformant line 243, carrying a single copy and thus segregating 3 to 1, similarly featured a high level of protection. Out of 33 S1 plants challenged, 21 plants (i.e. 64%) withstood the highly virulent E9 isolate from systemic infection and subsequent necrosis development, which is in accordance with the segregation ratio expected for a single dominant gene.

Progeny plants that resisted infection with ZYMV strain E15 were maintained and self-pollinated to produce S2 offspring. Transformant line 208 was omitted from further analysis, because of its high copy number and its complex inheritance. For the same reason, only plants carrying one of either loci as present within transformant line 343 were selected to be proceeded to the next generation (Fig. 3: plant numbers R4, R5, R7, and R9). A subset of S2 progeny populations was again analysed for resistance to both

ZYMV strains. As shown in Fig. 4, fixed homozygous lines showing high levels of resistance to ZYMV resistance were readily identified amongst the S2 populations descending from transformant 243 and 312. Upon mechanical inoculation with strain E9, homozygous S2 populations fully resisted infection under the conditions employed. When challenged with strain E15, homozygous populations displayed an incomplete level of resistance as some transgenic plants developed systemic symptoms. In case of transformant line 343, only progeny populations carrying both linked copies (343-R4 and 343-R5) displayed significant levels of protection. The S2 progeny descending from 343-R7 carrying the single copy locus showed only marginal levels of protection. Apparently, the protection to ZYMV infection observed for the S1 progeny of transformant 343 largely relies on the expression of one or both of the linked transgene copies, the contribution from the single copy locus being minor.

Discussion

The cultivation of melon suffers appreciable losses from viral infections. The most prevalent virus comprise a number of potyviruses including ZYMV, for which suitable sources of natural resistance are lacking. In such cases the concept of pathogen-derived resistance employing the use of synthetic resistance genes offers a powerful alternative (Sanford & Johnston, 1985). In this respect, the transgenic expression of viral coat protein genes is well-known to confer protection against the homologous virus (Beachy *et al.*, 1992), and nowadays is widely exploited to protect crops from viral infection. Likewise, this chapter describes the successful exploitation of the ZYMV CP gene to engineer high levels of resistance to ZYMV infections in cultivated melon. The transformation of melon with the ZYMV CP gene was shown to yield transgenic lines with high levels of resistance to ZYMV infection when challenged by mechanical inoculation. Resistance was not only observed for gene cassette pZU193, which carries a functional derivative of the ZYMV CP gene that is potentially expressed at the protein level, but also for pZU194 and pZU195 that only express the ZYMV CP gene at the transcript level, in either sense or antisense orientation. Evidently, it is the transcript rather than the coat protein itself that constitutes the active entity underlying the observed resistance. This result confirms earlier reports illustrating the dominant role of the transgene transcript in establishing engineered potyvirus resistance (reviewed by Lindbo *et al.*, 1993a). Since any recognition events based on protein-protein interactions are not involved, the transgenic resistance will not easily be overcome by mutant ZYMV strains that carry point mutations in their CP gene. Hence, transgenic resistance genes based

upon the RNA expression of the ZYMV CP gene may reasonably be expected to provide durable and reliable sources of genetic resistance to ZYMV infections.

When challenged with the virus, transgenic lines carrying homozygous loci of the ZYMV CP gene displayed high levels of resistance, the majority of plants remaining free of systemic symptoms. Such extreme resistance levels suggest a mechanism other than antisense effects, as the latter generally generate merely moderate levels of protection. A more likely explanation for the observed resistance would be the induction of a virus-specific antiviral state as postulated by Dougherty and co-workers (Lindbo *et al.*, 1993b). This model proposes the existence of a cytoplasmic activity that targets transcripts that have accumulated to a critical threshold level for degradation (Smith *et al.*, 1994). Once activated, the sequence specific degradation of the overexpressed transcript would also cause the elimination of homologous viral sequences from which the transgenic transcript is derived. Consistent with this assumption, virus resistance was shown to be correlated with high transcription rates, but low steady state levels of the mRNA (Lindbo *et al.*, 1993b; Smith *et al.*, 1994). Whether this is the case for the ZYMV resistant melon lines needs further experimentation, which is beyond the scope of this study.

In addition to ZYMV, the commercial culture of melon is frequently affected by watermelon virus II (WMV-II) and papaya ringspot virus (PRSV), two other potyviruses that both cause significant yield losses. Since engineered resistance based on the transgenic expression of CP gene-derived sequences has now been described for a substantial number of potyviruses, including ZYMV (Fang & Grumet, 1993), PVY (van der Vlugt *et al.*, 1992) and tobacco etch virus (Lindbo & Dougherty, 1992a and 1992b), it is expected that the same strategy will confer resistance to these two potyviruses as well. The subsequent combination of such synthetic resistance genes through classical breeding then presents the next challenge on the way to the development of melon cultivars carrying multiple resistances to viral infections. Considering the current intent to reduce the utilisation of pesticides in modern agriculture, the subsequent integration and stacking of pathogen-derived resistance genes within vegetable breeding programs constitutes a highly attractive approach towards a more sustainable agriculture.

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7 Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus

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Abstract

For a still growing number of positive-strand RNA viruses, it has been demonstrated that transformation of host plants with viral sequences confers resistance to the corresponding virus. In this chapter it is demonstrated that high levels of genetically engineered resistance can be obtained to tomato spotted wilt virus, an enveloped virus with a negative-strand RNA genome, by transforming tobacco with the viral nucleoprotein (N) gene. Since a translationally deficient N gene cassette was shown to generate similar levels of resistance, the observed resistance appears to be primarily RNA-mediated, due to the expression of the N gene at the RNA level. Transgenic tobacco plants are only protected to isolates or strains of TSWV and not to other tospovirus species that share considerable nucleotide homology in their N genes to TSWV. In addition to being protected upon mechanical inoculation, transgenic tobacco plants are also resistant to inoculation using viruliferous thrips, i.e. *Frankliniella occidentalis* (Perg.), the most important natural vector species. As such, this approach based on the transgenic expression of the viral nucleoprotein might be applicable for creating plants resistant to infection by other negative-strand RNA viruses.

This chapter summarises two earlier publications: Gielen, J.J.L., P. de Haan, A.J. Kool, D. Peters, M.Q.J.M. van Grinsven & R.W. Goldbach, 1991. Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. *Bio/Technology* 9: 1363-1367; de Haan, P., J.J.L. Gielen, M. Prins, I.G. Wijkamp, A. van Schepen, D. Peters, M.Q.J.M. van Grinsven & R. Goldbach, 1992. Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. *Bio/Technology* 10:1133-1137.

Introduction

Tomato spotted wilt virus (TSWV) represents the type species of the genus *Tospovirus*, which is classified within the arthropod-borne Bunyaviridae, a large family of negative-strand RNA viruses (Elliot, 1990). Other species that have hitherto been recognised as members of this genus are impatiens necrotic spot virus (Law *et al.*, 1991), groundnut ringspot virus and tomato chlorotic spot virus (de Ávila *et al.*, 1993b), while recent studies indicated the existence of even more tospovirus species (Reddy *et al.*, 1992; Heinze *et al.*, 1995; Yeh & Chang, 1995). Among plant viruses tospoviruses are unique in their particle morphology and genome structure, and moreover, they represent the only plant viruses biologically transmitted by thrips (reviewed by German *et al.*, 1992). Virus particles are enveloped, spherically shaped (80-110 nm in diameter) and are studded with surface projections. The genome consists of three species of linear single stranded RNA, denoted S, M and L, that are individually wrapped with nucleoproteins (N) to form pseudo-circular nucleocapsids.

The TSWV S RNA segment is 2.9 kb long and encodes two proteins in an ambisense gene arrangement (de Haan *et al.*, 1990). The nucleoprotein (29 kD) is encoded by the N gene in viral complementary sense, whereas the non-structural (NSs) protein (52 kD) is encoded in viral sense (Fig. 1A). The M RNA comprises 4.8 kb and also exhibits an ambisense character encoding the putative viral movement (NSm) protein (33 kD) and two membrane glycoproteins, G1 (78 kD) and G2 (58 kD), which constitute the envelope spikes (Kormelink *et al.*, 1992). The L RNA is 8.9 kb in length and is completely of negative polarity. It encodes one primary translation product of 332 kD corresponding to the viral polymerase present in the virus particles (de Haan *et al.*, 1991; van Poelwijk *et al.*, 1993).

The world-wide distribution of TSWV, together with the current dramatic expansion of one of its major vectors, the Western Flower thrips (*Frankliniella occidentalis* Perg.), makes this virus one of the most harmful plant viruses (Goldbach & Peters, 1994). To date more than 400 plant species, both mono- and dicotyledons, have been reported to be susceptible to TSWV infection, and considerable yield losses have been reported in the cultivation of many important crops, including tomato, tobacco, lettuce, groundnut, pepper and ornamentals such as *Impatiens*, *Cyclamen* and *Chrysanthemum* (Cho *et al.*, 1986; Peters *et al.*, 1991). Mainly due to resistance of thrips species to insecticides, routine sanitary measures are no longer adequate for controlling the incidence of TSWV infections.

The cloning and characterisation of the TSWV genome allowed us to address the question whether engineered resistance to TSWV infections could be achieved by the

transgenic expression of viral sequences, the viral N gene in particular. The rationale behind this approach is the dual function of the nucleoprotein. As demonstrated for various negative-strand viruses (Ihara *et al.*, 1985; Beaton & Krug, 1986; Franze-Fernandez *et al.*, 1987; Vidal & Kolakofski, 1989), the nucleoprotein is not only involved in wrapping the viral RNAs to yield nucleocapsids, but also in regulating transcription and replication during the infection cycle. At early stages of infection, when the titer of free, unassembled nucleoprotein is low, the viral polymerase is active as a transcriptase, while at later stages of infection when the titer of the nucleoprotein increases, the viral polymerase is switched into the replication mode. Therefore, it is reasoned that the constitutive accumulation of the TSWV nucleoprotein in a susceptible host plant like tobacco might cause premature switching of the viral polymerase resulting in the abortive replication of incoming viral RNAs.

Materials and methods

Virus and plant material. TSWV isolate BR-01 was originally isolated from tomato in Brazil. The viral N gene used in transformation experiments was derived from this virus isolate (de Haan *et al.*, 1990). Two other tospoviruses, groundnut ringspot virus (GRSV) isolated from groundnut in South Africa, and tomato chlorotic spot virus (TCSV) isolated from tomato in Brazil, have been described previously (Ávila *et al.*, 1990; Ávila *et al.*, 1993a). Virus isolates were maintained in tomato by grafting, or in *Nicotiana rustica* var. America. *Nicotiana tabacum* var. SR1 was used as recipient in transformation experiments. Transgenic tobacco plants were grown under certified greenhouse conditions (PKII), according to the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM).

Construction of plant gene cassettes for the TSWV N gene. All manipulations involving DNA were essentially performed according to standard procedures (Ausubel *et al.*, 1987). A cDNA fragment containing the TSWV N gene, 124 nucleotides of its 5'-untranslated leader sequence and 6 nucleotides of its 3'-trailer sequence (de Haan *et al.*, 1990) was provided with *Pst* I linkers and subsequently cloned into the expression vector pZU139, yielding pTSWVN-A. Expression vector pZU139 contained the cauliflower mosaic virus (CaMV) 35S promoter sequences and the terminator sequences from the nopaline synthase (*nos*) gene, separated by the recognition sequence of *Pst* I. Chimaeric gene cassette pTSWVN-B essentially differs from pTSWVN-A in that the 5'-untranslated leader of the TSWV N gene was replaced by that from tobacco mosaic

virus. To this purpose the TSWV N gene was amplified by means of the polymerase chain reaction (PCR) from a cDNA clone containing the full-length N gene (de Haan *et al.*, 1990), using oligomer primers 1823B (5' GGGCTGCAGCTGCTT**TCA**AGCAAGTTC 3') and 1824 (5' TTACGATATC**ATG**TCTAAGG 3'). Primer 1824 hybridised to the ATG region of the N gene and introduced the recognition sequence of *Eco*R V immediately upstream of the ATG initiation codon; primer 1823B was complementary to the carboxy terminus of the N gene and introduced a *Pst* I site at 6 nucleotides downstream of the TGA stop codon. The PCR-amplified fragment of 0.8 kb was digested with *Eco*R V and *Pst* I and ligated into expression vector pZU029, yielding gene cassette pTSWVN-B. Expression vector pZU029 contained the CaMV 35S promoter fused to the 5'-untranslated leader sequence from tobacco mosaic virus, and the *nos* polyadenylation signal separated from the promoter fragment by the recognition sequence of *Sma* I and *Pst* I. The translationally defective gene cassette pTSWVN-C was obtained in analogy to pTSWVN-B, applying PCR-mediated mutagenesis using oligonucleotide primer 1823B in combination with oligonucleotide primer Jan030 (5' TACGATATC**CTG**TCTAGA-GGTT 3'). Within this latter primer the ATG translation initiation codon was mutated into CTG and the translational reading frame of the N gene disrupted through the introduction of a frame shift mutation (insertion of an additional G residue at position +5). Upon their assembly, chimaeric gene cassettes were cloned as *Xba* I fragments into binary transformation vector pBIN19 (Bevan, 1984).

