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Development of a strategy to induce RNA-silencing in squash against virus diseases by genetic transformation

Dissertation

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Was wir wissen ist ein Tropfen,
was wir nicht wissen, ein Ozean.

Isaac Newton (1643-1727)

To my parents, wife and children

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Abbreviations

2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Acetosyringone	3, 5-Dimethoxy-4-Hydroxyacetophenon
BA or BAP	6-Benzylaminopurine (cytokinin)
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Centigrade
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl pyrocarbonate
dsDNA	Double strand DNA
EDTA	Ethylene diamine tetraacetic acid
IAA	Indole-3-acetic acid
Kb	Kilo base
KDa	Kilo Dalton
mM	Millimolar
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
Nos-pro	Nopaline synthase promoter
Nos-term.	Nopaline synthase terminater
nt	Nucleotides
RNase	Ribonuclease
rpm	Rounds per minute
SDS	Sodiumdodecylsulfate
SSC	Standard saline citrate
ssDNA	Single strand DNA
Tris	Tris-(hyydroxymethyl-amino methane)
Triton x100	Octylphenylpoly-(ethylenglycolether)
Tween 20	Poly xyethylen-sorbitan-monolaurate
UV	Ultraviolet light
V/V	Volume per volume
W/V	Weight per volume

1 Introduction

1.1 Taxonomy and economic importance of Cucurbits

Squash (*Cucurbita pepo* L.) is a member of the family *Cucurbitaceae* that consists of about 118 genera and 825 species, according to the last taxonomic treatment of Jeffery (1990). The family *Cucurbitaceae* is divided into five sub-families: *Fevilleae*, *Melothrieae*, *Cucurbitaceae*, *Sicyodeae*, and *Cyclanthereae* (Müller and Pax, 1894). The most important cultivated genera are *Cucurbita*, *Cucumis*, *Citrullus*, *Lagenaria* and *Luffa* found in the sub-family *Cucurbitaceae* and *Sechium* found in the sub-family Sicyoiodeae (Whitaker and Davis, 1962).

Three genera are cultivated worldwide in the sub-family *Cucurbitaceae* in a variety of environmental conditions. The genus *Cucurbita* consists of about 30 species of annual, tendril-bearing plants of the family *Cucurbitaceae* (Hutchinson, 1967). Four species are commonly cultivated: *C. maxima, C. mixta, C. moschata, and C. pepo* (Kernick, 1961). The fruits of these four species, and consequently the plants, are called squash, pumpkin, summer squash, winter squash, and host of other names, based solely on the culinary or other human uses for which the plants are used, and with no regard to proper species taxonomy (Everett, 1981). The genus *Cucumis*, to which the cantaloupe, cucumbers, and several melons belong, includes about 70 species; two major crop species are commonly cultivated: melon (C. *melo* L.) and cucumber (*C. sativus*). The genus *Citrullus* includes watermelon (*C. lanatus* Thunb.) (Table 1).

Cucurbits are among the largest and most diverse plant families have a large range in plant and fruit characteristics, include shape, size, colour, taste, aromn, sugar content, sex expression and parthenocarpy, ploidy and season (Bates *et al*., 1990). Various cultivars of *Cucurbita pepo* are called summer squash, winter squash, pumpkin, vegetable marrow, zucchini, spaghetti squash (Purseglove, 1968; Terrell *et al*., 1986).

According of the United Nations Food and Agriculture Organization (FAO) estimation, production of *Cucurbita* crops worldwide in 2002 was as following: 81 million tonnes for watermelon, 36 million tonnes for cucumber and gherkins, 22 million tonnes for melon and cantaloupe, and 17 million tonnes for pumpkins, squash and gourds. Total cucurbit production worldwide 156 million tonnes was greater than of tomato 108 million tonnes and about half than of potato 568 million tonnes.

Table 1: Latin and common names, diploid chromosome number and area of origin of the most important species of cultivated cucurbits, modified from Bisognin (2002)**.**

Latin name	Common name*	Chromosome number (2*n*)	Area of origin
Benincasa hispida (Thunb.)	Wax or ash groud	24	India
Citrullus lanatus (Thunb.) Matsumura. & Nakai. Syn. *C. vulgaris* (schrad)	Watermelon	22	Africa and India
Cucumis sativus L.	Cucumber	14	India
Cucumis melo L.	Melon, cantalope	24	Africa
Cucumis melo var ***momordica*** **L**	Muskmelon, snapmelon	24	Africa and India
Cucurbita ficifolia Bouché.	Fig-leaf gourd	40	Mexico, Central and South Americas
Cucurbita argyrosperma Huber. syn. ***C. mixta*** Pang.	Winter squash	40	Mexico, Central America
Cucurbita maxima Duchesne	Winter squash, pumpkin	40	South America
Cucurbita moschata (Duch. ex. Lam.) Duch. & Poir.	Winter squash, pumpkin	40	Mexico, north and south America
Cucurbita pepo L.	Summer squash, pumpkin, marrow, zucchini, courgette	40	North and central Americas, southern united states of America to northern parts of Costa Rica, Mexico
Lagenaria siceraria (Mol.) Standl.	Porongo, white-flowered gourd, bottle gourd	22	Africa, India
Luffa aegyptiaca Mill. *syn.* ***L. cylindrica*** (L.) Roem.	Smoth loofah, sponge gourd	26	Africa, India
Luffa acutangula (L.) Roxb.	Angled loofah, Ride gourd, Ribbed gourd	26	Africa, India
Momordica dioica Roxb.	Teasle groud	28	India
Momordica charantia (L.) *Syn.* ***M. charantea*** Linn.	Bitter gourd, karalla	22	India , Africa, tropical America, China
Sechium edule (Jacq.) Swartz	Chayote, Christophine	24	Southern Mexico, Central America
Trichosanthes anguina (L.)	Snake groud	24	India
Trichosanthes dioica Roxb.	Pointed gourd	24	India

* Most usual common names found in the literature.

The plants belonging to the family *Cucurbitaceae* provide a major portion of vegetable and they need to be investigated for maximum utilization (Halder and Gadgil, 1981). Cucurbits are among the most important plant families that supply human with edible products and useful fibers (Bisognin, 2002). The soluble fibers may help to reduce cholesterol and risk of heart disease; the fibres are important for keeping digestive tract operating properly by preventing constipation, hemorrhoids and diverticular disease (Sultana and Beri, 2003). Sucrose, glucose, and fructose are the three major constituents of ripe melon fruit soluble sugars (Rosa, 1928; Pratt, 1971; Hughes and Yamaguch, 1983; Lester and Dunlap, 1985; Schaffer *et al*., 1987 McCollum *et al*., 1988; Hubbard *et al*., 1989; Schaffer *et al*., 1996). The fruit flesh is a significant source of carbohydrates, iron carotene, folic acid, potassium, vitamin A, vitamin C also known as ascorbic acid, oxalic acid, phosphorus and mineral water and seeds are rich in oil and protein (Martyn and Miller, 1996; Richter, 2000; Nabi *et al*., 2002; Sultana and Bari, 2003; Sultana and Bari Miah, 2003; Naseem and Anis, 2005). All cultivars of *Cucurbita pepo* are eaten as a vegetable, fed to livestock, or used for ornament (Cobley, 1976).

1.2 Viral diseases in cucurbits

Viral diseases are the main problem in the production of cucurbit plants compared to diseases caused by other agents leading to total loss and prevent the growth in certain areas of some cucurbit crops. Cucurbit crops are susceptible to many viruses belonging to several virus families (Lovisolo, 1980; Nameth *et al.*, 1986; Purcifull *et al.*, 1988; Provvidenti, 1993; Zitter *et al*., 1996). Almost 35 different viruses have been isolated from Cucurbitaceae (Provvidenti, 1996) and virus resistance is therefore of major agricultural importance. Three viruses belonging to the genus potyvirus are *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV; formerly termed *Watermelon mosaic virus* II), and *Papaya ringspot virus* W (PRSV-W, formerly termed *Watermelon mosaic virus* I) and one belonging to genus Ipomovirus is *Cucumber vein yellowing* (CVYV) in the family *Potyviridae*; two viruses belonging to the family *Tobamoviridae* are *Cucumber green mottle mosaic virus* (CGMMV) and *Cucumber fruit mottle mosaic virus* (CFMMV). In addition to *Cucurbit yellow stunting disorder crinivirus* (CYSDV), *Cucumber mosaic virus* (CMV), *Squash mosaic virus* (sqMV) and *Melon necrotic spot carmovirus* (MNSV) in the families *Closteroviridae, Cucumoviridae,*

Comoviridae and *Tombusviridae, respectively*. As well as *Squash leaf curl begomovirus* (SqLCV) and *Watermelon chlorotic stunt begomovirus* (WCSV) in the family *Geminiviridae*.

On a worldwide basis, the three potyvirus species (ZYMV, WMV and PRSV-W) are the most commonly and economically important, reported in surveys of virus infecting cucurbits in different parts of the world (Lecoq, 1992; Shukla *et al.,* 1994; Rivera *et al.,* Grafton–Cardwell *et al.,* 1996; Rubies–Antonell *et al.,* 1996; Dahal *et al.,* 1997; Luis Arteaga *et al*., 1998). ZYMV is particularly notorious (Lecoq *et al.,* 1983) the virus was first discovered in Europe in Italy 1973 and France 1979, described in zucchini squash in Italy and France (Lisa *et al.,*1981) and it was also known as muskmelon yellow stunt virus (Lecoq *et al*.,1981). The virus was also described in Germany 1983 (Lesemann *et al*., 1983) and in Egypt 1983 (Provvidenti *et al.,* 1984). Afterwards, the presence of the virus was reported in almost all countries of the world (Table 2) where cucurbits are grown, under Mediterranean, subtropical and tropical regions.

Table 2: Some examples of the distribution of ZYMV, host and year of first description.

Country	First description	Reference
Europe		
France	Muskmelon 1979	Lecoq *et al*., 1981
Germany	1983	Lesemann *et al*., 1983
Italy	Zucchini 1973	Lisa *et al*., 1981
Africa		
Egypt	1983	Provvidenti *et al*., 1984
Nigeria	*Cucumeropsis edulis* 1978	Igwegbe, 1983
Asia		
Taiwan	Cucumber 1982	Hseu *et al*., 1985
Turkey	Squash 1983	Davis and Yilmaz, 1984
Oceania		
Australia	Pumpkin, zucchini 1981	Greber *et al*.,1987
America		
USA (Florida)	Squash 1981	Purcifull *et al*., 1984
Mexico	1984	Nameth *et al*., 1985

WMV-2 infects under natural conditions mainly cucurbits but also certain species belonging to other families, some of which are reported to be important sources of infection (Lovisolo, 1980). These families include *Amaranthaceae, Apocynaceae, Chenopodiaceae, Compositae Convolvulaceae, Cruciferae, Euphorbiaceae, Leguminosae, Caesalpinioideae,*

Papilionoideae, Malvaceae, Pedaliaceae, Scrophulariaceae, Solanaceae, Tetragoniaceae, Umbelliferae, Valerianaceae. This virus is wide spread in temperate, subtropical and tropical regions of the world (Milne, 1987).

1.2.1 Transmission and disease symptoms

The most common symptoms with the ZYMV and WMV in infected plants are mosaic, vein clearing, vein banding, chlorotic mottles, as well as leaf distortion and flower abortion resulting in reduction in fruit quality and quantity, especially if plants are infected in early growth stages (Blua and Perring, 1989; Alonso-Prados *et al.,* 1997).

The ZYMV and WMV are naturally transmitted by aphids in a non-persistent manner (Pirone and Harris, 1970). Transmission of viruses in the stylet-borne manner by aphids has also been reported by Lisa *et al*. (1981); Adlerz (1987); Francki and Habili (1990); Castle *et al*. (1992); Shukla *et al*. (1994). Non-colonizing aphids (i.e., aphids which probe then reject a plant as a host) can be important in the ecology of potyviruses infecting cucurbits (Zitter, 1977). The viruses have no latent period within the vector and have an infectivity of a few hours or days. The aphids can acquire the virus in seconds to minutes by probing an infected plant and immediately transmit or inoculate the virus to a healthy plant. Once the aphid acquires the virus, it does not retain it for long. The length of virus retention ranges from minutes to one day, depending on aphid behaviour. These characterizations of virus-vector relationship make control of viruses transmitted in a non-persistent manner by application of insecticide extremely difficult (Raccah, 1986). Pesticides aimed at reducing aphid vector populations seldom help to control virus spread for several reason. First, aphids can transmit the virus so quickly that the damage is done before the pesticide can kill the insect. Second, many of the most important vector species do not colonize or live on the crop. Instead, the transient aphids that land briefly and probe crop plants while searching for an appropriate host are the most serious vectors. Thus, the most important insect species will not be targeted by spraying a pesticide on the crop. Finally, many pesticides cause aphid activity to increase. Any treatment that increases aphid activity also increases movement between plants. In consequence, when the aphids move between plants they also move the virus. Hence, pesticide treatment may result in increased virus spread. In addition, frequent use of insecticides leads to increased production costs, increases potential of insects to be insecticide resistant.