Transformation of tobacco. The pBIN19 derived transformation vectors were mated into the non-oncogenic *A. tumefaciens* strain LBA4404 (Ooms *et al.*, 1981) by triparental mating using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). The resulting recombinant *A. tumefaciens* strains were checked for the integrity of the transformation vectors by Southern blot analysis. Transformation and regeneration of *in vitro*-grown *Nicotiana tabacum* var. SR1 was performed by the leaf disk method, essentially according to Horsch *et al.* (1985). Transgenic tobacco shoots, selected for resistance to kanamycin (100 µg/ml), were rooted, potted in soil and subsequently transferred to the greenhouse.

Serological analysis of transgenic tobacco plants. The amounts of the TSWV nucleoprotein accumulating in transgenic tobacco plants were quantified by double-antibody-sandwich (DAS) ELISA, using a rabbit polyclonal antiserum raised against purified TSWV nucleocapsids (Resende *et al.*, 1991). Protein samples were prepared by grinding leaf material in phosphate-buffered saline supplemented with 0.1% Tween-20 (PBS-T) and 2% insoluble polyvinylpolypyrrolidone, and incubated overnight at 4 °C in

microtiter plate wells (Nunc-Immuno Plate MaxiSorp™). The wells had previously been coated overnight at 4 °C with antiserum diluted to 1 µg/ml in coating buffer (50 mM sodium carbonate buffer pH9.6) and blocked with 1% BSA in PBS-T for 1 hour at room temperature. Bound antigen was detected by incubation with alkaline phosphate-conjugated antiserum (1µg/ml in PBS-T) for 3 hours at 37 °C, followed by para-nitrophenyl phosphate substrate development (1 mg/ml in 50 mM diethanolamine buffer pH9.8). The absorbance of each well was measured at 405 nm. Between all incubation steps the wells were thoroughly rinsed with PBS-T. Purified nucleoprotein was included as standard, while the total soluble protein content of leaf extracts was determined using the Bio-Rad protein assay.

The integrity of the TSWV nucleoprotein accumulating in transgenic tobacco plants was verified by Western blot analysis. Leaf tissue was homogenised in PBS-T and 25 µg of soluble protein was fractionated by electrophoresis in 12.5% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were blotted to Immobilon-P membranes (Millipore) by semi-dry blotting in semi-dry transfer buffer (29 mM glycine, 48 mM Tris, 0.0375% SDS and 20% methanol) for 1 hour at 0.8 mA/cm^2. Membranes were blocked for 3 hours at 37 °C in PBS-T containing 3% BSA, and subsequently incubated with polyclonal antiserum conjugated with alkaline phosphatase (Resende *et al.*, 1991), diluted to 1 µg/ml in PBS-T supplemented with 0.3% BSA. The immunoblot was further processed using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) as substrate according to the supplier's instruction (ImmunoSelect™, Life Technologies Inc.). Between subsequent treatments the membrane was washed with PBS-T containing 0.3% BSA.

Analysis of protection to TSWV and related tospoviruses. Prior to inoculation, progeny populations of self-pollinated tobacco transformants were analysed for the accumulation of TSWV nucleoprotein by DAS-ELISA, at least when carrying N gene cassettes pTSWVN-A or -B. Non-transgenic progeny plants which did not inherit the N gene cassette because of segregation, were used as controls. When in their two leaves stage, approximately 6 weeks after sowing, plants were dusted with carborundum and the largest leaf was inoculated with 25 µl inoculum, containing approximately 5-10 µg virus. Inocula were prepared by grinding 1 gram of systemically infected *Nicotiana rustica* leaves in 5 ml 0.1 M sodium phosphate buffer (pH7.0) supplemented with 0.01 M Na_2SO_3. Since TSWV and tospoviruses in general belong to the most unstable plant viruses known (Ie, 1970; Francki *et al.*, 1985), all inocula were prepared freshly and kept on ice. Transgenic plants were inoculated first, followed by the control plants. After inoculation, leaves were rinsed with water and plants were monitored regularly for the

development of systemic symptoms. Systemically infected leaves typically exhibited a yellow mosaic that later on developed into a severe necrosis that caused infected plants to die. Plants were scored susceptible when any leaf younger than the inoculated leaf showed systemic symptoms. Visually healthy plants were analysed for the presence of virus by ELISA using virus specific polyclonal antisera raised against purified nucleocapsids, or using monoclonal antibodies directed to the membrane glycoproteins (de Ávila *et al.*, 1990).

Inoculation using viruliferous thrips. Thrips cultures of *Frankliniella occidentalis* (Perg.) were maintained on bean pods (*Phaseolus vulgaris* L.) in modified Tashiro cages (Tashiro, 1967) at 27 °C and a photoperiod of 16 hours. Prior to each experiment, first-instar larvae (L1, 0-12 hours old) were allowed to feed on *Datura stramonium* (L.) plants infected with TSWV BR-01 for three days. The larvae were subsequently fed on healthy *Datura* plants that were changed regularly. Upon their emergence, adult thrips were individually tested for their infectivity on *Petunia hybrida* (L.) (Allen *et al.*, 1991). Viruliferous adults were transferred to 20 transgenic and 10 non-transgenic 4-weeks old tobacco seedlings (three thrips per plant). After an inoculation access period (IAP) of three days, the thrips were killed by spraying with a 10% solution of dichloorvos (Schering/Aagrunol), and tobacco plants were monitored daily for the development of local and systemic symptoms.

Results

Transformation of tobacco with the TSWV nucleoprotein gene.

Starting from a full-length cDNA clone of the TSWV nucleoprotein (N) gene, chimaeric gene cassette pTSWVN-A was constructed consisting of the cauliflower mosaic virus (CaMV) 35S promoter, the viral N gene with 124 nucleotides of its 5'-untranslated leader sequence, and the polyadenylation signal derived from the 3' flanking region of the nopaline synthase (*nos*) gene (Fig. 1B). In order to further enhance expression levels of the N gene, the 5'-leader region of the N gene was replaced by the 5'-untranslated leader from tobacco mosaic virus (TMV), which is known to function as a translational enhancer (Gallie *et al.*, 1987). To this purpose, the N gene was amplified from the cDNA clone harbouring the complete viral gene by means of PCR. The primers used in the amplification reaction carried appropriate restriction sites to facilitate the assembly of plant gene cassette pTSWVN-B, which comprises the 5'-untranslated leader sequence from tobacco mosaic virus (TMV) immediately downstream of the transcription

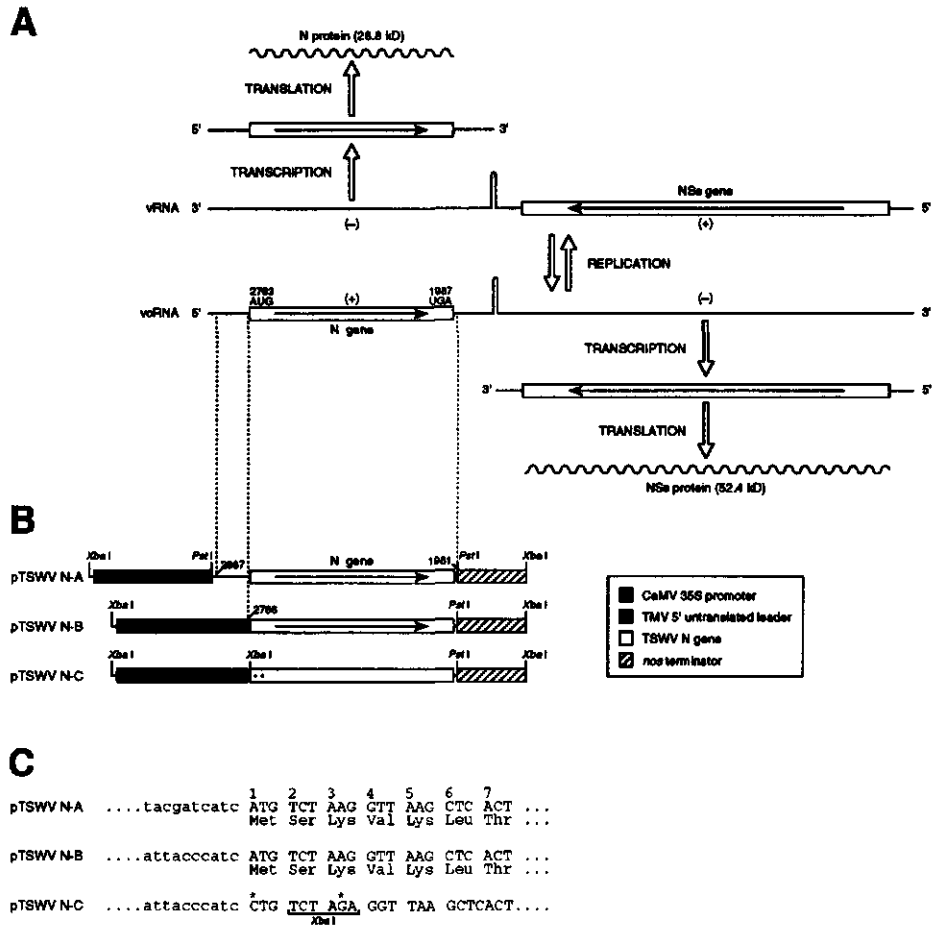

Fig. 1. Schematic representation of the structure of the ambisense TSWV S RNA (panel A), and of the chimaeric TSWV N gene cassettes (panel B). vRNA: viral RNA; vcRNA: viral complementary RNA. Sense and complementary sense regions of the vRNA and vcRNA strand of the ambisense S RNA are indicated with (+) and (-) respectively. The positions of the translation initiation and termination codon of the N gene are numbered from the 5' end of the vRNA (de Haan *et al.*, 1990). The point mutations within the nucleotide sequence surrounding the ATG initiation codon of the translationally defective gene cassette pTSWVN-C are shown below (panel C).

initiation site of the CaMV 35S promoter (Fig. 1B). A third chimaeric gene cassette, pTSWVN-C, was derived from pTSWVN-B by disrupting the translational reading frame of the N gene through site-directed mutagenesis, rendering this gene cassette translationally defective (Fig. 1C). Upon assembly, chimaeric gene cassettes were cloned into the binary transformation vector pBIN19 and subsequently transformed to *Nicotiana tabacum* var. SR1 by means of *A. tumefaciens*-mediated leaf disc transformation.

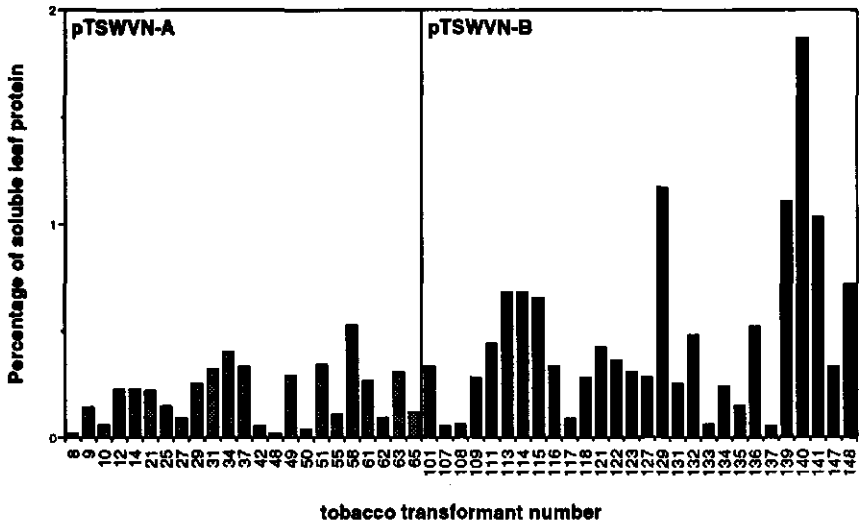

Fig. 2. Accumulation of the TSWV nucleoprotein in transgenic tobacco plants. Accumulation levels were quantified by DAS-ELISA using an antiserum raised against purified nucleocapsids, and presented as the percentage of the total soluble leaf protein content. Numbers refer to primary tobacco transformants.

Expression of the TSWV N gene in transgenic tobacco.

The expression of the TSWV N gene cassette in transgenic tobacco plants carrying functional gene cassette pTSWVN-A or pTSWVN-B was analysed and quantified by ELISA (Fig. 2). Between individual transformants, the accumulation levels of the nucleoprotein ranged from below detection level to 1.5% of the soluble leaf protein fraction. Such differences in the amount of nucleoprotein accumulating in independent transformants are probably due to position effects, originating from the modulation that the local genomic environment exerts on the expression of the transgene (Peach & Velten, 1991). Despite the inter-transformant variability, the average amount of nucleoprotein accumulating in pTSWVN-B transformants was approximately twice as high as in TSWVN-A transformants. The integrity of the nucleoprotein accumulating in transgenic plants was verified by Western blot analysis, showing that the transgenically expressed protein co-migrated with that extracted from tobacco plants systemically infected with TSWV (Fig. 3).