Potyviruses are attached to aphid with the aid of a helper component protein (HC-Pro) and coat protein (CP). Thornbury *et al*. (1985) identified the HC as a virus-coded non-structural protein, now known as HC-Pro, which binds the virus particles to aphid mouthparts, especially in the maxillary food canal and, to some extent, the sucking pump (Berger and Pirone, 1986; Ammar *et al*., 1994). A domain near the N-terminus of the virus coat protein, including the amino-acid triplet DAG, is crucial (Harrison and Robinson, 1988; Atreya *et al*., 1990), as also are two domains in HC-Pro, one of which is involved in binding to the DAG domain and the other probably in linking HC-Pro to the epicuticle of aphid stylets (Blanc *et al*., 1997 and 1998; Peng *et al*., 1998).

The availability of alternative hosts is partially responsible for the differential geographic distribution. Overwintered weed species were tested for the presence of the viruses. Svoboda and Polak (2002) found that ZYMV was detected by ELISA in one plant of *Tripleurospermum maritimum* out of 46 tested, in two plants of Stellaria *media* out of 29 tested in 2001 and 2002 they repeated such tests, found three plants of *T. maritium* out of 45 tested, three plants of *S. media* out of 52, and two plants of *Trifolium repens* out of 17 tested. The virus was successfully transmitted from *T. maritium, S. media and T. repens* to indicator plants of *Cucurbita pepo* convar. giromontiina. WMV overwinters in many common weeds found in temperate region, while PRSV-W and ZYMV have more restricted host ranges and overwinter mainly in winter cucurbit crops or in wild cucurbit species not found in temperate regions (Purcifull *et al.*, 1984; Lecoq, 1992; Desbiez and Lecoq, 1997). However, occasionally and often locally, severe ZYMV or PRSV-W epidemics are also reported in temperate growing regions where these viruses are usually not detected every year (Grafton-Cardwell *et al.*, 1996; Rubies-Antonell *et al.*, 1996; Luis Arteaga *et al.*, 1998).

Seed transmission may also contribute to potyvirus introduction into new production areas by creating primary infection sites from which these viruses could be efficiently disseminated by aphids. However, no seed transmission has been reported so far for PRSV-W (Purcifull *et al.*, 1984). ZYMV seed transmission has been observed in *Cucurbita* species in some laboratories (Schrijnwerkers *et al.*, 1991) but has not been confirmed by others (Gleason and Provvidenti, 1990; Robinson *et al.*, 1993). Recently, ZYMV was reported to be seed transmitted in a study with oilseed pumpkin (Tobias and Kovacs, 2001; Tobias *et al.*, 2003). On another study, ZYMV was not detected by observing approximately 70,000 seedlings originating from 12

Cucurbita cultivars infected by four ZYMV isolates (Lecoq *et al.,* 2003). It seems that, seed transmission rates are virus strain and host genotype depended.

1.2.2 Viral synergism

Infection of plants by single or multiple viruses is a common phenomenon (Davis and Mizuki, 1987; Falk and Bruening, 1994; Luis-Arteaga, 1998), when two plant viruses infect a plant simultaneously, viral interaction often occur resulting in devastating diseases, the outcome, depending upon the combination, is considered either antagonistic or synergistic (Hull, 2002). Antagonism usually occurs when the co-infecting viruses are related; resulting in interference or cross-protection (Hull and Plaskitt, 1970; Watts and Dawson, 1980; Sakai *et al*., 1983; Khan *et al*., 1994). Synergism normally occurs in mixed infection when the pair of viruses involved are unrelated, resulting in increased symptoms severity and greater virus accumulation than those produced by single infections (Hull and Plaskitt, 1970; Walkey and Payne, 1990; Khan *et al*., 1994; Jensen *et al*., 1996; Cho *et al*., 2000; Hull, 2002). In nature, mixed viral infection of many crops has had biological and epidemiological implication, which have been realized and addressed in many studies (Kassanis, 1963; Rochow, 1972; Pruss *et al.,* 1997; Sano and Kojima, 1989; Wang *et al*., 2002).

Plants may have barriers that block systemic spread of viruses between any of the cell types in the vascular system. These barriers sometimes allow limited or reduced rates of movement (Nelson and Van Bel, 1998; Hull, 2002). In some cases, mixed infection by two viruses has been shown to overcome barriers to the cell-to cell or long-distance movement of one of the viruses (Atabekov and Taliansky, 1990; Nelson and Van Bel, 1998; Hull, 2002). In synergism, it is generally recognized that one virus, which is not increased in accumulation (i.e. functioning essentially as catalyst), act as an up-regulator of the replication and/ or movement of an unrelated virus, usually increasing the intensity of symptoms induced compared with single infection by either virus alone (Rochow and Ross, 1955; Calvert and Ghabrial, 1983; Poopol and Inouye, 1986a,b; Goldberg and Brakke, 1987; Sano and Kojima, 1989; Vance, 1991; Anjos *et al.,* 1992; Bourdin and Lecoq, 1994; Pruss *et al.,* 1997). In most of the above cases of synergism the “catalytic virus” is a potyvirus. In the potyvirus incited synergisms it has been found that the central region of the HC-Pro is the mediator of synergism, and the same region also suppresses the host RNA silencing mechanism, suggesting that these two phenomenons are linked (Anjos *et al*., 1992; Vance *et al*., 1995;

Maia *et al*., 1996; Pruss *et al*., 1997; Shi *et al.*, 1997 Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Savenkov and Valkonen, 2001).

It has been reported that the potyvirus involved in a synergistic mixed infection induced its own cytopathological effects in host cell in addition to those characteristic of each virus (Carr and Kim, 1983). Interaction of Cucurbit species by potyvirus such as ZYMV, or by CMV are very common and cause considerable damage worldwide in severe epidemics in Cucurbit fields either in single or mixed infection (Grafton-Cardwell *et al.,* 1996; Luis-Arteage *et al.*, 1998). When plants were inoculated with mixture of the two viruses (ZYMV and WMV), the frequency of plants resistant to ZYMV was lower than expected, indicated that WMV infection may reduce the ability of a plant to resist ZYMV **(**Xu *et al*., 2004)**.** Zucchini squash and melon plants doubly infected with the potyvirus ZYMV and CMV have been shown to exhibit a severe synergistic pathological response and showed a strong increase in the level of accumulation of CMV positive- strand RNA and CP (increasing in titer), with no increase in the accumulation of ZYMV (Choi, 2002; Wang *et al.*, 2002; Fattouh, 2003).

1.3 Potyvirus genome structure and infection cycle

The genus potyvirus of the family *Potyviridae* belong to the *Picornaviridae* supergroup of animal and plant viruses (Koonin and Dolja, 1993), and contains 30% of all known plant viruses (Ward and Shukla, 1991). Potyviruses have flexuous filamentous particles 680-900 nm long and 11-15 nm in diameter (Dougherty and Carrington, 1988). Their genomes are single-strand positive-sense RNA molecules of approximately 10,000 bases (Brakke and van Pelt, 1970; Allison *et al*., 1986; Domier *et al*., 1986), encapsulated by nearly 2000 subunits of a single type of coat protein (CP) (Shukla *et al.,* 1994; Martin and Gelie, 1997). The viral RNA is translated into a large polyprotein (340-370 KDa) (Riechman *et al*., 1992; Riechmann *et al*., 1995; Urcuqui-Inchima *et al*., 2000) that is proteolytically processed to or cleaved into 8-10 functional proteins by three virus-encoded proteinases (or proteases): P1, HC-Pro and NIa (Riechmann *et al*., 1991; Revers *et al*., 1999). P1 and HC-Pro cleave only respective C-termini (Verchot *et al*., 1991). The NIa resembles the picornaviral 3c-like proteases and processes the remainder of polyprotein (Gorbalenya *et al*., 1989). The RNA genome has a 3´ poly (A) tail covalently and the 5´ end of the viral RNA does not have a cap structure (m GpppN, where N is any nucleotide), Figure 1. But it is covalently linked to a virus-encoded protein termed VPg via a tyrosine residue (Ambros and Baltimore, 1978; Rothberg *et al*.,

1978; Murphy *et al.*, 1991; Koonin and Dolja, 1993; Murphy *et al*., 1996; Oruetxebarria *et al*., 2001).

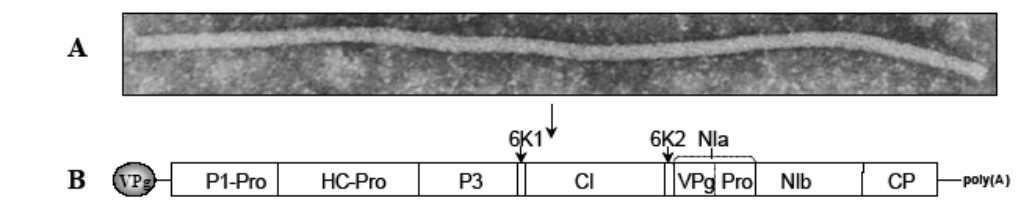


Figure 1: Schematic representation of potyvirus genome (Shukla *et al*., 1994). The particle consists of flexuous filamentous rods (A) that ecapsidate a single positive stranded RNA molecule (B). The genome contains one open reading frame and names of final protein products are indicated as boxes, separated by lines that indicate the putative cleavage sites of the polyproteins. The 5´- and 3´-untranslated regions are represented by single lines. The RNA genome is 3´-polyadenlated and has a viral protein (VPg) linked to the 5´-end.

Plant viruses have to evolve specific mechanism for successful transmission to host plant, because plant has thick cell walls. Successful plant viruses must develop strategies to move both cell-to cell and through the plant systemically. Once the virus entered the host cell, the potyvirus RNA is released from its coat protein subunits. This process is termed uncoating or disassembly. The mechanism by which uncoating of the potyvirus occur is not well documented. Because the potyviruses have a single strand plus-polarity RNA genome, the RNA can act directly as a messenger RNA (mRNA). The potyvirus also carries a viral replicase gene [a nuclear inclusion gene which is termed RNA-dependent RNA polymerase (RdRp)]. The RdRp of ZYMV was shown to bind poly (A) binding protein, indicating a direct role for the poly (A) tail in recruiting the replicase to the 3´end of potyviral RNA (Wang *et al.*, 2000). As they do not encode translation factor or ribosome, viruses must use or sequester host translational machinery to synthesize their proteins (Bushell and Sarnow, 2002; Schneider and Mohr, 2003). The 5´untranslated region (UTR) of the genomic RNA of several *Picornaviridae* viruses contains internal ribosome entry sites (IRES) that are composed of a complex group of stem-loop structures (Pelletier and Sonenberg, 1988; Carrington and Freed, 1990; Basso *et al.*, 1994). VPg is involved in multiple functions during the viral life cycle, it is critical for replication, translation, and phloem loading, as well as in a virulence determination (Rajmäki *et al*., 2004). The central domain of the VPg is involved in the ability to overcome resistances associated with recessive genes (Nicaise *et al*., 2003). However, the phenotypes of corresponding resistances differ depending on the host and potyvirus partners (Nicaise *et al*., 2003). VPg has been suggested to play a role in recruiting the eukaryotic

translation initiation factor 4E (eIF4E) (a cap-pending protein) of Arabidopsis, thereby functionally replacing the cap structure in the creation of a protein bridge between the 5´and 3´ends of the RNAs resulting in mRNA circularization and translation initiation (Wittmann *et al*., 1997; Pestova *et al*., 2001). In support of this model, the *Turnip mosaic potyvirus* (TuMV) and *Tobacco etch potyvirus* (TEV) VPgs were shown to interact with eIF(iso)4E of tomato and eIF4E of Arabidopsis, respectively (Wittmann *et al*., 1997; Leonard *et al*., 2000; Schaad *et al*., 2000; Leonard *et al*., 2004). The TuMV VPg could compete with a cap analogue for interaction with eIF(iso)4E in vitro (Leonard *et al*., 2000). In planta, eIF(iso)4E and poly A binding protein (PABP), which is a translation factor binds to the poly A tail of the RNAs, are associated with the TuMV VPg-eIF(iso)4E complex suggesting that the interaction of VPg with eIF(iso)4E promotes the recruitment of other translation factor (Leonard *et al*., 2004).