ELISA analysis of S1 progenies obtained by self-pollination of the primary transformants, revealed the segregation ratios of the active N gene cassettes from which the nucleoprotein is expressed. In most cases a segregation ration of 3 to 1 (expressor versus non-expressor) was obtained, indicating that the N gene cassette behaved as a single dominant gene. Within progeny populations, a strict correlation between the level

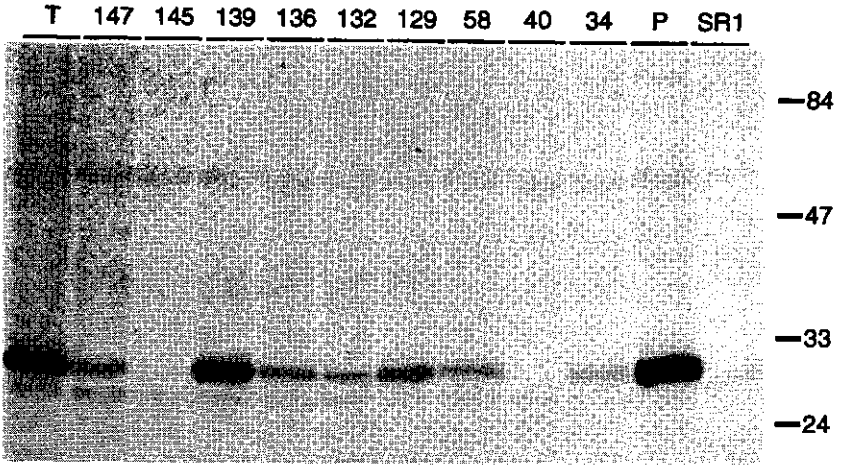

Fig. 3. Western blot analysis of tobacco transformants accumulating the TSWV nucleoprotein. Leaf protein samples were separated on 12.5% SDS-polyacrylamide gels, blotted to Immobilon-P membranes and the TSWV nucleoprotein detected using a polyclonal antiserum. Molecular weight markers are indicated on the right and numbers refer to primary tobacco transformants; T: tobacco systemically infected with TSWV; P: pepper systemically infected with TSWV; SR1: non-transgenic cceptor genotype.

of expression and the zygosity of the N gene cassette (homozygous or hemizygous) could not be observed. As anticipated, tobacco transformants carrying the translationally defective N gene cassette pTSWVN-C did not accumulate any nucleoprotein, at least not to detectable levels in DAS-ELISA. The expression of the defective N gene cassette at the transcript level, however, was confirmed by Northern blot analysis (data not shown).

Protection of transgenic tobacco against TSWV infections.

To evaluate the protection of transformant tobacco lines to TSWV infections, S1 progeny plants descending from 15 independent transformants accumulating the TSWV nucleoprotein were challenged by mechanical inoculation with the virus. Prior to inoculation, S1 progeny plants were analysed for the accumulation of the nucleoprotein to identify those individuals that inherited the N gene cassette. Segregant progeny plants that did not inherit the N gene cassette were used as susceptible controls. Over three separate experiments, 80 progeny plants of each selected line were inoculated with 5 to 10 µg virus and subsequently monitored for the appearance of systemic symptoms. Inocula were prepared from *Nicotiana rustica* leaves systemically infected with TSWV isolate BR-01. Observed levels of protection were arbitrarily classified into three categories of resistance: category A, over 60% of the progeny plants accumulating the

Table I. Resistance to TSWV infection upon mechanical inoculation of transgenic tobacco lines accumulating the TSWV nucleoprotein. Prior to mechanical inoculation, progeny plants were analysed for the accumulation of the transgenically expressed nucleoprotein. Non-transgenic progeny plants that did not inherit a copy of the TSWV N gene cassette functioned as susceptible controls and were omitted from the final scores of the transgenic progeny populations.

Gene construct	Transformant line	Accumulation level of the nucleoprotein[2]	Resistance category[1]
pTSWVN-A	12	0.3	A
	29	0.3	C
	61	0.3	C
	63	0.4	B
	58	0.6	C
pTSWVN-B	107	0.1	C
	108	0.1	C
	117	0.1	C
	109	0.3	C
	121	0.4	C
	115	0.7	C
	148	0.7	A
	141	1.0	C
	139	1.1	A
	129	1.2	B

[1]The accumulation levels of the transgenically expressed nucleoprotein are presented as percentages of total soluble leaf protein. [2]Category A, more than 60% of the plants accumulating the nucleoprotein resistant; category B, 20-60% of the transgenic plants resistant; category C, less than 20% of the plants resistant to TSWV infection.

TSWV nucleoprotein resistant to TSWV infection; category B, 20-60% resistant; category C, less than 20% resistant (Table I). Out of 15 transformant lines tested, 6 lines showed significant levels of protection to TSWV infection, in that they belonged to category A or B. A typical inoculation experiment is shown in Fig. 4, for transformant lines 129, 139 and 141. The initial transformants of these transformant lines accumulated equal amounts of the transgenically expressed nucleoprotein to roughly 1.0% of total soluble protein (Fig. 2). Their progeny populations, however, display different levels of resistance. Approximately 90% of the progeny plants descending from transformant line 139 (category A) resisted infection, while transformant line 141 (category C) merely showed a delay in systemic symptom development. Transformant line 129 (category B) displayed an intermediate level of resistance as only about 25% of the progeny plants accumulating nucleoprotein remained free of systemic symptoms. Evidently, a correlation between the observed levels of protection and the amount of nucleoprotein accumulating in transgenic tobacco plants could not be observed. When analysed by ELISA using monoclonal antibodies to the membrane glycoproteins of the virus (de

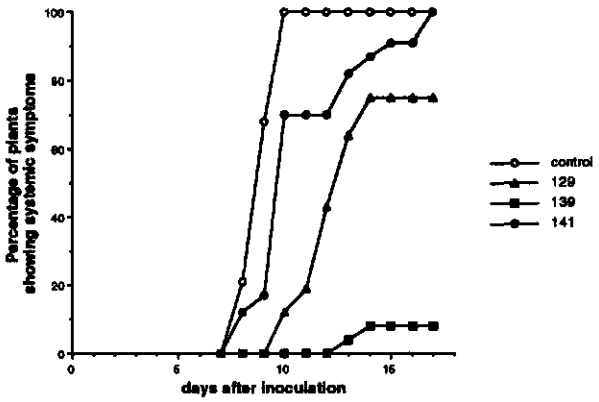

Fig. 4. Development of systemic disease symptoms in transgenic S1 progeny plants upon mechanical inoculation with TSWV. Non-transgenic progeny plants that did not inherit the TSWV N gene cassette because of segregation functioned as susceptible controls. Plants were inoculated with approximately 5-10 µg of virus and subsequently monitored for the appearance of systemic symptoms.

Ávila *et al.*, 1990), protected plants were shown to be completely free of virus, except for the few primary infection spots that appeared on the inoculated leaves.

Mechanical inoculation of S1 progeny populations descending from 23 independent pTSWVN-C transformants surprisingly revealed 4 lines that showed resistance to TSWV (Fig. 5). Within these S1 progeny populations segregating for the transgene, 30 to 80% of the plants resisted infection with TSWV and were shown to be free of virus when tested by DAS-ELISA. Other transformant lines were as susceptible as non-transformed control plants, reaching 100% infection within 10 days. In all, these results demonstrate the transgenic expression of the TSWV N gene to confer high levels of resistance to TSWV infection in tobacco. Yet, the engineered resistance is primarily mediated by the accumulation of the N gene transcript, rather than by the accumulation of the nucleoprotein itself.

Characterisation of resistance to TSWV infection.

Progeny plants that resisted TSWV infection were maintained and self-pollinated to produce S2 offspring. Fixed S2 progenies descending from primary transformations 12 and 129 carrying single homozygous copies of the N gene cassette were identified by ELISA. Challenging fixed homozygous progeny populations by mechanical inoculation revealed levels of resistance significantly increased compared to segregating populations, comprising hemizygous as well as homozygous individuals (Fig. 6). Levels of resistance in homozygous S2 progenies reached 100%, while non-fixed populations merely showed

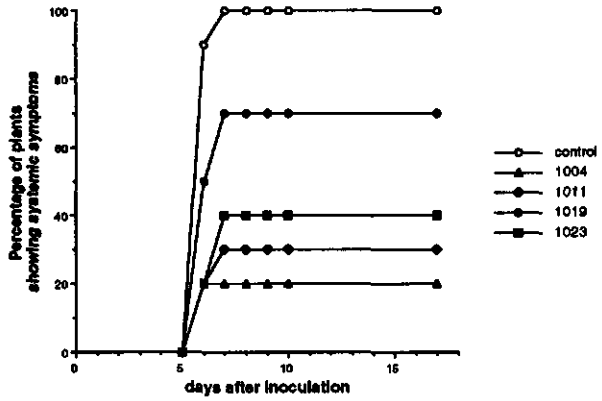

Fig. 5. Resistance to TSWV infection of S1 progeny populations segregating for the translationally defective gene cassette pTSWVN-C. For each transformant line twenty progeny plants were challenged by mechanical inoculation with approximately 5-10 µg of virus and subsequently monitored for the development of systemic symptoms. Control plants consisted of non-transformed SR1 tobacco plants.

intermediate levels of resistance (70% and 40% for transformant lines 12 and 129 respectively).

Recently, several laboratories described the occurrence of novel distinct tospoviruses (Law *et al.*, 1991; Reddy *et al.*, 1992; de Ávila *et al.*, 1993b; Heinze *et al.*, 1995; Yeh & Chang, 1995). Amongst these, tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV) have a host range similar to TSWV and share approximately 80% nucleotide sequence homology with the TSWV N gene (de Ávila *et al.*, 1993b). In order to evaluate the spectrum of resistance generated by the TSWV N gene cassette, homozygous S2 progenies descending from transformant lines 12 and 129 were challenged by mechanical inoculation with TCSV and GRSV (Fig. 7). Both viruses however, escaped from the protection engendered by the transgenic expression of the TSWV N gene, and transgenic tobacco plants normally developed systemic symptoms characteristic for both viruses.

Protection to TSWV infection upon inoculation by viruliferous thrips.

In nature, TSWV is obligatory transmitted by thrips species, of which *Frankliniella occidentalis* (Perg.) is the most important. To assess the engineered resistance upon thrips-mediated inoculation, transgenic tobacco plants were challenged by inoculation using viruliferous thrips. When three adult thrips were fed for three days on four-weeks old tobacco plants, typical feeding scars caused by mechanical damage of the leaf tissue were observed on all plants. Non-transgenic control plants became systemically infected

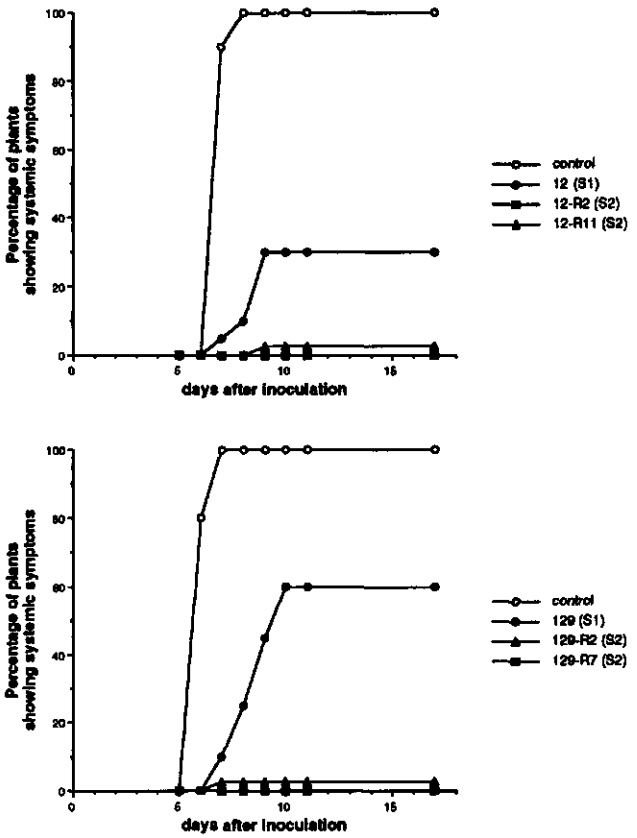

Fig. 6. Resistance to TSWV infection of fixed S2 progeny populations carrying single homozygous copies of the N gene cassette compared to segregating progeny populations comprising hemizygous as well as homozygous individuals. Plants were first analysed for the accumulation of the transgenically expressed nucleoprotein, and subsequently challenged by mechanical inoculation. Control plants consisted of non-transformed tobacco plants including progeny plants that did not inherit the N gene cassette through segregation.

within four to five days after inoculation. In contrast, homozygous transformant lines 12-R2 and 129-R7, both accumulating the transgenically expressed nucleoprotein, as well as line 1004-R2 accumulating the translationally defective mRNA transcript, all remained completely free of symptoms, except for the thrips feeding scars (Fig. 8). Evidently, the transgenic expression of the N gene not only confers resistance upon mechanical inoculation with virus, but also when challenged by inoculation with the thrips vector.

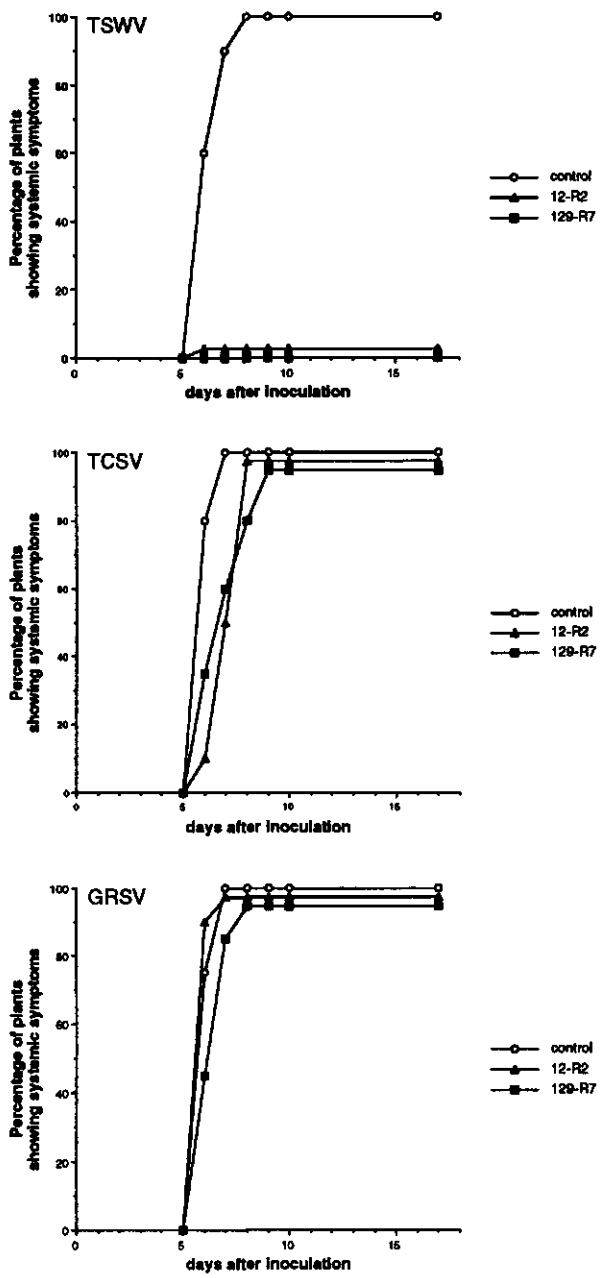

Fig. 7. Development of systemic symptoms in homozygous transformant lines upon mechanical inoculation with TSWV and related tospoviruses TCSV and GRSV. Transformant plants were challenged by mechanical inoculation with an inoculum of approximately 5-10 µg virus prepared from *Nicotiana rustica* plants systemically infected with TSWV, TCSV or GRSV. Control plants consisted of non-transformed SR1 tobacco plants.

Discussion

The transformation of tobacco with the TSWV N gene provides the first example of genetically engineered resistance to a negative-strand RNA virus. Upon mechanical inoculation, protected plants resisted infection and remained free of systemic symptoms, while those plants that did become infected showed a delay in systemic symptom development. When the transgene was fixed in homozygous progeny populations, levels of engineered resistance further increased, reaching virtual immunity as diseased plants could no longer be observed (100% resistance). As importantly, the engineered resistance was shown to be maintained when transgenic tobacco plants were challenged by inoculation using viruliferous thrips. In transgenic tobacco plants that resisted infection the presence of virus could not be demonstrated, except for the primary infection spots on the inoculated leaves.

The transgenically expressed nucleoprotein was shown to accumulate up to exceptionally high levels of approximately 1.5% of the total soluble leaf protein, without having any deleterious effects on phenotype or fertility of the tobacco transformants. In spite of such high accumulation levels, the engineered resistance is considered to be primarily mediated at the RNA level, since the transgenic expression of a translationally defective gene cassette of the TSWV N gene generated similar levels of resistance. Evidently, it is the N gene transcript rather than its translation product that represents the active entity underlying the engineered resistance.

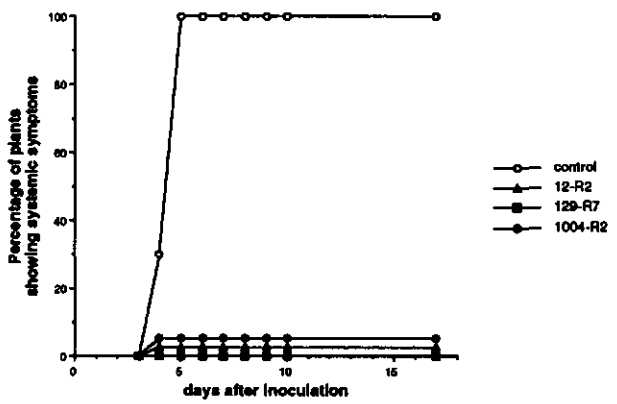

Fig. 8. Resistance of homozygous S2 progeny populations to TSWV infection upon inoculation using viruliferous thrips. First-instar larvae were fed on *Datura stramonium* (L.) plants infected with TSWV and subsequently maintained on several changes of healthy *Datura* plants until they reached the adult stage. Individual tobacco plants were challenged with the virus by inoculation with three adult thrips, that were allowed to feed on the tobacco plants for three days. Control plants consisted of non-transformed SR1 tobacco plants.

The N gene transcript as expressed in transgenic tobacco plants is of antigenomic polarity and might therefore exhibit antisense activity, blocking virus replication and multiplication. Alternatively, or additionally, N gene transcripts may compete for viral and/or host-encoded replication factors. Engineered resistance mediated by the accumulation of mRNA transcripts encoding structural viral proteins has been reported for potyviruses as well, including tobacco etch viruses (Lindbo & Dougherty, 1992), potato virus Y (van der Vlugt *et al.*, 1992) and zucchini yellow mosaic virus (Fang & Grumet, 1993; Chapter 6 of this thesis). The high levels of protection as generally observed for engineered resistances mediated at the transcript level, however, suggest a mechanism other than RNA-RNA interactions or competition effects. In this light, the existence of an virus-specific antiviral state as postulated by Dougherty and co-workers seems to provide a more plausible explanation (Lindbo *et al.*, 1993). As this model implies the accelerated break down of RNA sequences that surpass a critical threshold value for maximum accumulation, the overexpression of the N gene transcript then coincides with the immediate degradation of incoming viral sequences, yielding the virus resistant phenotype (Smith *et al.*, 1994).

Since the engineered resistance to TSWV infection is RNA-mediated, a certain minimum level of nucleotide sequence homology between the transgenically expressed N gene and that of the invading virus is required in order to obtain resistance. Accordingly, the TSWV N gene, which shares approximately 80% nucleotide sequence homology with the N gene from TCSV and GRSV (de Ávila *et al.*, 1993b), was unable to confer protection to these related tospoviruses. However, the N gene of TSWV was effective in protecting against different strains or isolates of TSWV with much less heterogeneity in their N gene sequences. The requirement for a minimal level of sequence homology higher than 80% has of course practical consequences if one aims at N gene-mediated resistance to multiple tospoviruses. In analogy to TSWV, however, it may reasonably be expected that the transgenic expression of N-gene derived sequences from other tospoviruses will likewise generate high levels of engineered resistance.

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8 Resistance to tomato spotted wilt virus in transgenic tomato hybrids

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Abstract

Tomato spotted wilt virus (TSWV) infections cause significant economic losses in the commercial culture of tomato (*Lycopersicon esculentum* Mill.). Culture practices and introgression of natural sources of resistance to TSWV have only been marginally effective in controlling the TSWV disease. Recently however, high levels of protection against TSWV have been obtained by transforming tobacco with a chimaeric gene cassette comprising the TSWV nucleoprotein gene (Chapter 7 of this thesis). This chapter demonstrates the successful application of this newly created TSWV resistance gene in cultivated tomato. Transformation of an inbred tomato line with the TSWV nucleoprotein gene cassette resulted in high levels of resistance to TSWV infections that were maintained in transgenic hybrids challenged with the virus by natural infection in open field. Therefore, transformant lines carrying the synthetic TSWV resistance gene make suitable progenitors for TSWV resistance to be incorporated into the breeding programs of tomato.

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Introduction

The commercial culture of tomato (*Lycopersicon esculentum* Mill.) is seriously affected by tomato spotted wilt virus (TSWV) infections causing significant yield losses. Characteristic symptoms associated with TSWV infections include plant stunting, bronzing or chlorosis of leaves, and the development of chlorotic or necrotic ringspots on the fruits. The virus is naturally transmitted by a number of thrips species, of which the Western flower thrips (*Frankliniella occidentalis* Perg.) is the most important. The dramatic expansion of *F. occidentalis* from North-America over the western hemisphere and its rapid acquisition of resistance to pesticides are the major reasons for recent outbreaks of the TSWV disease, not only in tomato but also in other crops such as pepper and lettuce (Goldbach & Peters, 1994).

Among plant viruses TSWV is unique in its genomic organisation and its particle morphology. The virus particle is bounded by a spherical shaped lipoprotein envelope enclosing a core of nucleocapsids, consisting of three genomic RNA segments that are individually associated with nucleoprotein, and that exhibit either negative or ambisense gene arrangements (Fig. 1) (de Haan *et al.*, 1990; de Haan *et al.*, 1991; Kormelink *et al.*, 1992). On the basis of these properties, TSWV has been classified as the type member of the genus *Tospovirus*, the only genus within the Bunyaviridae family that infects plants (for an overview on tospoviruses, see German *et al.*, 1992). Over the past few years, a number of distinct virus species has been identified and classified as novel tospoviruses (Law *et al.*, 1991; Reddy *et al.*, 1992; de Ávila *et al.*, 1993; Heinze *et al.*, 1995; Yeh & Chang, 1995) and it is expected that the number of tospoviruses will further increase in the near future.

Culture practices such as rotation, control of the thrips vector and removal of alternate weed hosts have only been marginally effective in the management of the TSWV disease (Cho *et al.*, 1989). Consequently, host plant resistance to the virus is the most promising means of controlling the disease in the long term. Several accessions of *Lycopersicon* germplasm and tomato cultivars descending from such accessions have been reported to be resistant to TSWV (Smith, 1944; Finlay *et al.*, 1952; Finlay *et al.*, 1953; Paterson *et al.*, 1989; Kumar *et al.*, 1993). The inheritance of a source of resistance to TSWV derived from *L. peruvianum* Mill. was found to behave as a single dominant gene (*Sw-5*) (Stevens *et al.*, 1992; Boiteux & Giordano, 1993). However, in the field, plants carrying the *Sw-5* gene frequently still accumulate virus resulting in the development of disease symptoms on the fruits (personal observation), which renders the utilisation of this source questionable. The fact that tomato cultivars carrying a reliable source of genetic resistance to TSWV have still not reached the market illustrates the limited applicability

or the complex inheritance of resistance sources identified thus far. Moreover, the emergence of new tospoviruses that infect tomato constitutes a serious threat and urges the need for identification or development of new sources of resistance to be incorporated into tomato breeding programs, especially when considering proposed reductions in the application of insecticides for reasons of environment protection.

Over the past decade numerous publications have demonstrated the successful generation of virus resistance through transgenic expression of viral sequences in plants (reviewed by Hull & Davies, 1992; Scholthof *et al.*, 1993; Wilson, 1993). Transformation of tobacco with the TSWV nucleoprotein gene (N gene) has likewise been shown to result in resistance against TSWV infections (Gielen *et al.*, 1991; MacKenzie & Ellis, 1992; Pang *et al.*, 1992; Chapter 7 of this thesis). Expression of a translationally defective N gene cassette generated similar levels of resistance, which indicates that the accumulation of nucleoprotein is not required to obtain TSWV resistance and that the observed resistance is primarily RNA-mediated (de Haan *et al.*, 1992; Chapter 7 of this thesis). To study the application of this technology in crops of agronomic importance, the TSWV N gene cassette was transformed to an inbred tomato line used in the production of fresh market hybrids. Upon mechanical inoculation resistant transformant lines were identified which were then cross-pollinated to produce experimental hybrids. The transgenic TSWV resistance is successfully maintained in the hybrid when challenged by thrips-mediated infection under field conditions, thereby indicating that transgenic parental lines can serve as progenitors for TSWV resistance in tomato breeding programs.

Materials and methods

Virus and plant material. TSWV isolate BR-01, originally isolated from tomato in Brazil, was maintained in tomato by grafting to prevent the generation of defective mutants by repeated mechanical passages (Resende *et al.*, 1991a). The tobacco (*Nicotiana tabacum* cv. Xanthi) cell suspension used in the transformation procedure of tomato was grown in the dark at 26 °C on a shaking platform and maintained through weekly subculturing in Xanthi medium: MS medium (Murashige & Skoog, 1962), supplemented with 30 g/l sucrose, 100 mg/l inositol, 200 mg/l KH_2PO_4, 1.3 mg/l thiamine, 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/l kinetin. Parental tomato line ATV847 was used as recipient in transformation experiments. This inbred line is used as male parent in the production of a number of hybrids for the South European market, that represent fresh market tomatoes of the indeterminant type. Transgenic tomato plants were grown under certified greenhouse conditions according to

the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM).

Construction of the plant transformation vector. All manipulations involving DNA were essentially performed according to standard procedures (Ausubel *et al.*, 1987). The construction of plant gene cassette pTSWVN-B carrying the TSWV N gene has already been described in Chapter 7. Upon its assembly the complete gene cassette was released as a *Xba* I fragment and cloned into the binary plant transformation vector pBIN19 (Bevan, 1984). The resulting transformation vector was subsequently introduced into the non-oncogenic *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.*, 1981) by triparental mating using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). The recombinant *A. tumefaciens* strain was checked for the integrity of the transformation vector by Southern blot analysis.