Plant viruses must accomplish three main steps to complete their infection cycle: (i) replication inside the cell, (ii) cell-to-cell movement through plasmodesmata, (iii) long-distance movement through the vascular tissue. Genomic RNA of potyvirus replicates in association with the endoplasmic reticulum (ER) in the cytoplasm of the infected cell. The 6K protein is largely responsible for the membrane modification and contains a highly hydrophobic domain that directs its association with intracellular membranes (Restrepo-Hartwing and Carrington, 1996; Schaad *et al*., 1997). The entire viral genome is first copied into a negative sense strand by the nuclear inclusion protein b (NIb) which is the viral RNA-dependent RNA polymerase RdRp) (Allison *et al*., 1986). The polyadenylated genomes of the potyvirus contain stem-loop in their 3´-UTR that are required for replication (Hsue and Masters, 1997; Haldeman-Cahill *et al*., 1998) which is recognized by the RdRp and act as promoters for the synthesis of the (-)-strand RNA. The cylindrical inclusion protein (C1) contains nucleoside triphosphatase and RNA unwinding activity and shares sequence similarity with other RNA helicases (Lain *et al*., 1990; Eagles *et al*., 1994; Fernandez *et al*., 1997). The produced (-)-strand RNA serve as a template for the synthesis of the progeny (+)-strand RNA (Kadare and Haenni, 1997). It was suggested that VPg may play a role in priming viral synthesis (Puustinen and Mäkinen, 2004). The P3 protein is thought to be involved in virus replication (Merits *et al*., 1999), accumulation (Klein *et al*., 1994) symptomatology (Chu *et al*., 1997; Saeaz *et al*., 2000), resistance breaking (Johansen *et al*., 2001; Hjulsager *et al*., 2002; Jenner *et al*., 2002, 2003; Desbiez *et al*., 2003) and cell-to-cell movement (Dallot *et al*., 2001; Johansen *et al*., 2001).

Systemic infection of plants with viruses involves virus replication in the initially infected epidermal or mesophyll cells; cell-to-cell movement through intercellular connections (plasmodesmata) into and through vascular tissue; loading into and long-distance transport through sieve elements (SE) in the phloem following partitioning of photoassimilates; and unloading from SE followed by cell-to-cell movement and infection of other parts (Carrington *et al*., 1996; Santa Curz, 1999; Ruiz-Medrano *et al*., 2001). The virus-encoded movement proteins (MPs) are needed for intracellular transfer of the virus to plasmodesmata and for plasmodesmal regulation to allow passage of virus to the next cell (Ding, 1998; Oparka and Turgeon, 1999; Crawford and Zambryski, 2001). The potyviruses do not encode dedicated MPs but have several multifunctional proteins involved in both viral movement and other functions (Urcuqui-Inchima *et al*., 2001). The HC-Pro, C1, 6k2, VPg and CP are movement-associated proteins (Rajamäki *et al*., 2004). Potyviral CP and HC-Pro are able to move cell-to-cell through the plasmodesmata and prossess RNA binding properties. The CP interacts with unkown plant proteins on the plasmodesmata to facilitate viral movement (Rodrignez-Cerezo *et al*., 1997; Rojas *et al*., 1997). C1 protein has a direct but transient role in the transfer of potyvirus genetic material through virally modified plasmodesmata (Rodrignez-Cerezo *et al*., 1997; Roberts *et al*., 1998). The CP, HC-Pro and VPg proteins are involved in long-distance movement in the phloem (Revers *et al*., 1999). Entry into and exit from the vascular system may be carried out by the HC-Pro protein (Cronin *et al*., 1995).

1.4 Strategies to control or engineer virus diseases

1.4.1 Conventional breeding

Plants, in nature, are generally resistant to most pathogen. The ability of a pathogen to cause disease in a host plant is usually the exception, not the rule. Different interactions are generated between the plant and the virus during each stage of the viral cycle. If the viral particle is not recognized by the host plant, a compatible interaction between the plant and the virus is established, this interaction may be favorable for the virus (Hammond-Kosack and Jones, 2000). However, if the plant recognizes the viral particle, an incompatible interaction that is unfavorable for the virus is established. It is known that plants can recognize the virus, limiting it to the site of infection leading to cell death a phenomenon kown as hypersensitive

response (HR) (Pontier *et al*., 1998). A series of complex cascade defense reactions can be induced, limiting virus replication and virus movement within the host plant (Hammond-Kosack and Jones, 2000). This model of plant-pathogen interaction was kown as "gene for gene" mechanism or RNA-mediated defense (Voinnet, 2001). Activation of hypersensitive resistance (HR) is depending on the recognition of the pathogen mostly by a single specific, dominant genes (Fraser, 1992). In most common situation, a given plant is simply not a suitable biological substrate for multiplication of a given microorganism, resulting in passive resistance. In the case of obligatory parasites such as viruses, absence or inadequacy of a single host factor may lead to the inability for the pathogen to multiply in the host or to systemically invade it (Ishikawa *et al*., 1997; Yamanaka *et al*., 2000).

A large number of naturally occuring dominant and recessive potyvirus resistance genes have been identified (Provvidenti and Hampton, 1991). The nature of these resistances and the relationship between resistances within a host species or between different plant species are generally not known (Johansen *et al* 2001). Recessive resistance to infection is relatively common for potyviruses, comprising as many as 40% of all known resistances (Provvidenti and Hampton, 1991; Diaz-Pendon *et al*., 2004). Recessive resistance is often strain specific, and this has led to identification of VPg as the host-specific pathogenicity determinant (Nicolas *et al*., 1997; Schaad *et al*.,1997; Keller *et al*., 1998; Johansen, 2001). The most likely functional interpretation of recessive resistance is that it reflects the lack of an essential factor required for virus infection and/or the development of disease, i.e. dominant susceptibility gene. These resistances often occur as cluster of resistance specificities targeted against distinct potyviruses or pathotypes of the same virus (Gao *et al*., 2004). The first evidence that recessive resistance may actually be caused by the lack of a host factor came from a study demonstrating that the resistance gene *va* in *Nicotiana tabacum* is caused by loss of a large chromosomal fragment (Noguchi *et al*., 1999). The recessive gene *mlo*, which confers resistance against powdery mildo in barley is, however, an example demonstrating that recessive resistance can also be caused by lack of a host factor that suppresses a resistance response (Büschges *et al*., 1997).

Resistance to WMV-2 has been observed in wild *Cucurbita* species, *C. ecuadorensis* and *C. foetidissima* (Provvidenti *et al*., 1978), *C. pedatifolia*, *C. ficifolia* and *C. moschata* (Nigerian local or Menina) (Providenti, 1986; Gilbert-Albertini *et al*., 1993). The resistance to WMV-2 and to certain other viruses (PRSV-W, ZYMV and CMV) was transferred from *C.*

ecuadorensis to *C. maxima* to produce the cultivar Redlands Trailblazar (Herrington *et al*., 1991). A selection from a Chinese cultivar of *C. maxima* (p1419081) also possesses a good level of resistance (Provvidenti, 1982). Inheritance of resistance in watermelon (*Citrullus lanatus var lanatus)* to ZYMV-china (ZYMV-CH) was conferred by a single resessive gene and tolerance to WMV was controlled by at least two recessive genes (Xu *et al*., 2004). Resistance genes to ZYMV and WMV-2 have been identified in germplasm collections of *Cucurbitaceae*, but no resistance to these two viruses has been found in *C. pepo* (Fuchs and Gonsalves, 1995). Breeding virus resistant varieties is generally slow and inefficient due to two reasons. First, environmental conditions may have a large effect on the expression of virus symptoms. Second, many viruses have multiple strains, some are able to overcome resistance genes. Breeding for virus resistance can be augmented by the use of DNA markers linked to the resistance genes (Brigneti *et al*., 1997; Hämäläinen *et al*., 1997; Sorri *et al*., 1999) and is expected to make breeding for virus resistance more efficient and will lead to faster development of resistant cultivars (Danin-Poleg *et al*., 2000). Forthermore, molecular mapping of these resistance genes aims at cloning them, which will enable their use as resistance factors through genetic transformation.

1.4.2 Cross-protection mediated resistance

Cross protection is a phenomenon in which systemic infection of plant with one virus (the protector) prevents infection with another virus (the challenger) (McKinney, 1929). Cross protection can be used in virus disease management by inoculating attenuated virus strains to crop plants to prevent infection with more severe strains (Rast, 1972; Gonsalves, 1998; Rezende and Pacheco, 1998). Its occurrence between virus strains also has been used as an indicator of relatedness (Matthews, 1991; Bos, 1999). However, the viral genes and genomic sequences playing a role in cross-protection and the reasons for its narrow effectiveness to related viruses (strains) are unknown in most cases. Cross protection viewed as a biological method, offers several advantages for viral disease control. It does not pollute, does not constitute a risk to growers or consumers, and does not interfere with any other practice applied for disease management. Furthermore, application is simple and inexpensive (Rezende and Pacheco, 1998). However, several authors have pointed out some risks associated with the use of mild strains in the field, such as the synergistic effect with other viruses, breakdown of cross-protection, and mutation of the mild strain to a severe form (Fulton, 1986; Posnette and Todd, 1955; Rezende and Müller, 1995).

There are reports on successful use of cross-protection with mild strain of ZYMV in France (Lecoq *et al*., 1991), Taiwan (Wang *et al*., 1991), the UK (Walkey *et al*., 1992), Lebanon (El-Zammer *et al*., 2001), Turkey (Yilmaz *et al*., 1994), California (Perring *et al*., 1995). However, in cucurbits, the comparative efficacy of cross-protection and other management practices has received limited attention.

The cross-protection method has not been extensively used due to the difficulties of obtaining useful mild strains and the fear that the live strain could spread to other crops or mutate into severe form (Fuchs *et al*., 1997).

1.4.3 Coat protein mediated resistance

In 1985, Sanford and Johnston put forward the concept of pathogen derived resistance (PDR), whereby expression, in susceptible organisms, of proteins derived from a pathogen would interfere with the biology of this pathogen. Also, the term coat protein–mediated resistance (CPMR) the resistance comes from the expression of the viral CP gene in transgenic plants. Accumulation of the CP in transgenic plants has been shown to confer resistance to infection by the virus from which the CP gene is derived and by related viruses. The CP gene was the first transgene successfully used to confer virus resistance to transgenic plants (Powell *et al*., 1986). Since then, the approach has been extensively used for resistance against numerous viruses belonging to different groups in many crop plants (Pang *et al*., 2000; Lehman *et al*., 2003; Liao *et al*., 2004; Yang *et al*., 2004).

Coat protein-mediated resistance has been demonstrated in melon (Fang and Grumet, 1993; Gonsalves and Slightom, 1993; Fuchs *et al*., 1997) and in cucumber (Gonsalves *et al*., 1992). In some cases, the resistance in transgenic plants to virus infection was effective (Gonsalves *et al*., 1992; Fang and Grumet, 1993; Fuchs *et al*., 1997) or delayed the appearance of viral disease symptoms (Gonsalves *et al*., 1994). Expression of the coat protein gene of ZYMV in *Nicotiana benthamiana* plants protected the plants against seven different potyviruses [WMV-2, Bean yellow mosaic virus (BYMV), *pea mosaic virus* (PeaMV), *peper mottle virus* (PeMV), *Potato virus Y* (PVY), clover yellow vein virus (CIYVV), and *Tobacco etch virus* (TEV)]. The level of protection depended on the virus and the inoculum's concentration, and expression of the coat protein gene of WMV-II in the study in *Nicotiana benthamiana* showed better protection against these potyviruses than those expressing the ZYMV CP gene (Namba *et al.,* 1992). Fang and Grumet (1993) introduced several constructs derived from the ZYMV

coat protein gene into muskmelon and tobacco plants: the full-length coat protein gene, a conserved central "core" of the coat protein gene, and in antisense version. Transgenic melon plants expressing the full-length coat protein were highly resistant to ZYMV infection. Transgenic plants expressing only the core part of the coat protein showed a limited protection against ZYMV. The antisence construct allowed variable levels of protection, correlated with transcript level i.e. increasing the level of CP expression enhances the plant virus resistance response (Fuchs *et al*., 1997). The approach of CP-mediated resistance was also used to produce transgenic hybrid squash ZW-20 resists to ZYMV and WMV-2 (Fuchs and Gonsalves, 1995). The transgenic F1 hybrid squash containing the ZYMV and WMV-2 CP genes showed excellent resistance to mixed infection by both potyviruses (Arce-Ochoq *et al.*, 1995; Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Tricoli *et al*., 1995). In 1995, this hybrid, marked as cultivar Freedom II, became the first virus-resistant transgenic crop released commercially in the USA. Another transgenic squash line designated CZW-3 and containing the CP genes of CMV, ZYMV, and WMV-2 is resistant to these three viruses (Tricoli *et al*., 1995). The coat protein genes of CMV, ZYMV, and WMV-2 were introduced into cantaloupe line CZW-30, transgenic plants showed high resistance under high disease pressure by mechanical inoculation and /or natural challenge inoculation by indigenous aphid vectors. Only 8 % of these plants were infected by two or three viruses while 99 % of non-transformed plants were mixed infection.