Transformation of parental tomato line ATV847. The transformation method described hereinafter is based on the application of tobacco feeder layer cells during preculture and cocultivation of the cotyledon explants (Shahin *et al.*, 1986; Fillatti *et al.*, 1987; Yoder *et al.*, 1988). Thin layers of a tobacco Xanthi cell suspension were poured onto petri dishes containing Xanthi medium solidified with 10 g/l micro agar (Duchefa, Haarlem). The feeder plates were incubated at 26 °C in the dark for 24 hrs. Directly before use, a sterile Whatman filter was placed on top of the feeder cells. Cotyledon explants were prepared from 8 to 10 days old *in vitro* grown seedlings of parental tomato line ATV847. The cotyledons were cut across the base and top to provide two cut surfaces for infection. The resulting explants were subsequently placed on the feeder plates with the abaxial side up and precultured at 24 °C in the dark for 24 hours. An overnight culture of the recombinant *Agrobacterium* strain carrying transformation vector pTSWVN-B was diluted in liquid MS medium containing 30 g/l sucrose to a density of $5x10^7$ cells/ml. The explants were incubated for 5 to 15 minutes in the *Agrobacterium* suspension, dried on a sterile Whatman filter and placed back onto the feeder plates. After 48 hours of cocultivation in the dark at 26 °C, the explants were transferred to selection medium (MS medium, 10 g/l sucrose, 10 g/l glucose, 2.0 mg/l zeatin, 0.02 mg/l IAA, 250 mg/l carbenicillin, 100 mg/l kanamycin, 10 g/l micro-agar) with the axial side up. After 5 days in the dark, plates were transferred to the light (1500-2000 Lux) and explants were subcultured every 2 weeks. From 4 to 8 weeks after cocultivation, shoot primordia were cut from the explants and elongated on MS10 medium (MS medium, 10 g/l sucrose, 250 mg/l carbenicillin, 10 g/l micro-agar). Shoots were rooted on MS10 medium supplemented with 25 mg/l kanamycin, potted in soil and transferred to the greenhouse.

Serological analysis of transgenic tomato plants. The accumulation of transgene-derived TSWV nucleoprotein in transgenic tomato was detected by means of double-antibody-sandwich (DAS) ELISA or Western blot analysis essentially as described in Chapter 7, using a rabbit polyclonal antiserum raised against purified TSWV nucleocapsids (Resende *et al.*, 1991b).

Flow cytometric analysis of ploidy levels. Tomato transformants accumulating TSWV nucleoprotein were analysed for their ploidy level by flow cytometry. Intact nuclei were stained with 4',6-diamidino-2-phenylindol (DAPI) by chopping leaf tissue with a sharp razor blade in a commercial staining solution (Partec GmbH). Nuclei samples were filtered through nylon cloth (30 µm), kept on ice for at least 15 minutes and subsequently run through the flow cytometer (PAS-II, Partec GmbH) to determine their relative DNA-content (de Laat *et al.*, 1987). Nuclei prepared from leaf tissue of diploid broccoli were used as internal standard.

Southern blot analysis. Total DNA was extracted from transgenic tomato plants essentially as described by Doyle & Doyle (1990), using an isolation buffer containing 2% hexadecyltrimethylammoniumbromide (CTAB). Portions of 10 µg DNA were digested with *Eco*R V, *Hin*d III or *Xba* I, fractionated by electrophoresis in 0.8% agarose gels and transferred to Hybond-N membranes (Amersham) by capillary blotting in alkaline transfer buffer (Ausubel *et al.*, 1987). The blot membranes were subsequently hybridised to a ^{32}P-labelled DNA fragment containing the TSWV N gene in a SSC based hybridisation buffer containing 10% dextran sulphate (Wahl *et al.*, 1979).

Analysis of protection to TSWV infection after mechanical inoculation. Prior to inoculation offspring populations were analysed for the accumulation of nucleoprotein by DAS-ELISA to identify those progeny plants expressing the N gene cassette. After emergence of the first leaf, about 3 to 4 weeks after sowing, seedlings were dusted with carborundum powder and wiped with cotton-wool dipped in the virus inoculum. Since TSWV is highly unstable upon homogenisation, the inocula were freshly prepared by grinding 1 gram of systemically infected tomato leaves in 10 ml of 0.1 M sodium phosphate buffer (pH7.0) supplemented with 1% Na_2SO_3 and kept on ice. Transgenic plants were inoculated first, followed by non-transformed control plants to check the inocula for their infectivity at the end of inoculation. All accessions were organised in a randomised block design with five or six replications. One week after the first inoculation the tomato plants were inoculated for a second time to achieve maximum disease incidence. After inoculation plants were rinsed with water. The extent of the

TSWV infection was monitored by visual observation for the development of systemic symptoms. Plants with aberrant phenotypes were omitted from the notations. Normally, susceptible tomato plants develop systemic symptoms within 2 to 4 weeks after mechanical inoculation with TSWV. Plants were scored as being susceptible when any leaf younger than the inoculated leaves showed typical systemic symptoms such as chlorosis or bronzing of the leaves. In addition plant stunting and wrinkling or curling of the top leaves could be observed on systemically infected plants. The absence of virus in symptomless transgenic plants was checked by direct ELISA using a polyclonal antiserum raised against purified NSs protein, a nonstructural viral protein that accumulates to high levels in TSWV infected plant cells (Kormelink *et al.*, 1991).

Analysis of protection in transgenic hybrids upon natural inoculation in open field. In the summer of 1994, an open field trial was executed on the premises of S&G Semillas in El Ejido, Spain, conformable to the legislation and regulations imposed by the Spanish authorities. Early April, when plants were approximately 20 cm in height, transgenic *Astrid* hybrids were planted in soil in netted greenhouses, organised in four replications of 10 to 12 plants each. Non-transgenic hybrids were included as susceptible control and plants were exposed to thrips-mediated inoculation throughout their crop cycle. The trial was managed like a normal tomato crop, except that the chemical control of thrips and white flies was delayed until susceptible controls reached a TSWV infection percentage of 85%. At the end of the trial (end of June 1994), when bearing several clusters of fruits, plants were scored visually for the presence of disease symptoms incited by TSWV infections. Only plants that were completely free of any symptoms were scored healthy.

Results

Construction of the TSWV nucleoprotein gene cassette.

The TSWV nucleoprotein (N) gene was amplified using PCR from a cDNA clone harbouring the complete viral gene. The primers used in the amplification reaction carried appropriate restriction sites to facilitate the cloning of the N gene into expression vector pZU029 (Fig. 1). Since the N gene was obtained by PCR-amplification the cloned fragment was sequenced to exclude the possibility of mutations generated by the *Taq* polymerase. The resulting gene cassette, pTSWVN-B, comprises the CaMV 35S promoter, the viral N gene and the polyadenylation signal derived from the 3' flanking region of the nopaline synthase (*nos*) gene. The CaMV promoter was modified by fusing the 5'-untranslated leader sequence from tobacco mosaic virus (TMV) immediately

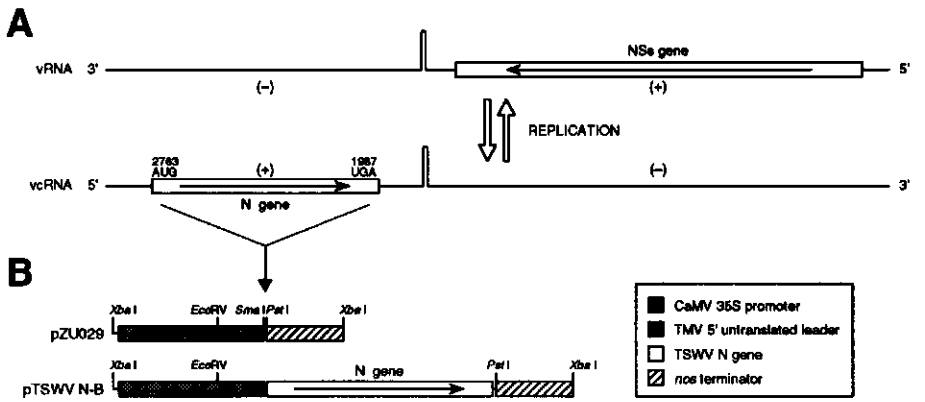

Fig. 1. Schematic representation of the small genomic RNA (S RNA) of TSWV and of the construction of the chimaeric TSWV N gene cassette. vRNA: viral RNA; vcRNA: viral complementary RNA. Sense and complementary sense regions of the vRNA and vcRNA strand of the ambisense S RNA are indicated with (+) and (-) respectively. The complete N gene cassette was cloned into binary transformation vector pBIN19 as a *Xba* I fragment. E: *Eco*R V; P: *Pst* I; S: *Sma* I; X: *Xba* I.

downstream of the transcription initiation site. The TMV leader is known to function as a translational enhancer (Gallie *et al.*, 1987). The chimaeric gene cassette was subsequently cloned into the binary transformation vector pBIN19 as a *Xba* I fragment and transferred to *Agrobacterium tumefaciens* strain LBA4404. In tobacco, the N gene cassette pTSWVN-B already proved to confer TSWV resistance, not only upon mechanical inoculation but also upon inoculation using viruliferous thrips (Gielen *et al.*, 1991; de Haan *et al.*, 1992; Chapter 7 of this thesis).

Transformation of parental tomato line ATV847.

Transgenic tomato plants were obtained by means of *Agrobacterium*-mediated leaf disc transformation (Shahin *et al.*, 1986; Fillatti *et al.*, 1987; Yoder *et al.*, 1988), using genotype ATV847 as acceptor. This inbred tomato line is used as male parent in the production of a number of fresh market hybrids of the indeterminant type. The transformation protocol was optimised to obtain maximum frequencies of transformation for the parental tomato line, using kanamycin resistance as selectable marker. After eight weeks about 20% of the cotyledon explants gave rise to shoot primordia, which were cut from the explants and rooted in the presence of 25 mg/l kanamycin. Rooted shoots were transferred to the greenhouse and about 45% of the transformants accumulated the TSWV nucleoprotein at detectable levels in an ELISA assay. Western blot analysis showed that this transgenically expressed protein comigrated with that extracted from tomato plants systemically infected with TSWV, thereby demonstrating the integrity of

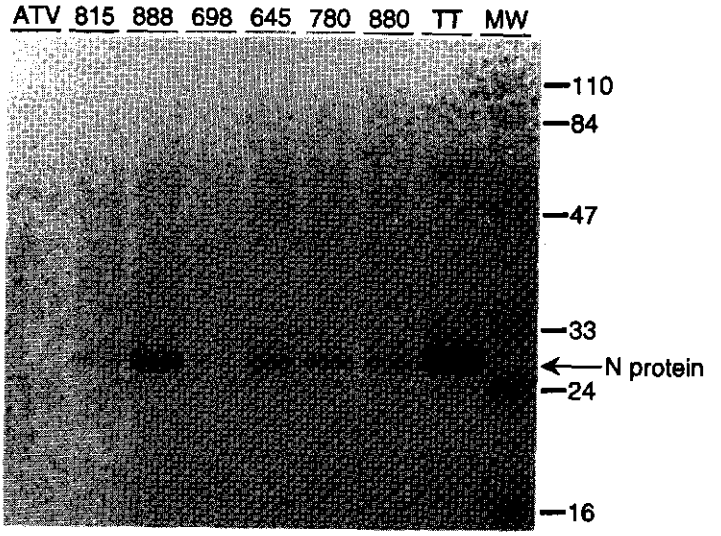

Fig. 2. Western blot analysis of tomato transformants accumulating TSWV nucleoprotein. Leaf protein samples were subjected to SDS-polyacrylamide gel electrophoresis, blotted to Immobilon-P membranes and TSWV nucleoprotein was detected using an antiserum raised against purified TSWV nucleocapsids. Molecular weight markers are indicated on the right and numbers refer to the tomato transformant lines; TT: tomato systemically infected with TSWV; ATV: non-transformed tomato line ATV847 used as recipient in transformation experiments.

the nucleoprotein produced in transgenic plants (Fig. 2). In order to identify transformants with aberrant ploidy levels, transgenic tomato plants accumulating varying levels of nucleoprotein were analysed for their ploidy level by means of flow cytometry. Only 60% of the expressors retained the diploid ploidy level and were subsequently maintained to produce offspring by self-pollination. The effective transformation frequency, expressed as the percentage of explants that gives rise to independent diploid transformants accumulating nucleoprotein, was calculated at 5%. None of the transformants or their progeny populations exhibited phenotypic aberrations that could be assigned to the accumulation of the nucleoprotein or to the insertion of the N gene cassette into the genome.

Protection of transgenic tomato against TSWV infections.