The molecular mechanism of this viral "cross- protection" has remained elusive and controversial. In some cases, the coat protein (CP) of the protective virus was thought to be primarily responsible, either by preventing particle disassembly or by re-encapsidating the incoming genome of the more severe challenge virus, which therefore cannot function (Wilson, 1993; Beachy, 1999; Goldbach *et al*., 2003).

A potential risk of CP-expressing transgenes is that their expression product can coat the nucleic acid of a related incoming virus, giving complete (transcapsidation) or partial (phenotypic mixing) encapsidation of challenging virus RNA by the transgenic CP. This could modify the epidemiological behaviour of the incoming virus (Timmerman-Vaughan, 1998; Hammond *et al*., 1999). Heteroencapsidation can take place in the presence of mixed infections in non-transgenic plants and in transgenic plants expressing a viral CP. In normal plants transcapsidation has been reported to occur between related luteoviruses (Rochow, 1972; Hu *et al*., 1988; Creamer and Falk, 1990), Potyviruses, (Milne *et al.*, 1980) and

vitiviruses (Milne *et al*., 1984). Moreover, non-aphid transmissible potyvirus strains can become transmissible when phenotypic mixing develops in doubly infected plants (Hobbs and McLaughlin, 1990; Bourdin and Lecoq, 1991). Phenotypic mixing has also been experimentally detected in several plants expressing transgenic potyvirus CP and inoculated with a closely related virus (Lecoq *et al*., 1993; Hammond and Dienelt, 1997). Interaction between defective RNAs and CPs in transgenic plant, than abolish or complement virus assembly and/or aphid transmission have also been described (Osbourne *et al*., 1989; Jacquet *et al*., 1998; Varrelmann and Maiss, 2000). Transient co-expression of a functional viral gene and a mutated infectious transcript of potato virus X transferred by particle bombardment has been reported to restore virus functionality (Morozov *et al.*, 1997).

1.4.4 Post-transcriptional gene silencing

Gene silencing and its role in pathogenesis and resistance and by control of gene expression is the major discovery of the past decade in the field of virology, plant biology and biotechnology. RNA silencing, known also as post-transcriptional gene silencing (PTGS) and RNA interference (RNAi), represents a novel cellular pathway conserved in plants and animals that destroys RNA in a sequence-specific manner (Ding, 2000; Matzke *et al.,* 2001; Waterhouse *et al.,* 2001; Hannon, 2002). The phenomenon was first discovered after introducing chalcone synthase gene into petunias in order to increase pigmentation of the flowers (Napoli *et al*., 1990; Van der Krol *et al*., 1990). Surprisingly no increasing in colour was observed as expected, instead, it was shown variegated pigmentation or no colour completely (white flowers) (Jorgensen, 1995). This phenomenon, then called co-suppression, indicated that not only the transgene itself was inactive, but also that the transgene sequences somehow affected the expression of the endogenous loci. It was proposed that the products of degradation of the double-stranded RNA region in the CHS gene might be related to this post-transcriptional gene silencing (PTGS) (Van der Krol *et al*., 1990; Jorgensen *et al.,* 1996). When plants were transformed with homologous of endogenous genes the transgene and the endogenous gene were co-suppressed in some of the lines (Napoli *et al.,* 1990; Jorgensen, 1995), many examples of co-suppression were caused by a post-transcriptional event that resulted in reduced steady-state mRNA levels of the silenced gene (Metzlaff *et al.,* 1997). The silencing does not depend on the presence of endogenously encoded homologous loci; silencing can occur between related transgenes, and it is not only triggered by transgenes but can also be initiated by viruses (Angell and Baulcomble, 1997), known as virus induced gene

silencing (VIGS). Virus induced gene silencing (VIGS) was first used to describe the phenomenon of recovery from virus infection (Van Kammen, 1997). However, the term of VIGS has since been applied almost exclusively to suppress gene expression by means of recombinant viruses (Ruiz *et al*., 1998; Baulcombe, 1999).

RNA interference (RNAi) was initially described in plants and *Caenorhabditis elegans*, when it was discovered that double-stranded RNA (ds RNA) was much more potent at silencing gene expression than either sense or antisense single-stranded RNA (ssRNA) (Fire, *et al.,* 1998; Sharp, 2001; Zamore, 2001). RNA interference (RNAi) has recently been identified as a natural mechanism for the regulation of gene expression in all higher organisms from plants to human (Baulcombe, 2004). In the field of virus resistance, induction of silencing mechanism is triggered by formation of dsRNA within plant cells which has homology with invading virus sequences. Transgene constructs can be arranged as inverted repeats, producing double-stranded RNA (dsRNA), which efficiently trigger silencing of homologous genes (Smith *et al.,* 2000). It was shown that RNA silencing process is sequence specific when Hamilton and Baulcombe (1999) found small dsRNA molecules of around 25nt in transgenic plants corresponding to the transgene. It was shown that the dsRNA silencing trigger is processed to RNA segments of 21-23nt in length named small interfering RNAs (siRNAs) (Zamore *et al*., 2000). The cleaving of the dsRNA into siRNAs occurs by a member of the RNAse III family of enzymes termed Dicer (Bernstein *et al*., 2001; Ketting *et al*., 2001). The siRNA were shown to be associated with nuclease, named RNA-induced silencing complex (RISC) (Figure 2). Analysis of the chemical structure of siRNA shown that they were double-stranded and contained 5-phosphorylated termini and 2 nt 3`-overhangs (Elbashir *et al*., 2001). For each siRNA duplex, only one strand, the so-called guide strand, is present in the active, mature RISC, where as the other strand, named passenger strand, is destroyed, or serve as a primer for invading virus sequence by the help of RNA-depended RNA polymerase to make more dsRNA. This enables siRNA-mediated silencing to spread throughout the plant, by cell-to-cell movement of silencing associated RNA (Yoo *et al*., 2004), resulting in the resistance of the plant against further viral infection. The single-stranded siRNA in RISC guides the sequence-specific degradation of complementary target mRNA. The mRNA is cleaved at a position 10 nt from 5`-end of the guiding siRNA (Elbashir *et al*., 2001; Nykänen *et al*., 2001; Martinez *et al*., 2002). Subsequently, the 5´-mRNA fragment generated by RISC cleavage is rapidly degraded from its 3´-end by the exosome, whereas the 3´-fragment is degraded from its 5´-end by XRNI (Orban and Izaurralde, 2005).

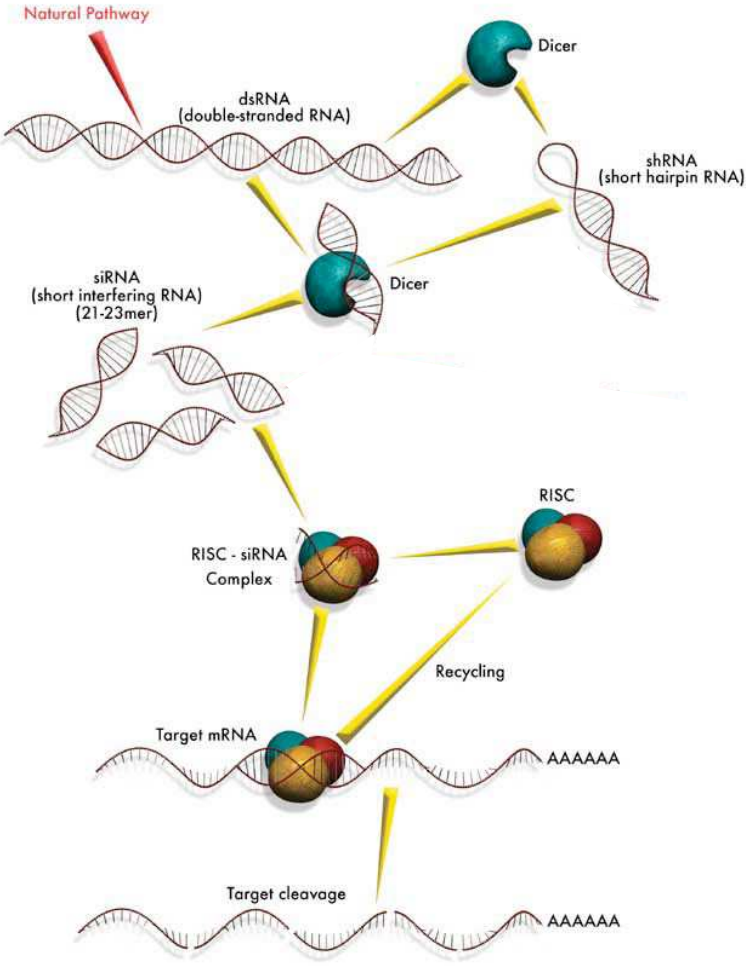


Figure 2: The pathway of RNAi. The endonuclease dicer initiates RNAi by processing long dsRNAs or short hairpin RNAs to 21 nt long dsRNAs with 2 nt 3´- overhangs. The siRNA is then incorporated into RISC and unwinded. The antisence strand guides the active RISC ti the target position on the target mRNA which in turn is sequence specifically cleaved and degraded. (http://webdoc.sub.gwdg.de/diss/2005/gruber/gruber.pdf.)

1.4.4.1 Influence of construct design on the silencing efficiency

Stable RNAi has been developed in a variety of organisms including protozoa (Shi *et al*., 2000), algae (Fuhrmann *et al*., 2001; Soupene *et al*., 2004), fungi (Liu *et al*., 2002), plants (Chuang and Meyerowitz, 2000; Smith *et al*., 2000; Wesley *et al*., 2001; Waterhouse and Helliwell, 2003) and Metazoans (Kennerdell and Carthew, 2000; Tavernarakis *et al*., 2000; Piccin *et al*., 2001; Paddison and Hannon, 2002; Brown *et al*., 2003; Dykxhoorn *et al*., 2003).

It is commonly believed that during cytoplasmic replication of positive-sense single stranded (ss) RNA viruses, the replicative form and replicative intermediates may represent the pool of dsRNA that trigger VIGS (Voinnet, 2001). The discovery that double-stranded RNA (dsRNA) is a more effective inducer of PTGS than either sense or antisense RNA in both plants and animals (Fire *et al*., 1998; Kennerdell and Carthew, 1998; Waterhouse *et al*., 1998; Sanchez-Alvarado and Newmark, 1999) has prompted the development of improved gene-silencing methodology.

It was shown that transgenes designed to express double-stranded or single-stranded self complementary (hairpin) RNA have a similar post-transcriptional silencing effect in plants (Waterhouse *et al*., 1998; Wang and Waterhouse, 2000) and that, in at least two example, almost 100% of plants transformed with an intron-containing hairpin RNA construct showed silencing (Smith *et al*., 2000).

Several lines of evidence indicate that double-stranded RNAs (dsRNAs) or self-complementary hairpin RNAs (hpRNAs) that were produced during the intermediate steps of viral genome replication are the key triggers of the RNA degradation mechanism (Hamilton *et al*., 1998; Waterhouse *et al*., 1998; Bass, 2000). Expression of hairpin RNAs corresponding to viral sequences induced virus resistance in almost 100% of the transgenic plants (Lindbo and Dougherty, 1992; Waterhouse *et al*., 1998; Smith *et al*., 2000). Hairpin RNA-mediated gene silencing exploits the cellular mechanisms that recognize double-stranded RNA (dsRNA) and subject homologous mRNA to sequence-specific degredation. Recent work has demonstrated the potential for hpRNA constructs that produce dsRNA to efficiently silence targeted gene expression (Wesley *et al*., 2003; Akashi *et al*., 2004; Scattat *et al*., 2004). Intron-containing constructs [e.g., Intron hairpin RNA (ihpRNA)] are most effective in inducing gene silencing and can produce independent transgenic lines of which 90-100% exhibit silencing (Wesley *et al*., 2001; Cazzonelli and Velten, 2004).