Prior to inoculation with TSWV, S1 progeny plants were analysed for the accumulation of viral nucleoprotein to identify those individuals that inherited the N gene cassette. Non-expressing segregants were used as susceptible controls in the inoculation experiments, in addition to non-transformed ATV847 plants. Upon emergence of the first

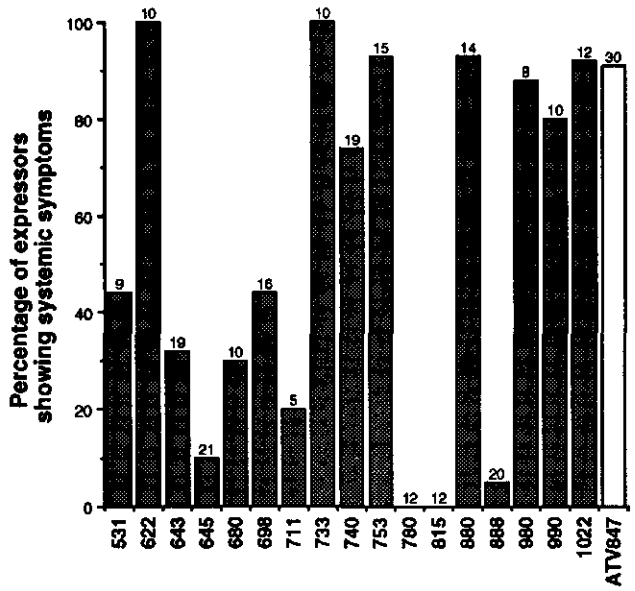

Fig. 3. Resistance to TSWV infection of S1 progeny populations upon mechanical inoculation. Plants were challenged twice with an inoculum prepared from systemically infected tomato plants. Control plants consisted of non-transformed ATV847 acceptor plants. Figures on top of the bars refer to the number of tomato plants accumulating nucleoprotein that have been scored for systemic symptoms in the final observation about eight weeks after the first inoculation. Progeny plants that did not inherit the nucleoprotein gene cassette through segregation were omitted from the analyses.

true leaf tomato seedlings were mechanically inoculated using an inoculum prepared from systemically infected tomato plants. The inoculation was repeated one week later and plants were subsequently monitored for the development of systemic disease symptoms, such as chlorosis and bronzing of the non-inoculated leaves and wrinkling of the youngest leaves. In later stages of infection diseased plants became stunted and reduced in height compared to mock inoculated plants. Control plants reached infection percentages of 90% or higher within two to four weeks. The final observation was made six weeks after the first inoculation and is shown in Fig. 3. Out of 24 progeny populations challenged with the virus, 11 transformant lines could be identified which showed reduced susceptibility to TSWV infection, ranging from complete resistance in transformant lines 780 and 815 to moderate levels of resistance in lines 531 and 698. All other transformant lines were as susceptible as the controls. Protected tomato plants were free of virus when tested by ELISA using an antiserum raised against a non-structural viral protein (NSs) that accumulates to high levels in TSWV infected plant cells (Kormelink *et al.*, 1991). ELISA values of protected transformants never exceeded the mean ELISA value of negative controls plus three times the standard deviation (0.101

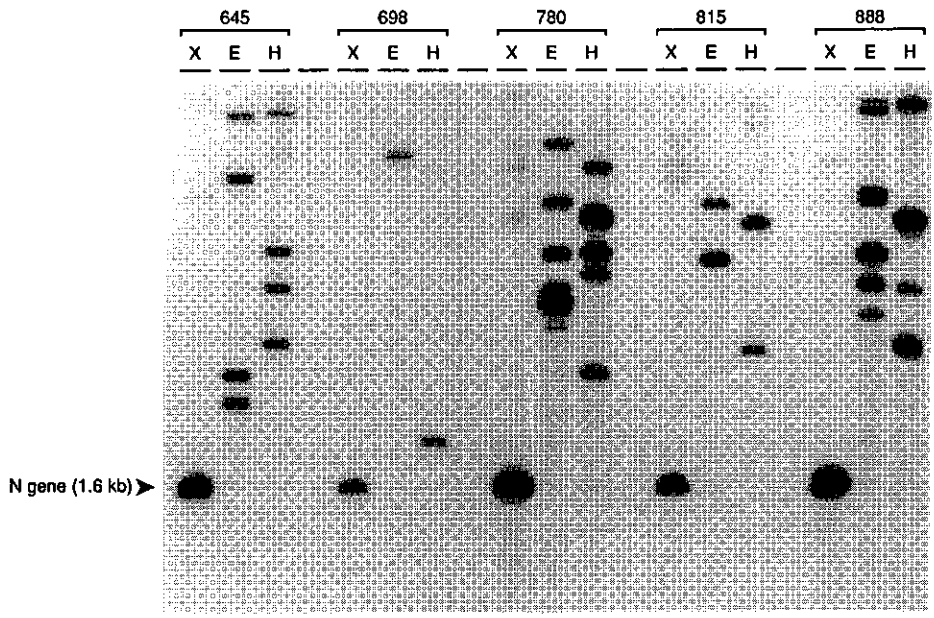

Fig. 4. Southern blot analysis of S1 progeny plants that resisted TSWV infection. Total DNA was extracted from leaf tissue, digested with *Eco*R V (E), *Hin*d III (H) or *Xba* I (X), fractionated by agarose gel electrophoresis and blotted to Hybond-N membranes. Genome fragments that comprised the TSWV N gene were detected by hybridisation with a ^{32}P-labelled DNA fragment derived from the structural N gene. Numbers refer to the primary tomato transformants from which the S1 progeny plants descend. The *Xba* I digest releases the TSWV N gene cassette as a fragment of 1.6 kb; the *Eco*R V and *Hin*d III digests release a number of border fragments correlated with the copy number of the N gene cassette.

plus 3 times 0.010), while ELISA readings of infected controls were out of range. The absence of detectable amounts of virus indicates that protected plants are probably immune rather than tolerant to infection. From each S1 transformant line which showed reduced susceptibility against TSWV infections a number of individual plants was maintained and self-pollinated to produce S2 offspring. The copy number of the N gene in selected S1 plants was determined by Southern blot analysis (Fig. 4). The majority appeared to carry multiple copies of the transgene, except for transformant line 698 which carried a single copy. Transformant line 815 carried two copies of the transgene, probably residing on the same chromosome, as could be deduced from the 3:1 segregation ratio observed for the expression of the N gene cassette in the S1 progeny (data not shown).

The complex inheritance of multiple independent transgene copies hampers their fixation within homozygous lines. Therefore, only transformants carrying minimal copy numbers were proceeded to produce S2 progenies. Non-segregating S2 populations, as determined by ELISA for the accumulation of the TSWV nucleoprotein, were subsequently

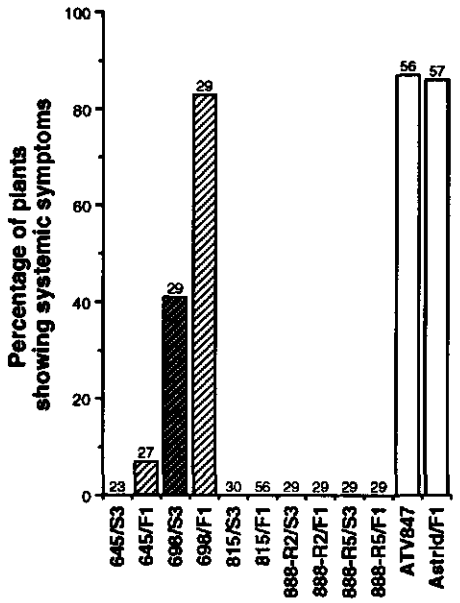

Fig. 5. Resistance to TSWV infection of homozygous S3 populations (double-hatched bars) and their corresponding hybrids (hatched bars). Control plants (white bars) consisted of non-transformed ATV847 acceptor plants and *Astrid* hybrids. Plants were challenged twice by mechanical inoculation with an inoculum prepared from systemically infected tomato plants. Figures on top of the bars refer to the number of tomato plants that have been scored for systemic symptoms in the final observation about eight weeks after the first inoculation.

subjected to Southern blot analysis to determine their copy number and to verify their non-segregating nature at the DNA level (data not shown). These analyses resulted in the identification of five progeny populations carrying homozygous copies for one or two linked integration events (Table I). Transformant lines 888-R2 and 888-R5 both descend from the same initial transformant, but represent different transformation events in view of the fact that both pairs of linked transgene copies segregate independently. To produce experimental hybrids, homozygous S2 lines were cross-pollinated with parental line ATX011. The novel hybrids, tentatively named *Astrid*, and their corresponding S3 lines were challenged with TSWV by mechanical inoculation (Fig. 5). The homozygous S3 lines and the experimental hybrids descending from transformant lines 815, 888-R2 and 888-R5 were all completely resistant to TSWV infection. Evidently, the transgenic expression of the TSWV N gene within these transformant lines suffices to generate complete resistance, even in the hemizygous hybrids derived from these lines. An example of protected hybrids descending from transformant line 815 is shown in Fig. 6. The homozygous S3 population and the experimental hybrid derived from transformant

Fig. 6. Resistance to TSWV infection upon mechanical inoculation in tomato hybrids derived from transformant line 815. Plants were photographed six weeks after the first inoculation. From left to right, transgenic *Astrid* hybrid expressing the TSWV N gene and non-transformed *Astrid* hybrid.

line 698, however, showed incomplete levels of resistance, as already observed for the S1 population. Within a population of 29 S3 plants that all carried one homozygous copy of the transgene, 12 plants developed systemic symptoms, resulting in an intermediate resistance level of 59%. The corresponding hemizygous hybrid featured a marginal resistance level of only 17%. Likewise, the hemizygous hybrid derived from transformant line 645-R6 did not equal the complete resistance observed for the homozygous S3 population. Apparently, the level of resistance within partially protected transformant lines is highly influenced by the zygotic state of the transgene, in that homozygous plants feature higher levels of resistance when compared to their hybrid counterparts.

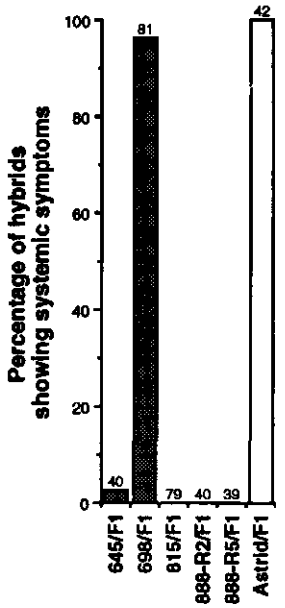

Fig. 7. Protection of hemizygous hybrids against TSWV infection upon natural inoculation by thrips and under field conditions. Control plants (white bars) consisted of non-transformed *Astrid* hybrids. The final notation for systemic disease symptoms was made when plants bore several clusters of fruits. Only plants devoid of any disease symptoms neither on vegetative plants parts nor on fruits were scored healthy.

Protection to TSWV infection upon natural inoculation under field conditions.

In nature, TSWV is transmitted by thrips species. To determine the level of protection upon natural infection, transgenic hybrids were planted in netted greenhouses in El Ejido, Spain, and exposed to continuous inoculation by the thrips vector. The El Ejido location is known for the high incidence of the TSWV disease in the local culture of tomato. The field trial comprised four replications of 10 to 12 hybrid plants that were planted in soil at their juvenile plant stage. At the end of the crop, when plants bore several clusters of fruits, individual hybrid plants were carefully inspected for visual disease symptoms, not only on the vegetative plant parts, but also on the fruits. At that time, susceptible control plants consisting of non-transgenic *Astrid* hybrids, reached an infection percentage of 100%, which illustrates the high incidence of the TSWV disease in the El Ejido region. Disease symptoms included the bronzing and necrosis of leaves, but also more general leaf malformations such as wrinkling and chlorosis, and early infected plants were severely stunted. Affected fruits showed chlorotic or necrotic ringspots, typical for TSWV infections. Only hybrid plants free of any symptoms were scored healthy (Fig. 7). Except for the hybrid descending from transformant line 698, hemizygous hybrids all exhibited high levels of resistance to TSWV infection. In case of hybrids 815, 888-R2 and 888-R5, any plants showing TSWV disease symptoms were not found, and hence appear virtual immune. This result unequivocally demonstrates the successful protection of hemizygous tomato hybrids against natural infection by TSWV. If both transgene

copies as present within these transformant lines are closely linked, preferably at the same locus, these transformant lines would carry a source for TSWV resistance that inherits as a single dominant trait, and thus would make excellent progenitors for TSWV resistance.

Contrary to the highly resistant transformant lines 645-R6, 815, 888-R2 and 888-R5, the intrinsic level of protection of transformant line 698 as observed in greenhouse assays employing mechanical inoculation (Fig. 5), appears to be too low for hemizygous hybrids to resist continuous inoculation with the virus by the thrips vector in open field. At the end of the trial most hybrid plants descending from transformant line 698 displayed systemic disease symptoms resulting from TSWV infection (Fig. 7).

Discussion

Breeding for disease resistance by introgression of genetic sources for resistance is generally regarded as the best strategy for sustainable crop protection. The exploitation of sources for host plant resistance that are naturally present within the gene pool of the crop involved, has contributed a great deal in breeding for disease resistance in crops that are cultivated in modern agriculture. In the past decade the concept of pathogen-derived resistance (Sanford & Johnston, 1985) has been put into practice to combat plant viral diseases, resulting in the development of synthetic resistance genes. Upon the introduction of chimaeric genes comprising plant viral sequences, transgenic plants show reduced susceptibility towards the corresponding virus, as has been described by many reports for a large number of host-virus combinations (Hull & Davies, 1992; Scholthof *et al.*, 1993; Wilson, 1993). Provided that the crop involved is amenable to genetic modification, pathogen-derived resistance genes represent novel sources of genetic resistance that are available to the breeder in addition to natural sources. In the case where natural resistance genes are lacking, transformation of crop plants with pathogen-derived resistance genes may even be the only way whereby genetic resistance can be achieved.

In tobacco it has previously been described that expression of the TSWV N gene confers resistance to TSWV infections (Gielen *et al.*, 1991; MacKenzie & Ellis, 1992; Pang *et al.*, 1992; Chapter 7 of this thesis). This chapter describes the successful use of the same TSWV N gene cassette to create tomato hybrids which are completely resistant to TSWV, thereby illustrating the broad applicability of the synthetic TSWV resistance gene. Accordingly, it may reasonably be assumed that the same gene will be useful in any other crop that suffers from TSWV infections, including pepper, lettuce and

ornamentals like *Chrysanthemum, Cyclamen* and *Impatiens* (Peters *et al.*, 1991). Since the transgenic TSWV resistance is primarily RNA-mediated (de Haan *et al.*, 1992; Chapter 7 of this thesis), the resistance will not easily be overcome by mutant TSWV strains that carry point mutations in their nucleoprotein gene. Hence, the synthetic TSWV resistance gene is expected to be a durable and reliable source of genetic resistance to TSWV.