Common approaches rely on the production of dsRNA by transcription from transgenes that generate complementary (sense and antisense) transcripts or from a single transcript unit consisting of two inverted repeats separated by a spacer (generating a hairpin-loop shaped RNA) (Chuang and Meyerowitz, 2000; Kennerdell and Carthew, 2000; Smith *et al*., 2000; Tavernarakis *et al*., 2000; Fuhrmann *et al*., 2001; Paddison and Hannon, 2002; Dykxhoorn *et al*., 2003; Waterhouse and Helliwell, 2003). However, the level of gene silencing is often

quite variable, depending on the type of construct, transgene copy number, site of integration and target gene (Chuang and Meyerowitz, 2000; Fuhrmann *et al.,* 2001; Piccin *et al.,* 2001; Kalidas and Smith, 2002; Liu *et al.,* 2002; Williams and Rubin, 2002; Waterhouse and Helliwell, 2003; Brown *et al.,* 2003; Kerschen *et al.,* 2004). Transforming plants with a single sense or antisense construct typically can promote PTGS (Van Houdt *et al*., 2000), however it results in only a small proportion of individuals with silenced (in case of viral transgenes: resistant) phenotypes, silenced lines were 15-30% (Smith *et al*., 2000; Wesley *et al*., 2001) or in no more than 10-20% gene silencing efficiency of a transgenic population using either antisense (Ecker and Davis, 1986; Rothstein *et al*., 1987; Sheehy *et al*., 1988: Smith *et al*., 1988) or sense constructs (Napoli *et al*., 1990; Smith *et al*., 1990 Van der Krol *et al*., 1990).

1.4.4.2 Viral silencing suppressor

RNA silencing is an evolutionarily conserved surveillance system that occurs in a broad range of eukaryotic organisms. In plant, RNA silencing acts as an antiviral system; thus virus infection requires suppression of gene silencing (Lakatos *et al*., 2004). As a counter-defensive strategy viruses have evolved suppressor proteins that inhibit various stages of silencing process. These suppressors are diverse in sequence and structure and appear to be encoded by virtually any type of plant virus (Moissiard and Voinnet, 2004). Many plant viruses have been known to evolve a suppressor(s) of RNA silencing to counteract RNA silencing (Lu *et al*., 2004; Qi *et al*., 2004; Roth *et al*., 2004; Silhavy and Burgyan, 2004) and they act at different steps in the PTGS pathway. For instance, the helper component protein (HC-Pro) of potyvirus, which interferes with RNA silencing at a step upstream of the production of siRNA (Brigneti *et al*., 1998; Llave *et al*., 2000; Mallory *et al*., 2001). It also affects micro RNA (miRNA) biogenesis and function (Kasschau *et al*., 2003; Waterhouse and Helliwell, 2003 Chapman *et al*., 2004; Silhavy and Burgyan, 2004), or it seem to impair the production or stability of siRNA (Mallory *et al*., 2002). These results suggest that the mechanism of HC-Pro function are complex and remain to be fully understood (Baulcombe, 2004). On the other hand, the 2b protein encoded by cucumber mosaic virus (CMV) could prevent spread of RNA silencing signals by blocking their translocation (Brigneti *et al*., 1998; Guo and Ding, 2002), or prevent the initiation of silencing (Kasschau and Carrington, 1998).

The p19 protein encoded by Tomato bushy stunt virus (TBSV) is a well-studied silencing suppressor protein. In an infiltration assay, p19 was found to be one of the most effective

suppressors of silencing and completely prevented the production/accumulation in plants of both short (21-22nt) and long (24-26nt) forms of siRNA (Hamilton *et al*., 2002; Havelda *et al*., 2003). The p19 protein of Cymbdium ringspot virus (CymRSV), a virus smilar to TBSV, was shown to bind in vitro specifically to (ds) siRNAs generated by PTGS but not to bind to singles-stranded (ss) siRNAs, long ss-, or dsRNA (Silhavy *et al*., 2002; Lakatos *et al*., 2004). The structure of p19 has been determined, revealing that the siRNA duplex is bound by a p19 homodimer and that conserved tryptophan residues cap either end of the (ds) siRNA, ensuring the binding of only 19 to 21nt RNAs (Vargason *et al*., 2003; Ye *et al*., 2003) or 20 to 22nt RNAs and if the siRNA duplexes more than 22 or less than 20nt in length they bind but in less efficiency (Baulcombe and Molnár, 2004). The affinity of p19 for 19-nt and 25-nt siRNAs is 320- and 37- fold lower than for the 21-nt species, respectively (Vargason *et al*., 2003). Thus, silencing suppressor p19 binds siRNAs and sequesters them, thereby preventing their incorporation into RISC. In addition, p19 is also known to be involved in several other aspects of viral infection. TBSV that does not express p19 is unable to move systemically in spinach, does move systemically in *Nicotiana benthamiana* but no longer induces systemic necrosis, does not induce local necrosis in *Nicotiana tabaccum*, and has impaired local, cell-to-cell movement in pepper (Scholthof *et al*., 1995a, 1995b; Turina *et al*., 2003). Silencing suppressor proteins of viruses can act not only to negate self-silencing, and silencing of co-infecting unrelated viruses. For example, the synergistic interaction of potato virus X (PVX) and potato virus Y (PVY), which is associated with increased replication of PVX, results from the action of the HC-Pro protein of potato virus Y in counteracting self-silencing of PVX replication (Pruss *et al*., 1997).

Agrobacterium-mediated transient gene expression has emerged as a valuable tool to study PTGS phenomena in plants (Van der Hoorn *et al*., 2000; Johansen *et al*., 2001). PTGS is an important factor in any transient gene expression system and significantly reduces the efficiency of *Agrobacterium*-mediated transient gene expression (Voinnet *et al*., 2003). This limitation can be at least partially overcome by using virus-encoded suppressor proteins, such as p19 and HC-Pro, which have been shown to reduce the effects of PTGS, enhancing reporter gene expression levels several fold (Brigneti *et al*., 1998; Voinnet *et al*., 1999).

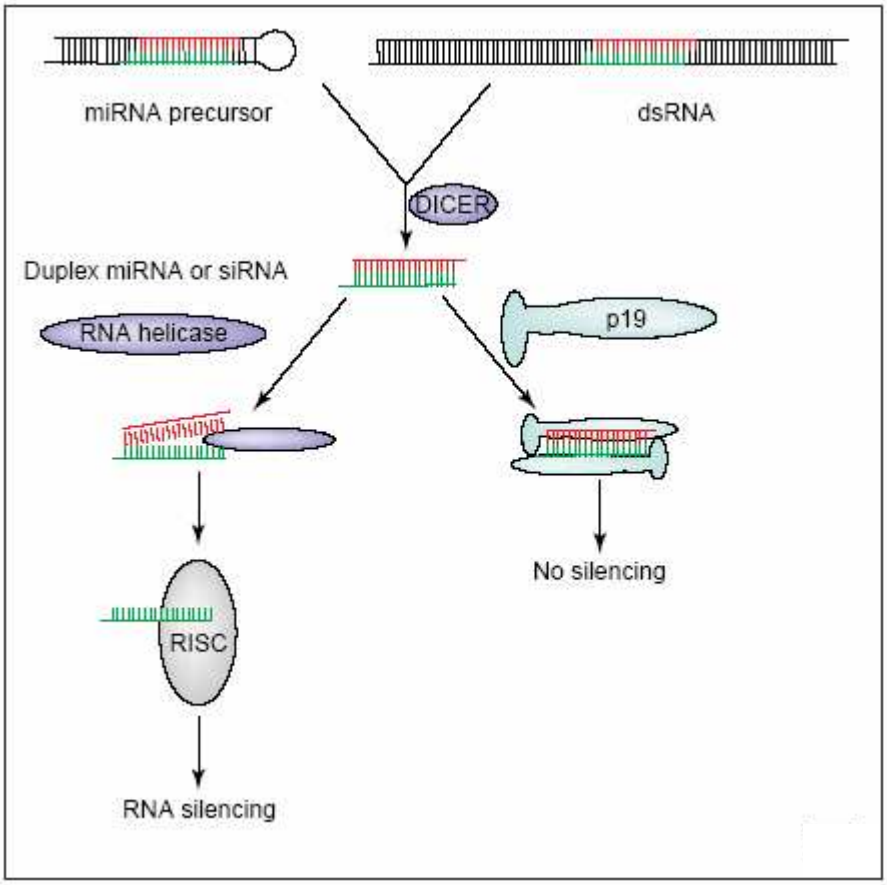


Figure 3: A model of RNA interference specificity complex (RISC) assembly in the presence or absence of p19. Dicing of double-stranded RNA (dsRNA) or of a micro RNA (miRNA) precursor is proposed to generate duplex small interfering (siRNA) or miRNA intermediates. The duplex RNA is a substrate for an RNA helicase that selects one of the two strands for incorporation into RISC; the other strand is degraded. In the presence of p19 the helicase is unable to access the duplex RNA and it is proposed that strand separation and RISC assembly does not occur (Baulcombe and Molnár, 2004).

1.5 Biotechnology and genetic engineering in plant

Two important discovers revolutionized plant biotechnology and enabled scientists to develop new procedures for crop improvement. The first discovery was the unique capability of single cell to regenerate and give rise to whole plant with all the genetic features of the parent (Birch, 1997). The second discovery was that gene transfer into a plant genome could be mediated by a plant-infecting bacterium, especially *Agrobacterium tumefaciens* (Van Larebeke *et al*., 1974; Zaenen *et al*., 1974). Together these discover enabled regeneration of

transgenic plants (from a single cell with stable expression of an introduced foreign gene. Transgenic plant technology had obvious advantages over conventional plant breeding approaches for crop improvement. Thus, one of the first target areas using transgenic plants was plant protection against disease, and several strategies were used to protect against pathogens or other disease-causing factors. Plants are regenerated from cell culture via two methods, somatic embryogenesis and organogenesis. Both are controlled by plant growth regulators, factors added to the culture medium, and tissue culture conditions.

1.5.1 Somatic embryogenesis

Somatic embryogenesis is a development process of somatic cells, which resembles morphologically zygotic embryogenesis (Neumann, 1995). The somatic embryo is an independent bipolar structure and is not viscerally attached to the tissue origin (Ammirato, 1987). The developmental switching of somatic embryogenesis involves different gene expression conferring on the somatic cells the ability to manifest the embryogenic potential (Raghavan, 1997; Raghavan, 2000). Somatic embryogenesis thus involves many of molecular events encompassing not only differential gene expression, but also various signal transduction pathway for activating/repressing numerous gene sets, may of which are yet to be identified and characterized (Chugh and Khurana, 2002). The process of somatic embryogenesis is similar to zygotic embryogenesis (Figure 4); it can be divided into a morphogenic phase, characterized by pattern formation, morphogenesis and differentiation of the basic tissue, followed by a maturation phase (Debeaujon and Branchard, 1993; Luo *et al*., 2001).

Production of somatic embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct occurring somatic embryogenesis involves the formation of an asexual embryo from a single cell or a group of cells on a part of the explant tissue without an intervening callus phase. The indirect embryogenesis consists of establishing an explant in culture, subsequent proliferation of embryogenic callus or suspension, initiation of pro-embryos and inducing bipolar embryo from pro-embryo initials (Sharp, *et al.,* 1980). The levels of embryo induction and plant regeneration from *in vitro* tissue cultures are basically influenced by genotype and physiological status of donor plant, the plant organ used as an explant, the culture medium and the interaction between them (Lazer, *et al*., 1984; Mathias and Simpson, 1986; Bregitzer, 1992). Somatic embryogenesis is important to the genetic

improvement of plants using biotechnology for the production of micropropagation, mutation breeding, cryopreservation and genetic transformation (Bhojwani and Razadan, 1996).

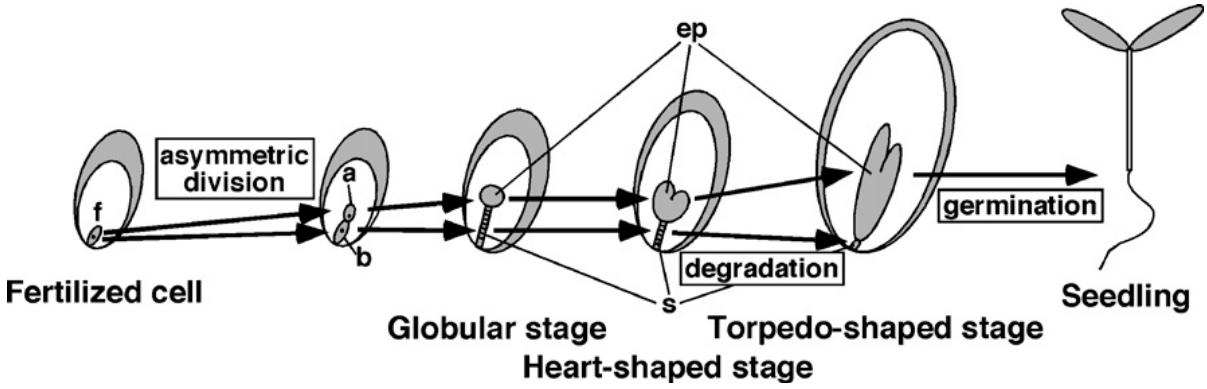


Figure 4: Model scheme of zygotic embryogenesis in angiosperms. a, apical cell; b, basal cell; ep, embryo proper; f, fertilized cell; s, suspensor (Umehara and Kamada, 2005).