Upon mechanical inoculation, 11 out of 24 tested transformant lines, expressing the TSWV N gene, showed protection to TSWV infection, ranging from virtual immunity to intermediate levels of resistance. Intermediate resistance levels are to some extent due to incomplete dominance of the transgene. Incomplete dominance is characterised by the fact that the expression of the transgene does not necessarily confer resistance, as illustrated by the discrepancy between the physical inheritance of a single transgene and the inheritance of the resistance trait observed for the homozygous population descending from transformant line 698. Gene dosage effects that result from the partial contribution of multiple transgene copies to the level of resistance may underlie the modulation of resistance levels in transformant lines carrying multiple transgene copies, as well as the difference in resistance levels observed between homozygous lines and hemizygous hybrids descending from the same primary transformant (Fig. 5). The occurrence of incomplete dominance and gene dosage effects urges the screening of large numbers of transformant lines in order to identify suitable progenitors for the TSWV resistance trait.

The observation that the transgene zygosity state affects the level of resistance is in line with the model of a virus-specific antiviral state as postulated by Dougherty and co-workers (Lindbo *et al.*, 1993). This model proposes the existence of a cytoplasmic activity that targets transcripts that have accumulated to a critical threshold level for accelerated degradation (Smith *et al.*, 1994). This down-regulation of the steady-state level of the transgenic transcript would then coincide with the elimination of viral sequences from which the transgenic transcript is derived, thereby yielding the virus resistant phenotype. Consistent with this assumption, virus resistance was shown to be correlated with high transcription rates, but low steady-state levels of the mRNA transcript. Since the transgene transcript is expected to accumulate to higher levels in homozygous transformant lines than in hemizygous hybrids, the decreased level of resistance observed for hybrids 645-R6 and 698 may be explained according to 'threshold' model (Smith *et al.*, 1994), in that the accumulation level of the transgene transcript in the hybrids drops below the threshold level. Within transformant lines 815, 888-R2 and 888-R5 that exhibit virtual immunity, the transcription rates of the transgene

probably are sufficiently high to compensate for such zygosity effects in the hemizygous hybrid, that equally exhibit complete resistance.

The introgression of pathogen-derived resistance genes in breeding programs is facilitated by the possibility of tracing the transgene by means of simple molecular techniques such as Southern blot or PCR analysis. Application of these techniques in backcross programs eliminates the need for repeated and laborious resistance screenings of progeny populations. In case of the TSWV resistance gene, the transgene can also be traced by ELISA for the accumulation of nucleoprotein.

Transgenic tobacco plants expressing the TSWV N gene are protected against TSWV infection, but remain susceptible to other tospoviruses (de Haan *et al.*, 1992; Chapter 7 of this thesis). The emergence of new tospovirus species, some of which infect tomato (de Ávila *et al.*, 1993), emphasises the need for identification or development of additional sources of genetic resistance against such viruses. From our experience with TSWV, it is anticipated that expression of the N gene from other tospoviruses in transgenic plants will also generate resistance. Therefore, synthetic resistance genes based on tospovirus N genes represent sources of genetic resistance that are available to the breeder as soon as novel tospoviruses emerge. Summarising, it is assumed that the technology developed to obtain TSWV resistance can be applied to generate resistance to any tospovirus in any crop susceptible to the corresponding tospovirus.

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9 Summary and concluding remarks

Viral diseases cause significant economic losses in most, if not all, crop species throughout the world. Total cost is not only restricted to reduction in crop yield and quality, but also include the development and application of a wide array of disease control measures. Routinely employed culture practices include quarantine measures, eradication of infected plants and weed hosts, crop rotation and the use of certified virus-free seed or planting stock. Additionally, the use of pesticides to control insect vector populations implicated in transmission of the virus, represents an important tool to limit the incidence of viral disease outbreaks. However, none of these non-genetic control measures is likely to provide the long-term answer to combat viral diseases, because of their expense and their sometimes questionable effectiveness and reliability. Moreover, current concern about pollution and food safety is forcing hazardous pesticides of the market.

As key control pesticides are progressively abandoned, there is a growing urgency for the development of alternative methods to control viral diseases. Breeding for virus resistance generally provides the best prospects for virus control in the long term. In the past, the introgression of genetic sources for host plant resistance that are naturally present within the gene pool of the crop involved, has been successfully applied to develop virus resistant crop cultivars for a considerable number of agronomically important crops. Although plant breeding for virus resistance still is of great potential, there are limitations to this conventional approach. An appropriate source of resistance may not be available in interfertile relatives, the source may be tightly linked to undesirable traits, or may be multigenic and as such difficult to advance in breeding programs. Consequently, the major barrier inherent to plant breeding for virus resistance is the scarcity of suitable sources of host resistance.

The limitations of conventional breeding and routine culture practices urge the need for the development of alternative forms of virus control that can be fully integrated within traditional methods. In this perspective, the concept of pathogen-derived resistance as elaborated by Sanford & Johnston (1985), provides an attractive strategy to produce novel, but genetic forms of virus control, by transforming crop plants with nucleotide sequences derived from the viral genome. Major progress in the molecular

characterisation of plant virus genomes and the stable transformation of plant species (Fisk & Dandekar, 1993) opened the avenue for molecular breeding to produce transgenic progenitors carrying novel and 'green' sources of virus resistance, that can be incorporated in routine crop breeding programs.

Molecular breeding for virus resistance relies on two basic disciplines. Firstly, the design and construction of pathogen-derived resistance genes (Chapter 3), and secondly, the introduction and expression of such synthetic resistance genes in transgenic plants (Chapter 2). For dicotyledonous plant species, *Agrobacterium*-mediated transformation generally is the method of choice (Zambryski, 1992; Zupan & Zambryski, 1995), as is illustrated by the successful transformation of elite lines of lettuce, melon and tomato (Chapters 4, 6 and 8 respectively). In spite of the highly similar approaches employed, starting from seedling cotyledons for explant material and using kanamycin resistance as selectable marker, the efficacy of transformation appeared highly variable for the various crops. For tomato, the effective transformation frequency, defined as the percentage of explants yielding independent diploid transformants that express the transgene at measurable levels, was calculated at approximately five percent. The transformation of melon, on the other hand, was severely hampered by the emergence of false positives that escaped from kanamycin selection during regeneration. On average, merely ten percent of the shoots was truly transgenic, thereby decreasing the effective transformation frequency to values below 0.5%, but heavily increasing the resultant effort devoted to the selection of true transformants. General tissue culture conditions, however, comprising parameters such as the concentration and kind of plant hormones and plant vitamins, and the use of feeder layers, are known to have a significant influence on transformation efficiencies and do differ considerably for the transformation protocols of tomato, melon and lettuce. Additionally, there exist large differences in responsiveness of different crop genotypes to transformation and to *in vitro* tissue culture in general. Consequently, plant transformation remains a highly empirical science for which elaborated protocols do not exist (van Wordragen & Dons, 1992). Only trial and error, based on the knowledge and experience from existing protocols can contribute to the development and optimisation of transformation procedures.

For the design and construction of pathogen-derived resistance genes a plethora of strategies can be employed, only limited by the size and content of the viral genome concerned (Chapter 3). The viral coat protein (CP) gene, however, has thus far been more widely applied than any other viral sequence, because coat protein-mediated protection was the first described (Powell Abel *et al.*, 1986; Beachy *et al.*, 1990). Accordingly, the transformation of tomato with the CP gene from cucumber mosaic cucumovirus (CMV) was shown to generate high levels of protection to CMV infection in tomato hybrids, not

only when challenged by mechanical inoculation, but also when exposed to repeated inoculation by viruliferous aphids in open field (Chapter 5). On the contrary, the transgenic expression of the CP gene from beet western yellows luteovirus (BWYV) in lettuce only yielded marginal levels of protection (Chapter 4). Transgenic lettuce plants never resisted infection with BWYV, but merely showed a delay in systemic symptom development. Apparently, the transgenic expression of viral CP genes to engineer resistance against the homologous virus does not apply with equal success to all virus genera, or to all virus-crop combinations.

For potyviruses the protection engendered by the transgenic expression of the potyviral CP gene is known to be mediated at the transcript level, rather than by the accumulation of transgenically expressed coat protein (Lindbo *et al.*, 1993). Likewise, the transformation of melon with the CP gene from zucchini yellow mosaic potyvirus (ZYMV) proved to be established by some mechanism of RNA interference, since a translationally deficient gene cassette of the ZYMV CP gene equally generated high levels of protection (Chapter 6). As such, the general term *coat protein-mediated protection* (CPMP) suggesting a mechanism mediated by the accumulation of the coat protein, is heavily misleading, and should rather be replaced by *coat protein gene-derived protection*, or shorter *capsid gene-derived protection* (CGDP). A second example of engineered resistance mediated at the transcript level results from the transformation of tobacco with the nucleoprotein (N) gene from tomato spotted wilt tospovirus (TSWV), a negative strand RNA virus. The transgenic expression of a translationally defective N gene cassette similarly afforded high levels of resistance, reaching virtual immunity in transformant lines carrying homozygous copies of the N gene cassette (Chapter 7). Transformation of tomato with the same TSWV N gene likewise engendered high levels of resistance, culminating in the development of experimental hybrids that resisted TSWV infection upon continuous inoculation with the virus by the thrips vector in open field (Chapter 8). Hence, tomato transformant lines carrying the TSWV N gene make suitable progenitors for TSWV resistance that can be incorporated into classical breeding programs, as do tomato transformant lines carrying the CMV CP gene, or melon transformants carrying the ZYMV CP gene for resistance against their cognate viruses.

The use of engineered resistance genes has advantages over the use of host genes for virus resistance. A unique feature of genetically engineered virus resistance is their source, being the viral genome. In fact, it is the viral pathogen itself that supplies the basic constituents for engineering virus resistance, which can be cloned and identified fairly easily. This is an essential difference from conventional breeding which, of necessity, is limited to host genes that can be introgressed from interfertile relatives. Moreover, one and the same gene construct can be applied in multiple crops to confer

genetically engineered resistance against the virus from which the gene construct was derived. Once engineered resistance genes are incorporated into the crop species of interest, they can be forwarded into breeding programs and manipulated like any other Mendelian trait conferred by a single dominant gene. Their inheritance can easily be traced by means of simple molecular techniques such as Southern blot analysis or PCR analysis. Application of these techniques in backcross programs eliminates the need for repeated and laborious resistance screenings of progeny populations, and can even be adapted to discriminate between homozygous and hemizygous progeny plants. In this manner, the transgene functions as a molecular marker that is one hundred percent correlated to the resistance trait.

In contrast to cultivar resistance genes, any recognition events based on highly specific interactions between the protein products from host resistance genes and viral avirulence genes (Keen, 1990; Dawson & Hilf, 1992), are not involved in transgenic virus resistance mechanisms. Consequently, engineered resistance genes are not likely to be easily overcome by mutant virus strains carrying point mutations, and as such may reasonably be expected to provide reliable sources of genetic resistance to viral infections. This reasoning especially applies to engineered resistance mechanisms mediated at the transcript level that do not imply the transgenic expression of any protein product at all. Nevertheless, one consideration that must be taken into account in the deployment of any monogenic resistance source, is the general experience that viral pathogens do vary and may overcome single genes in resistance breeding. The development of resistance by the virus would quickly negate the significant environmental benefits, including the reduced use of pesticides, inherent to the employment of genetic sources of virus resistance and irrespective of their nature. As such, it is important to stack multiple resistance genes operating at different levels, so that if the virus overcomes one level, it will be faced by other levels of protection. The combination of multiple sources of resistance might additionally yield higher levels of protection through complementation. In this perspective, one feasible combination would be the viral coat protein gene and a replicase-derived gene, thereby creating an oligogenic type of 'green' resistance based on different underlying mechanisms preventing the homologous virus from systemic infection and concomitant disease development. Alternatively, the combination of host and engineered resistance genes provides a tempting approach in developing oligogenic, highly durable sources of virus resistance.

Although criteria for effective field resistance to viral infections can vary significantly between the crop and the virus concerned, it is of crucial importance to ascertain whether transgene expression effecting protection upon infection under controlled conditions commonly achieved by mechanical inoculation, also holds upon transmission by the

natural vector and under field conditions. The practical value of genetically modified progenitors for virus resistance in fact can only be evaluated by extensive field testing, thereby merging the efforts of plant molecular biology and plant breeding. Ultimately, it is the breeder who should decide whether transgenic progenitors can be considered elite material that meets the demands for its incorporation into the crop breeding program. In this respect the genetic stability of the novel resistance trait and the overall field performance are critical factors inherent to the development of transgenic elite lines or a final transgenic cultivar. Unfortunately, relatively few studies of field performance of genetically engineered plants have been published to date, at least when considering the tremendous number of field trials conducted over the past few years (Ahl Goy & Duesing, 1995). Successful field testing of genetically modified crop cultivars not only provides proof of their superiority over existing cultivars, but will also contribute to demonstrate their environmental safety in order to diminish public concern and scepticism (Rogers & Parkes, 1995).

In all, the ultimate commercialisation and profit of transgenic sources of virus resistance will depend on an array of factors including field performance, genetic stability, public acceptance and the resolution of environmental concerns and patent related issues. Comprehensive studies on the environmental impact, toxicity and other safety issues must first properly be addressed before releasing engineered virus resistant cultivars. As such, extensive field trials and associated studies are now required to adapt genetically engineered sources of virus resistance for their implementation into practical agriculture.

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Samenvatting

De moderne land- en tuinbouw wordt gekenmerkt door monocultures van genetisch identieke planten, die op korte onderlinge afstand en onder zo ideaal mogelijke cultuur omstandigheden worden geteelt. Dergelijke intensieve teelten bevorderen echter de uitbraak van ziekten en plagen, welke kunnen leiden tot grote verliezen in opbrengst en kwaliteit. Tot de cultuurmaatregelen ter bestrijding, of beter ter preventie van planteziekten behoren het gebruik van ziektevrij uitgangsmateriaal, de rotatie en diversificatie van opeenvolgende teelten, de verwijdering van besmette planten, maar ook de toepassing van gewasbeschermingsmiddelen. Het gebruik van gewasbeschermingsmiddelen komt echter meer en meer onder druk te staan vanwege hun milieubelasting en hun mogelijke toxiciteit.

De veredeling voor ziekteresistentie wordt algemeen beschouwd als de beste strategie voor de bestrijding van ziekten en plagen in cultuurgewassen. De introgressie van natuurlijke bronnen van resistentie in cultuurgewassen heeft geleid tot de ontwikkeling van een groot aantal ziekteresistente rassen en variëteiten, die het nut van de resistentieveredeling in de praktijk bewezen hebben. Helaas kent ook de resistentieveredeling haar beperkingen. Zo kan een geschikte bron van resistentie niet voorhanden zijn, of zijn geassocieerd met negatieve eigenschappen die zijn toepassing in cultuurvariëteiten uitsluiten. Bovendien leidt het voortdurend gebruik van een monogene bron van resistentie vaak tot de selectie van nieuwe pathotypen die de resistentiebron weten te omzeilen en waartegen de cultuurvariëteit dus niet langer beschermd is. Met andere woorden, de voornaamste beperking inherent aan de resistentieveredeling betreft het gelimiteerde aantal bronnen van resistentie dat de veredelaar ter beschikking staat.

De vooruitgang in plant weefselkweek technieken en in de plant moleculaire biologie hebben geleid tot de ontwikkeling van de moleculaire veredeling, ter aanvulling op de meer klassieke vorm van veredeling. Met name voor wat betreft de resistentieveredeling wordt de potentie van de moleculaire veredeling hoog ingeschat. In dat verband beschrijft dit proefschrift de ontwikkeling van virusresistentie voor een drietal groentegewassen en een viertal plantevirusen.

Aan de basis van de moleculaire planteveredeling staat de genetische modificatie van planten, welke de mogelijkheid biedt tot de introductie van nieuwe genen en eigenschappen, kortweg transformatie genoemd (Hoofdstuk 2). Voor de transformatie van tweezaadlobbigen wordt doorgaans gebruik gemaakt van *Agrobacterium*, een bodembacterie die van nature in staat is tot de overdracht van genetisch materiaal naar de plant. Daarnaast is er de ontwikkeling en constructie van virusresistentiegenen, welke het vermogen herbergen transgene planten te beschermen tegen virusinfecties (Hoofdstuk 3).

In de afgelopen tien jaar is reeds voor groot aantal plantevirusen aangetoond dat de transformatie van planten met diverse delen van het virale genoom kan leiden tot de ontwikkeling van resistentie tegen het betreffende virus. Deze techniek die bekend staat bekend als '*pathogeen-afgeleide resistentie*' is als eerste beschreven voor het manteleiwit gen van het tabaksmozaïekvirus en is sindsdien succesvol toegepast voor een groot aantal andere virale genen in een groot aantal plantesoorten. Het mechanisme dat aan de verschillende vormen van pathogeen-afgeleide resistentie ten grondslag ligt kan zeer verschillend zijn voor verschillende virusen en voor verschillende virale genen, maar het resultaat is min of meer identiek: de transgene planten zijn verminderd vatbaar voor het virus waarvan het resistentie gen is afgeleid.

Zoals zovele cultuurgewassen wordt ook de commerciële teelt van sla, tomaat en meloen bedreigd door een aantal virusziekten, waarvoor afdoende bronnen van natuurlijke resistentie ontbreken. In dergelijke gevallen kan de moleculaire veredeling voor virusresistentie uitkomst bieden. Zo resulteerde de transformatie van tomaat met het manteleiwit gen van het komkommermozaïekvirus in de ontwikkeling van een transgene tomatehybride met een verhoogd niveau van resistentie tegen dit virus, niet alleen na mechanische inoculatie, maar ook na natuurlijke infectie in het veld (Hoofdstuk 5). De transformatie van sla met het manteleiwit gen van het bietevergelingsvirus in tegenstelling, leverde slechts een marginale bescherming, van onvoldoende niveau voor verdere toepassing in de resistentieveredeling van sla (Hoofdstuk 4). Dit resultaat illustreert echter de verschillen in efficiëntie van manteleiwit gen-afgeleide bescherming voor verschillende virussoorten in verschillende gewassen.

Van potyvirusen, waartoe ook het courgettevergelingsvirus behoort, is bekend dat de expressie van het manteleiwit gen een vorm van resistentie genereert die wordt bewerkstelligd door het transcript, en niet zozeer door de accumulatie van het manteleiwit zelf. Zo ook resulteerde de transformatie van meloen met het manteleiwit gen van het courgettevergelingsvirus in hoge niveaus van resistentie, onafhankelijk van de accumulatie van het manteleiwit zelf, daar een translationeel deficiënt manteleiwit genconstruct evenzeer actief bleek (Hoofdstuk 6).

De transformatie van tabak met het nucleoproteïne gen afkomstig van het tomatebronsvlekkenvirus (tomato spotted wilt virus: TSWV) leverde een tweede voorbeeld van transgene virusresistentie gemedieerd op RNA niveau (Hoofdstuk 7). Expressie van een translationeel inactief genconstruct resulteerde ook hier in hoge niveaus van resistentie. Indien homozygoot voor het transgen, vertoonden resistente lijnen virtuele immuniteit, hetgeen zich openbaart in een volledige bescherming van dergelijke lijnen tegen virusinfectie en de totale afwezigheid van systemische symptomen. De aanwezigheid van het virus kan in dergelijke planten niet worden aangetoond. Toepassing van hetzelfde

genconstruct in tomaat leidde tot de ontwikkeling van een transgene, TSWV resistente hybride, die zowel in de kas als onder normale cultuur omstandigheden in het veld virtuele immuniteit vertoonde (Hoofdstuk 8). Als zodanig kan geconcludeerd worden dat dergelijk plantmateriaal van groot nut kan zijn in toekomstige veredelingsprogramma's, hetgeen evenzeer geldt voor de tomate- en meloenlijnen getransformeerd met het mantel-eiwit gen van het komkommermozaïekvirus, respectievelijk het courgettevergelingsvirus. Het gebruik van transgene resistentiegenen kent een aantal voordelen boven het gebruik van natuurlijke bronnen van resistentie. Het feit dat transgene virusresistentie doorgaans is afgeleid van het virale genoom zelf, maakt dat zodra het virale genoom is gekloneerd en in kaart gebracht, het virus zelf een bron van resistentie vormt. Dit gegeven is een belangrijk verschil met de klassieke resistentie veredeling, waarin de veredelaar afhankelijk is van bronnen van resistentie die van nature aanwezig in de genenbank van het betreffende gewas. Bovendien kan eenzelfde transgen in meerdere gewassen die van hetzelfde virus te lijden hebben worden toegepast. De introgressie van transgenen wordt bovendien nog eens vereenvoudigd door de gelijktijdige implementatie van moleculaire technieken, zoals Southern blot analyse of de polymerase kettingreactie, waarbij het transgen zelf functioneert als moleculaire merker voor de resistentie eigenschap.

De praktijkwaarde van een transgene bron van virusresistentie kan slechts worden bepaald door veldproeven, waarbij het virus door de natuurlijke vector en onder normale cultuuromstandigheden wordt overgedragen. De resistentie eigenschap dient daarbij niet alleen stabiel tot expressie te komen, maar ook te zijn gecombineerd met een onveranderde of een verbeterde veldprestatie van de transgene cultivar. De selectie van die transgene elite lijnen, die uiteindelijk als progeniteur voor de virusresistentie in toekomstige verdelingsprogramma's gebruikt zullen gaan worden, vereist dan ook de inspanning van zowel de veredelaar als de moleculair bioloog. De laatste jaren is het aantal veldproeven met genetisch gemodificeerde planten exponentieel toegenomen, met als voornaamste doelstelling de evaluatie van transgeen plantmateriaal onder praktijkomstandigheden. Behalve dat dergelijke veldproeven de superioriteit van transgene cultivars in de praktijk bewijzen, dragen zij tevens bij aan de evaluatie van vermeende risico's verbonden met de marktintroductie van transgene planten, hetgeen de publieke acceptatie van transgene planten alleen maar kan ondersteunen.

Monogene resistenties staan bekend om hun beperkte duurzaamheid. De grootschalige teelt van cultuurgewassen met monogene resistentiebronnen leidt doorgaans tot de selectie en ontwikkeling van nieuwe pathogeenstammen met een verandere specificiteit, waartegen het resistentie gen niet langer actief is. Met name virusen zijn zeer variabel en kunnen als zodanig zeer snel evolueren, resulterend in het ontstaan van nieuwe stammen. Teneinde de duurzaamheid van monogene resistentiegenen, waartoe ook transgene

bronnen van virusresistentie behoren, te waarborgen, dient de combinatie van meerdere bronnen van resistenties in een en dezelfde cultivar te worden overwogen. Wanneer beide bronnen zijn gebaseerd op verschillende mechanismen van resistentie zal complementatie plaatsvinden, wat enerzijds kan leiden tot hogere niveaus van bescherming, anderzijds de duurzaamheid van beide bronnen kan verlengen. De ontwikkeling van een virulente virusstam vereist nu immers de gelijktijdige doorbraak van twee resistentiegenen. Zo zouden twee verschillende transgenen voor virusresistentie met elkaar gecombineerd kunnen worden, maar evenzo een natuurlijke met een transgene bron van resistentie, beiden resulterend in de ontwikkeling van 'groene', oligogene en dus duurzame bronnen van virusresistentie.

De daadwerkelijke marktintroductie van transgene virus resistente cultuurgewassen is vooralsnog afhankelijk van een groot aantal vraagstukken van zeer uiteenlopende aard. Zo resteren er technische vragen omtrent de stabiliteit, de veldprestatie en de voedselveiligheid van transgene cultivars, ecologische vragen omtrent de invloed van transgenen op hun omgeving, alsook ethische vragen omtrent de eigendomsrechten en de publieke acceptatie van genetisch gemodificeerde planten. Deze vraagstukken vereisen gegronde studies en aandacht alvorens tot de marktintroductie van virus resistente gewassen besloten kan worden, zonder dat daarbij de enorme voordelen van transgene planten voor de hedendaagse land- en tuinbouw uit het oog verloren mogen worden.

Curriculum vitae

Johannes Jacobus Ludgerus Gielen, de auteur van dit proefschrift, werd op 25 augustus 1962 geboren te Roggebot Sluis in de Zuidelijke IJsselmeerpolders, het tegenwoordig grondgebied van de gemeente Dronten. Na het behalen van het VWO diploma aan het Christelijk Nassau-Veluwe College te Harderwijk, werd augustus 1980 aangevangen met de studie Biologie aan de Vrije Universiteit te Amsterdam. Tijdens de doctoraal fase vond verdere specialisatie plaats door middel van stages Plantenfysiologie, Microbiële Fysiologie, Phytopathologie en Moleculaire Genetica. Na voltooiing van zijn studie, trad de schrijver juli 1987 in dienst van *Zaadunie*, het tegenwoordige *S&G Seeds* te Enkhuizen, met als voornaamste taakstelling de ontwikkeling van virus resistentie in groentegewassen door middel van genetische modificatie. In dat kader werd een nauwe samenwerking tot stand gebracht met de vakgroep Virologie van de Landbouw Universiteit Wageningen, onder leiding van professor Goldbach, waarvan dit proefschrift het tastbaar resultaat vormt. Sinds november 1994 is de schrijver als 'Programme Leader Biotechnology' in dienst van *Hilleshög NK* te Saint-Sauveur, Frankrijk, dat evenals *S&G Seeds* deel uitmaakt van de *Sandoz Seeds* groep, Bazel.

Nawoord

Met de voltooiing van dit proefschrift sluit ik een periode van zeven jaar S&G Seeds en zeven jaar Enkhuizen af. Terugkijkend kan ik niet anders concluderen dan dat het zeven **vette** jaren zijn geweest, mede dankzij de prettige werksfeer zoals ik die door de jaren op Hercules drie en vier heb mogen ervaren. Zeven jaren van onderzoek ook, waaraan een groot aantal mensen in meer of mindere mate een bijdrage heeft geleverd, zoals wordt weerspiegelt door de diverse co-auteurs en de 'acknowledgements' bij de verschillende hoofdstukken. Dus bij dezen, een oprecht 'bedankt' aan een ieder die op wat voor manier dan ook, een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift. *En français, je tiens à remercier tous ceux qui ont d'une façon ou d'une autre, contribué à la réalisation de cette thèse. Merci.*

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André, waartoe een 'uitdagend' briefje al niet toe kan leiden. Jouw voorstellen tot verandering zijn van eminent belang geweest in mijn loopbaan tot nu toe, waarvan dit proefschrift een tastbaar bewijs vormt. 'Human resource management' in de volste zin van het woord. Gelukkig heeft jouw motivatievermogen mijn scepticisme tot nu toe altijd weten te overwinnen.

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