Several types and combination of plant growth regulators and explant sources have been tested for induction of somatic embryogenesis from various *Cucurbitaceae* species (Jelaska *et al*., 1985; Chee, 1991, Chee, 1992; Kintzios *et al*., 2002). Auxins are absolutely required for the induction of somatic embryogenesis from different *Cucurbitaceae* species (Debeaujon and Branchard, 1993). In Many studies somatic embryogenesis was obtained with a high concentration of an auxin source often in combination with other auxins or cytokinins (Jelaska *et al*., 1985; Juretic and Jelaska, 1991; Tabei *et al*., 1991; Kintzios *et al*., 2002). Maturation of squash somatic embryo was commonly conducted on medium without growth regulators (Kintzios *et al*., 2002).

1.5.2 *Agrobacterium*- mediated transformation

Members of the genus *Agrobacterium* are ubiquitous components of the soil microflora, the vast majority of which are saprophytic, surviving primarily on decaying organic matter. However, several species of Agrobacteria cause neoplastic diseases in plant, including *Agrobacterium rhizogenes* (hairy root disease), *Agrobacterium rubi* (cane gall disease), *Agrobacterium tumefaciens* (crown gall disease) and *Agrobacterium vitis* [formally termed *Bacillus ampelopsorae* (Cavara, 1897)] (crown gall of grape), as well as an virulent strain *Agrobacterium radiobacter*. Almost 100 years after its discovery as the causative agent of crown gall disease (Smith and Townsend, 1907), the Gram-negative *A. tumefaciens* is still

central to diverse fields of biological and biotechnological research, ranging from its use in plant genetic engineering (Newell, 2000) to representing a model system for studies of basic biological processes underlying genetic transformation (Gelvin, 2000; Tzfira and Citovsky, 2002). The natural host range of *Agrobacterium* among species of the plant kingdom is rather extensive and includes members of most of the plant families. This had already been determined in the early 1970s by infecting various plant species with wild-type of *A. tumefaciens* (De Cleene and De Ley, 1976) or *A. rhizogenes* (De Cleene and De Ley, 1981). Recently, *Agrobacterium* was found capable, under laboratory condition, of genetically transforming eukaryotic organisms that do not belong to the plant kingdom ranging from yeast (Bundock *et al*., 1995) to filamentous fungi and cultivated mushrooms (De Groot *et al*., 1998) to cultured human cells (Kunik *et al*., 2001). The crown gall disease or genetic transformation by *A. tumefaciens* results from the transfer of a specific DNA fragment (transferred DNA or T-DNA) located at bacterial Ti (tumour-inducing) plasmid within the bacterium to the plant cell, followed the integration into the host cell genome and expression in the transformed host cell (Gelvin, 1998). Three genetic elements, *Agrobacterium* chromosomal virulence genes (*chv),* T-DNA delimited by a right and left borders and Ti plasmid virulence genes (*vir*) constitute the T-DNA transfer machinery (Gelvin, 2000; Zupan *et al*., 2000; Tzfira and Citovsky, 2000).

Researchers have taken advantage of this naturally occurring transfer mechanism, and designed DNA vectors from the Ti plasmid DNA by replacing the T-DNA fragment that contained oncogenes, with a DNA of interest. This achievement has allowed development of a binary vector system to transfer foreign DNA into plants. Two plasmids are being used in the binary vector, i. e., the Ti plasmid contained the *vir* genes with oncogenes deleted (named disarmed or *vir* helper plasmid), and a genetically engineered T-DNA plasmid containing the desired genes (An *et al*., 1985). The binary vector system comprising an octopine-type *vir* helper strain such as LBA4404 (Hoekema *et al.,* 1983) that harbours the disarmed Ach5 Ti plasmid and a binary vector such as pBin19 (Bevan, 1984) is very commonly used for plant transformation. The available range of vir helper strains has been expanded with the nopaline-type MP90 (Konez and Schell, 1986) and the L, L-succinamopine-type EHA101 (Hood *et al*., 1986). The bacterial kanamycin resistance gene in EHA101 was deleted to develop the *vir* helper strain EHA 105 (Hood *et al.,* 1993). Higher transformation efficiency can be achieved with EHA101 and EHA105 harbouring the (supervirulent) genes in many recalcitrant plants such as rice (Rashid *et al.,* 1996), wheat (Cheng *et al*., 1997), and barley (Tingay *et al*., 1997).

A new *vir* helper strain pTiChry5 has been constructed from an *Agrobacterium* strain virulent on soybean (Torisky *et al*., 1997). This strain has good potential for legume transformation.

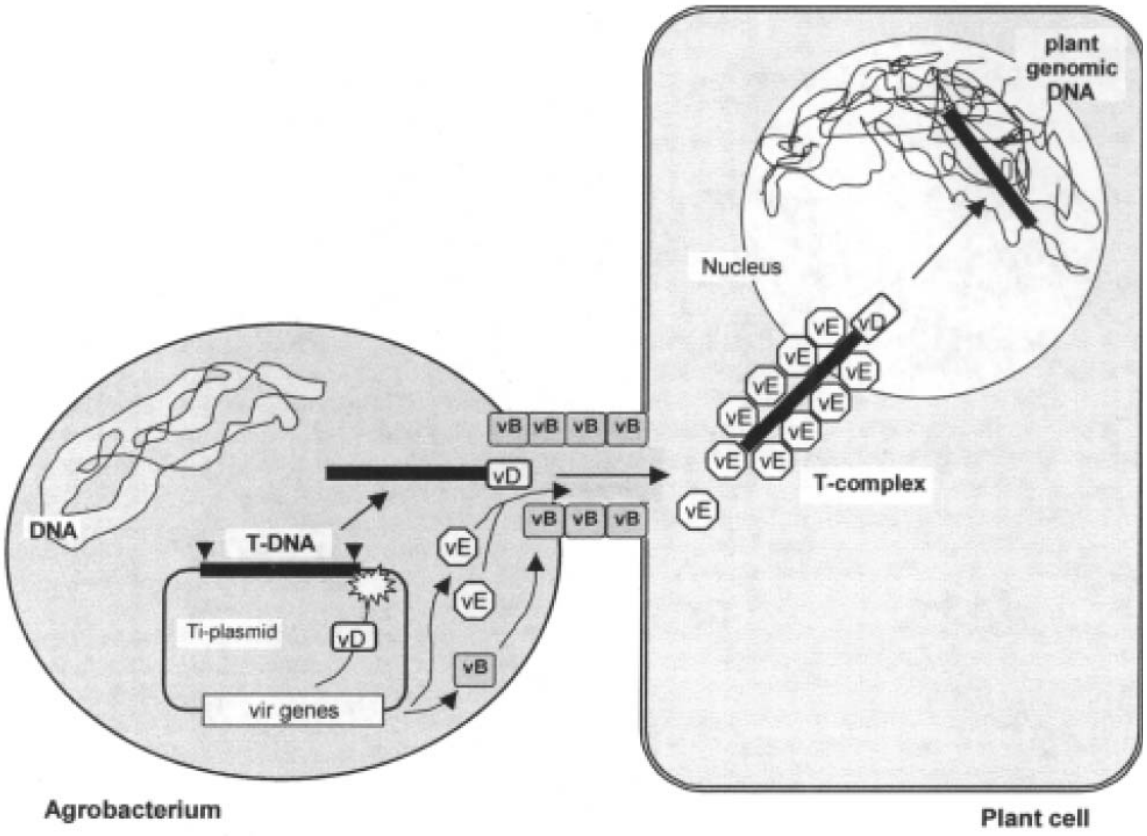


Figure 5: Schematic representation of T-DNA transfer from *Agrobacterium* to the plant genome (Wilson *et al.*, 2004).

Possessing of bacterial infection and T-DNA transfer is initiated by releasing amino acids, organic acids and sugars from wounded plant cells which act as chemoattractants to tumorigenic Agrobacteria, which bind to plant cells in a polar orientation upon reaching the wound site (Gelvin, 2000; Winans, 1992). Weak attachment to the plant cell is first achieved through synthesis of acetylated polysaccharides, followed by strong binding through the extrusion of cellulose fibrils (Gelvin, 2000). Simultaneously, the *vir* regulon, a set of operons required for the transfer of virulent DNA, is activated by the *vir*A/*vir*G two-component regulatory system (Stachel and Zambryski, 1986). The presence of acidic extracellular conditions (pH 5.0-5.5), phenolic compounds and monosaccharides at a plant wound site directly or indirectly induce autophosphorylation of the transmembrane receptor kinase *vir*A (Winans, 1992). Activated *vir*A transfers its phosphate to the cytoplasmic *vir*G protein, which

then binds to the vir box enhancer elements in the promoters of the *vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G operons, upregulating transcription (Rossi *et al.,* 1998). Through the co-operative action of the *Vir*D1 and *Vir*D2 proteins, a single-stranded DNA fragment (the T-strand) is synthesized from one or more regions of the tumor –inducing (Ti) plasmid delimited by specific 25 nucleotide repeat sequences (Winans, 1992). *Vir*D2 remains covalently bound to the 5′ end of the T-strand, which is subsequently coated by the *vir*E2 single-stranded DNA binding protein, although it is unclear whether *vir*E2 associates with the T-strand in the bacterial cell or in the plant cell (Vergunst, 2000).

The T-strand/*vir* protein complex (T-complex) is exported from *Agrobacterium* to the plant cell cytoplasm through a type IV bacterial secretion apparatus encoded by the *vir*B operon and *vir*D4 (Vergunst, 2000). Both *vir*E2 *vir*D2 possess nuclear localization sequences and interact with endogenous plant proteins thought to facilitate targeting of the T-complex to the nucleus, including an importin- a type 2C protein phosphatase and three cyclophilins (*vir*D2-interating factors), and VIP1 and VIP2 (VirE2-interating factors) (Gelvin, 2000; Tzfira and Citovsky, 2002). The *Agrobacterium* transferred DNA (T-DNA) can then integrate into the plant cell genome through non- homologous recombination in a process that appears to require plant-encoded proteins (probably enzymes related to DNA repair or recombination) (Ziemienowicz, *et al.,* 2000; Van Attikum, *et al.,* 2001).

1.5.3 Selectable marker genes

The incorporation of a selectable marker gene during transformation is essential to identify transgenic cells and to regenerate efficiently transgenic plants. The most commonly used selectable marker genes include neomycine phosphotransferase-II (*npt-II*) which was isolated from Tn5 transposon of *Escherichia coli* (Mazodier *et al*., 1985), hygromycin phosphotransferase *(hpt)* that was isolated from *E. coli* (Waldron *et al*., 1985) bialaphos resistance (*bar)* which was isolated from *Streptomyces hygroscopicus* (Thompson *et al*., 1987), and phosphinothricin acetyltransferase (*pat)* that was isolated from *Streptomyces viridochromogenes* (Strauch *et al*., 1988). The *npt-II* gene confers resistance to kanamycin and other related aminoglycoside antibiotics such as G418 and paromomycin sulphate, neomycin and amikacin (Bevan *et al*., 1983). The *hpt* gene confers resistance to hygromycin B (Van den Elzen *et al*., 1985). The *bar* and *pat* genes confer resistance to herbicide BASTA® and phosphinothricin (PPT), respectively (Murakami *et al*., 1986). PPT, the active agent of

the herbicide BASTA acts by inhibition of glutamine synthetase, an enzyme that produces glutamine from ammonia and glutamate (Tachibana and Kaneko, 1986). Accumulation of ammonia after exposure of plant cells to PPT could be the primary reason for PPT toxicity (Tachibana *et al*., 1986; Ziegler and Wild, 1989). Kanamycin is an aminoglycoside antibiotic isolated from the soil bacterium *Streptomyces kanamyceticus*. Aminoglycosides act primarily by binding to the 30S subunit of prokaryotic ribosomes and inhibiting protein synthesis (Mingeot-Leclercq *et al*., 1999). In eukaryotes they also inhibit protein synthesis by binding to the ribosomal complex (Vasquez, 1979; Bar-Nun *et al*., 1983), hence their usefulness as selection agents for plant and mammalian genetic transformation.

1.6 Aims of the study

The main objective of the current study is to introduce virus resistance against two squash infecting viruses, *Zucchini yellow mosaic virus* and *Watermelon mosaic virus*, into the genome of squash (*Cucurbita pepo* L.) by induction of RNAi in plant. This goal will be reached by the following milestones: (i) establishment of an in vitro regeneration procedure through somatic embryogenesis; (ii) establishment of a genetic transformation system through *Agrobacterium*-mediated transformation method; (iii) designing an inverted repeat construct from *Watermelon mosaic virus* and *Zucchini yellow mosaic virus* containing an Intron as a spacer; (iv) transfer of the inverted repeat construct into plant genome to obtain transgenic plants resistant to these viruses by gene silencing; and (v) screening for induced RNA interfering in the transgenic plants which is main point of resistance against virus invaders in plant.

2 Material and Methods

2.1 Biological material

2.1.1 Bacterial strains

Three E. coli strains were used for cloning experiments: DH5α (Invitogene and Gibco), JM110 (Stratagene and Promega) and Invα (Invitrogen) and the following four A. tumefaciens strains were used for squash transformation: AGL1 (Lazo et al., 1991) carries C58Rif^R as chromosolmal DNA and pEHA105 as helper plasmid, ATHV (Hood et al., 1986) carries C58C1Rif^R as chromosomal DNA and pEHA101 devoid from the bacterial Km^R gene as helper plasmid, GV3101pMp90RK (Koncz and Schell, 1986) carries C58Rif^R as chromosolmal DNA and pMP90RK as helper plasmid and LBA4404 (Hoekema et al., 1983) carries TiAchRif^R as chromosomal DNA and pAL4404 as helper plasmid.

2.1.2 Plasmids

Three plasmids: pTPCR (pUC19), pBluescript+/- and pRT101 were used for cloning and construction of inverted repeat constructs of WMV and ZYMV and one plasmid: pCatgfp was used for cloning of sensor constructs of WMV and ZYMV. Several binary vector plasmids pPBGUS, pPNgfp, pPBgfp, pPZP200, pPZPnpt, pPZPbar, pPin-mgfpER5, pbarp19B53His and pKBgfp were used in plant transformation. All used plasmids for cloning and binary vectors are outlined in the appendix.

2.1.3 Primers

Several primer pairs were used for RT-PCR, PCR amplification and DNA sequencing are outlined in the appendix (Table 4, 5 and 6).

2.1.4 DNA ladder

The DNA ladders were used to determine the size of separated DNA fragments in electrophoresis gel such as 1 Kb, 100 bp, or λ-digested with *Pst*I and the DNA molecular weight marker VII, digoxigenin-labeled (Roche).

2.1.5 Plant material

Seeds of three cultivars of summer squash of *Cucurbita* pepo were used: CX3005 and CX3006 were provided from Nunhems Netherlands BV Company and Dundoo ez F1 and *Nicotiana benthamiana* seeds from our institute.

2.2 Tissue culture methods

2.2.1 Induction and explant preparation

●Explants derived from mature seed

Seeds were surfaced sterilized according to method of Chee (1992) with 1% sodium hypochlorite containing 0.2% (2µl/ml) tween-20 for 10 minutes and then rinsed three times with sterile water. The seed coat were removed by gloved hand, sterilised again with 1% sodium hypochlorite containing 0.2% (2µl/ml) for 25 minutes, rinsed three times with sterile water, followed by 70% alcohol for one minute and rinsed three times with sterile water. Sterilized seeds were cut transversely into two unequal sections; one section consisted of the embryonic axis and one-third of the cotyledon and the remaining section contain two thirds of cotyledons. The explants including the embryonic axis and one-third of the cotyledon were cultured facing up and the explants containing only the cotyledons were cultured horizontally. The two sections were cultured together in perty dishes on MS medium (Murashige and Skoog, 1962); (two seeds/plate).

●Explants derived from seedling (shoot tip and cotyledon)

The sterilized seeds were incubated on MS medium in darkness at 25° C. After 10-15 days, shoot apices consisting of the apical domes and some supporting tissues were excised from the grown seedling. The apices were cut into longitudinal halves and cultured horizontally on the cut side on MS induction medium, at the same time, the cotyledon leaves were removed, divided into 1-cm pieces, and cultured horizontally on the MS medium in at 25° C in darkness.

● Explants derived from leaves

Sterilized seeds were cultured on MS medium containing 10 g/l agar and 20 g/l sucrose and germinated under diffuse light at 25°C. The plantlets were propagated successively; the leaves were excised and divided into 1-cm pieces long (8 to 10 pieces/plate).

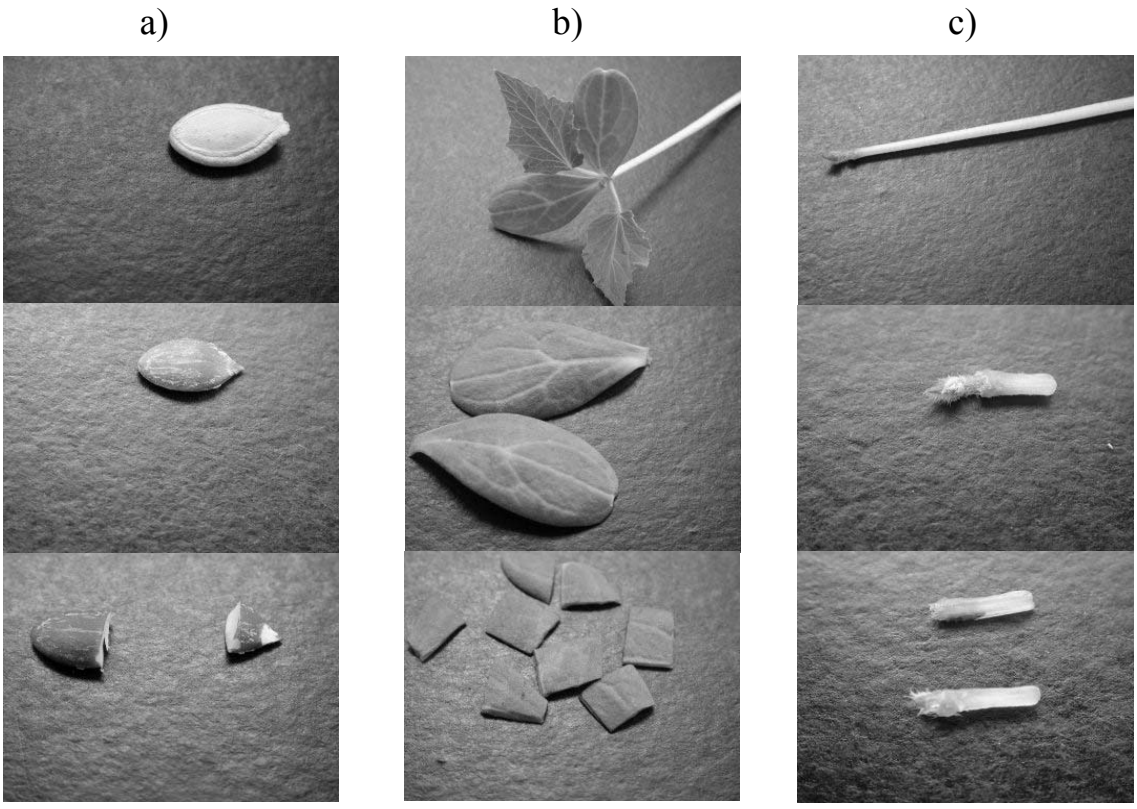


Figure 4: Preparation of explants; a) mature seed, b) cotyledons and leaves, c) shoot tips.

2.2.2 Media and subculture period for induction of somatic embryogenesis

The explants induction media for all the experiments contained 8 g/l micro agar and 30 g/l sucrose. The explants were sub-cultured every 4-5 weeks. The MS media was adjusted to 5.8 pH prior autoclaving at 110° C for 25 minutes. All the previous explants were incubated on MS medium containing different plant growth regulators as following: 2, 5, and 10 mg/l 2, 4-D as auxin alone or in combination with 1.5 or 3 mg/l kinetin as cytokinin, another experiment was carried out with a combination of 2,4,5-T, BAP and NAA as following: (0.6, 0.4, 0.05 mg/l), (1.2, 0.8, 0.1 mg/l) and (2.4, 1.6, 0.2 mg/l), respectively. The explants were sub-cultured every 4 to 5 weeks on a fresh medium.

2.2.3 Plant regeneration from somatic embryos

Somatic embryos induced on hormone medium were transferred onto propagation medium contained 5 mg/l of 2, 4-D and 3 mg/l of kinetin for nine weeks then they were transferred onto regeneration medium MS hormone free for 2 weeks and subcultured on MS medium supplemented with activated charcoal at 5 g/l for 4 weeks. Afterwards the embryos were sub-cultured again on fresh medium and moved to light/dark growth chamber 16/8 for another 4 weeks. The experiment was carried out with four plates each contains four callus clusters; each callus cluster was weight about 500 mg.

2.3 *Agrobacterium*-mediated transformation of squash

2.3.1 Transient expression of GUS gene in squash embryogenic calli

Agrobacterium strains harbouring pPBGUS as a binary plasmid which carries bar gene as a selectable marker gene were moved from -70°C and streaked onto LB agar plates supplemented with 300 mg/l spectinomycin (spec)/streptomycin (strep). The agar plates were incubated upside down at 28°C for 2 days. A single colony was picked and grown overnight at 37°C in liquid LB medium contains spec/strep 300mg/L with shaking at 220 rpm. The *Agrobacterium* culture was collected in falcon tubes 15 or 50 ml, centrifuged at 5000 rpm at room temperature for 13 min. The pellet was resuspended in ½ volumes in liquid MS

medium. To adjust the O.D., 0.1 ml of the resuspended bacteria was diluted in 0.5 ml (1:5) or 1 ml (1: 10) in centrifuge tubes (1.5 ml) and 1ml of MS medium was taken without bacteria as blank for zeroing the spectrophotometer. The absorbance at 600 nm was measured; the resusbended bacterial culture was diluted in MS liqied medium to bring 0.5 O.D. and 100 μM acetosyringone was added. An appropriate amount of this culture was mixed with squash embryogenic calli, then the calli were transferred onto solid MS medium continig 5 mg/l 2,4-D and 3 mg/l Kinetin. Five experiments were done as follows: i) the four indicated *Agrobacterium* strains were used in the transformation, ii) acetosyringone was added at different concentrations 0.1, 0.2, 0.5 and 1 mM to liquid infection medium in which the embryogenic calli were immersed for 1 h, and then the calli were transferred to solid MS contains the same acetosyringone concentration or MS acetosyringone free medium, iii) optical density was adjusted to three concentrations 0.1, 0.5 and 0.85, iv) different sub-culture length on fresh MS medium 1, 5, 9, 13 and 21 days, and v) washing and without washing of squash calli with MS liquid medium prior to *Agrobacterium* transformation. Eight calli per treatment were used and replicated two times for sub-culture length and acetosyringone concentration, three times for *Agrobacterium* concentration and four times for MS washing and *Agrobacterium* strains. After a three days co-cultivation period at 25°C in darkness; the calli were transferred onto MS medium containing 500 mg/l carbenicillin (cb) to inhibit *Agrobacterium* growth of ATHV, LBA4404, GV3101pMP90RK and cefotaxime/augmentin 200 mg/l both for AGL1 strain. The blue spots of GUS gene expression were visually estimated under binocular microscope.

2.3.2 Histochemical Gus assay for squash embryogenic calli

Histochemical Gus assay was performed three days after transfer of embryogenic calli to the selection medium (six days after infection) according to Jefferson; 1987, where one part of the X-Gluc A was added to 49 parts of Gus buffer, then the calli were immersed in this mixture, and incubated overnight at 37°C. The mixture was replaced with 70% ethanol and blue spots were accounted visually under binocular microscope.

Gus buffer

$NaH_2PO_4.H_2O$	6.0 g/l
Na-EDTA	0.37 g/l
$K_4Fe\ (CN)_{6.3H2O}$	0.422 g/l

$K_3Fe(CN)_6$	0.329 g/l
Triton X-100	0.1 %
pH	7.0

Filter sterilization and kept from light with aluminium foil in 4°C

X-glucoronide (X-Gluc A)

25mg/ml in Dimethyl sulfoxide (DMSO), kept in –20°C

2.3.3 Establishing of a selection system

To determine the optimal concentration of selection agents, several selection agents and different concentration were used with non-transformed calli as follows: 1) kanamycin at 25, 50 and 100 mg/l and phosphinothricin at 2, 10 and 20 mg/l. 2) kanamycin at 100, 200 and 300 mg/l, paromomycin at 25, 50 and 100 mg/l, and combination of kanamycin/paromomycin at 25/25, 50/50, and 100/100 mg/l for one month. Selection of transformed calli was carried out seven days after transformation of squash embryogenic calli with *Agrobacterium tumefaciens* ATHV strain. The *Agrobacterium* harboured either pPNgfp binary vector which carries *nptII* as selectable marker gene conferring resistance to kanamycin and paromomycin or pBargfp which carries *bar* as selectable marker gene conferring resistance to PPT. The selection agents were applied to select transformed cells as follows: Kanamycin at 50, 100, 150, 200 and 300 mg/l, paromoycin at the same concentration, kanamycin/paromomycin at 25/25, 50/50, 75/75, 100/100 mg/l and 150/150, and PPT at 2.5, 5.0, 7.5, and 12.5 mg/l. Eight calli were used per treatment for one time and sub-cultured every 4 weeks for four months. All selection agents were added to MS medium with 5 mg/l 2, 4-D and 3 mg/l kinetin and incubated in darkness at 25° C. The GFP expression was visually estimated with florescent microscope.

2.3.4 Transformation of *Nicotiana bentamiana*

Agrobacterium transformation ATHV strain harboured pPZPnpt binary vector was used for genetic transformation. This binary vector contained inverted repeat constructs of WMV-2 and ZYMV sequences under control of 35S promoter which were constructed in this study. The *Agrobacterium* was grown in LB media containing spec/strep 300 mg/l both. Leaf discs of wild type *Nicotiana bentamiana* plant was grown *in vitro* from seeds. Leaf discs were

mixed with *Agrobacterium* culture for 1 h and incubated for 2 days at 25 °C onto MS medium supplemented with 1mg/l BAP and 0.1 g/l NAA. Then the explants were transferred onto MS medium contained 500 mg/l carbenicillin, 100 mg/l kanamycin and the same photohormones. The explants were sub-cultured every three to four weeks onto fresh medium until callus and shoots began to form (6 to 8 weeks). Developed shoots were individually separated and transferred onto hormone free media. Regenerated plantlets formed roots on kanamycin medium and were transferred to soil.

2.4 Molecular biological methods

2.4.1 Total RNA extraction from squash leaf materials

Total RNA was extracted from leaves of putative infected squash plant tissues by using procedures described by Napoli *et al*., (1990) as follows: 100-200 mg leaf tissues were ground in liquid nitrogen by using autoclaved and pre-cooled mortar and pestles. The ground tissues were then homogenized in 500 µl of NE-buffer. The suspension was transferred to a reaction tube, 500 µl of phenol were added, vortexed and centrifuged for 2 min at 4C°at 13000 rpm. The upper phase was transferred to a fresh tube, combination of 500 µl of chloroform/ isoamylalcohol 24: 1 were added, vortexed, and centrifuged for 2 min at 4C° at 13000 rpm. The supernatant was afresh transferred to a new tube, for precipitation 0.2 volume of 8 M Lithium chloride LiCl pH 7.5 was added or LiAc if present, centrifuged for 30 min at 13000 rpm at 4C°. A pellet was resuspended in 100 µl DEPC H_2O. The precipitation was again done by adding 1/10 volume of 3M sodium acetate (NaAc) pH 5.2, 2.5 volume of 100% cold ethanol and incubated for 1h on ice. The pellet was again collected by centrifuging for 30 min at 1300 rpm at 4C°. To wash the salt 100 µl of 70% ethanol were added, vortexed, centrifuged for 5 min at 13000 rpm at 4C° and dried under vacuum or air-dried. Then the pellet was dissolved in 35 µl DEPC water. To check the presence of RNA, 4 µl were mixed with 4 µl FDE dye, incubated at 65° C for 10 min and loaded onto 1% agarose gel electrophoresis at 100 volt, 40mA for 200 min.

NE-buffer 100ml

Final conc.	*Conc. of stock solution*	*Volume of stock solution*
100 mM NaCl	5 M NaCl	2 ml
10 mM Tris/HCl (pH 7.5)	1 M Tris/HCl pH 7.5	1 ml
10 mM EDTA	0.5 M EDTA	0.2 ml up to 90 ml dH_2O and autoclaved
1% SDS	10% SDS	10 ml

FDE dye

900 µl formamid deionisirt

20 µl EDTA 0.5 M (pH8)

80µl DEPC-H_2O

2.4.2 Polymerase chain reaction (PCR)

PCR is the enzymatic method to make multiple copies of pre-selected segment of DNA via repetitive cycles of DNA synthesis. The reaction uses two specific oligonucleotides (primers), which hybridize to sense and antisense strands of the template DNA fragment, four deoxyribonucleotide triphosphates (dNTP`s) and a heat-stable DNA polymerase. Each cycle consists of three reactions which take place under different temperature. First, the double-stranded DNA is converted into two single strands (denaturation 94°C). They function as templates for the synthesis of new DNA. Second, the reaction is cooled (50-60°C) to allow the annealing (hybridization) of primers to the complementary DNA strands. Third, DNA polymerase extends both DNA strands at 72°C (synthesis) starting from the primers. Because the DNA molecules synthesized in each cycle can serve as a template in the next cycle. The number of target DNA copies approximately doubles by every cycle. After a certain number of cycles, the target DNA fragment between two primers is specifically synthesized. The repeating cycles of heating and cooling take place in a thermocycler.

The PCR was used heir to amplify 150bp in the previously synthesized sequences of WMV-2 and ZYMV by RT-PCR. The PCR amplification was carried out in 25 µl reactions as follows: about 20 ng (1µl) of DNA template (plasmid) which contains either WMV-2 or ZYMV sequences in separated reaction tubes; 12 pmol (0.25 µl) of two specific primers (sense and antisense); 1 µl of a mixture of four deoxyribonucleoside triphosphates (dNTPs); 0.25µl of Taq (Eppendorf) DNA polymerase, which adds a single deoxyadenosine (A) to the 3 ends of

the PCR product to allow it to be efficiently ligated to a linearized vector contains 3 deoxythymindine (T) overhangs; 2.5 µl of 10X Taq buffer and 20 µl of distilled water. The mixture was transferred to a 0.2 ml PCR tube. The reaction of cycling conditions was as shown below;

Temp.	**Time**	**Cycles**
94°C	5 min	1
94°C	30 sec	
52°C	30 sec	30
72°C	30 sec	

2.4.3 Reverse transcription-Polymerase chain reaction (RT-PCR)

RT-PCR was used to generate a segment of 700 and 1000 bp of coat protein gene of WMV-2 and ZYMV respectively using cDNA generated from the total RNA extracted from the virus infected squash plants. The reaction mixture consisted of 5µl total RNA (5µg), 4µl 5X M-MuLV reverse transcriptase buffer, 1µl 3'-primer (12pmol), 1µl 5'-primer (12pmol), the mixture was adjusted to a final volume of 20 µl with DEPC treated H_2O or RNase free water. The reaction was incubated at 70°C for 10 minutes, left at room temperature for 15 minutes, 1 µl of 2 mM deoxynucleoside triphosphates (dNTPs) was added, 0.5 µl RNase inhibitor (20u) and 1µl M-MuLV reverse transcriptase (200u) was added and incubated at 42°C for 60 min. The reaction was stopped by incubating the mixture at 70 °C for 10 min. PCR amplification after cDNA synthesis was as follows: 2µl cDNA, 2.5 µl 10X Eppendorf buffer, 1 µl 3'-primer (12pmol), 1µl 5'-primer (12pmol), 0.5 dNTPs (20mM), 0.4 µl eppendorf taq and double distilled water was added to a final volume of 25 µl. The reaction condition was performed in a PCR-Cycler as shown in 2.4.2. 2µl of RT-PCR product was loaded in gel electrophoresis to check the expected fragment size.

2.4.4 Electrophoresis of nucleic acids

For electrophoresis, gel plate and combs were first kept in 3% of H_2O_2 overnight before use to make them RNase free when working with RNA. Percentage of the agarose gel ranged from 1-2% (w/v) in 1X TAE or TBE for RNA, DNA and PCR product according to the size of

fragment. 0.1µg/ml fluorescent intercalating dye Ethidium bromide was added to the gel solution and electrophoresis running buffer prior to the experiment. The samples were supplemented with 1X loading dye. To determine the fragment sizes, a suitable DNA molecular weight standard was loaded neighbour the loaded samples in the gel lanes. Then, the bands were visualized using an ultraviolet (UV) transilluminator.

10X TAE buffer

Tris-HCl pH 8.0	48.4 g
Acetic acid	11.5 ml
0.5 M EDTA	25 ml
in 1 litre distilled H_2O,	
Autoclaved	

10X TBE buffer

Tris-HCl pH 8.0	48.4 g
Acetic acid	11.5 ml
0.5 M EDTA	25 ml
in 1 litre distilled H_2O,	
Autoclave	

10X loading dye

Sucrose	40%
Xylene cyanol	0.25%
Bromphenol blue	0.25%

2.4.5 DNA sequencing

Sequencing PCR was performed in a total reaction of 10 µl as follows: one sequencing reaction with 12 pmol (1 µl) of forward primer and another sequencing reaction with 12 pmol (1µl) of reverse primer; 2 µl of 5X sequencing buffer; 5 µl (200-500 ng) of plasmid DNA; 2 µl of Big Dye-Terminator enzyme premix (Genterprise, Mainz). The mixture was transferred to a 200 µl thin-walled PCR tube and placed in the programmed thermal-cycler. The reaction was run with cycle program shown below.

Temp.	Time	Cycles
95°C	30 sec	
55°C	4 min	30

The samples are then ready for sequencing analysis. In this study, the DNA sequencing was analyzed by Genterprise GmbH in Mainz or 4 base lab Reutlingen, Germany.
In this method, the premix solution contains four didesoxynucleotides (ddNTPs), each labelled with a different fluorescent dye, and unlabeled desoxynucleotides are mixed with the template plasmid and one specific primer. Therefore during the PCR reaction, the synthesized single strand DNA would be stopped randomly with the incorporation of ddNTP. Thus, DNA fragments of different size were labelled at their 3-ends with base specific fluorescent dyes, which could be analysed with the DNA sequencer.

2.4.6 Preparation of chemically- and electro-competent bacterial cells

2.4.6.1 KCM E. coli competent cells

A single colony of invα cells was harvested from an LB agar plate and grown overnight in 2 ml liquid LB at 37°C incubator with 220 rpm shaking. 200 µl of this culture was inoculated 50 ml of SOB medium, and allowed to grow to an OD_{600} of 0.3-0.4 at 37°C on a rotary shaker. The cells were centrifuged for 10 min at 3000 rpm at 4°C. The pellet was placed in ice and resuspended with 1/10 volume of cold TSB. Then the cells were again incubated on ice for 20 min, 100µl aliquots were frozen in liquid nitrogen and stored at -70°C.

Luria Bertani (LB) Medium

Tryptone/peptone	10 g
Yeast extracts	5.0 g
NaCl	5.0 g
Bacto-Agar	15 g
PH 7.2 with 0.1 NaOH or HCl	

SOB medium

Tryptone/ peptone	20 g
Yeast extracts	5.0 g
NaCl	0.6 g (10 mM)
KCl	0.2 g (2.5 mM)
$MgCL2/MgSO_4$ (Mg2+)	10 mM sterile filtrated, added post autoclaving

TSB medium

LB Medium pH 6.1	
PEG3300	10%
DMSO	10%
Mg2+	20 mM sterile filtrated, added after autoclaving

2.4.6.2 Rubidium chloride E. coli competent cells

E. coli bacterial strain invα competent cells were prepared for RbCl-mediated transformation by heat-shock as described by Hanahan (1985). An individual colony was inoculated in 5 ml liquid LB medium overnight. 1ml from this culture was inoculated into 100 ml LB broth (Psi) medium and incubated at 37°C, grown for 3-4 hour until OD_{600nm} reached of 0.5. The culture was kept on ice for 15 min and pellet was collected by centrifuging in 50 ml Falcon tubes at 5000 rpm at 4°C. The pellet was resuspended in 1/3 volume of TFB-I buffer by gentle hand shaking in ice, incubated 15 min on ice and recollected by centrifuging at 5000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended again in 1/30 volume of TFB-II. The cells were kept on ice for 15 min, and 100 µl aliquots were dispensed into pre-chilled micro centrifugation tube, immediately frozen in liquid nitrogen and stored at -70°C.

TFB I

Potassium acetate	30 mM
Rubidium chloride	100 mM
Calcium chloride	10 mM
Manganese chloride	50 mM
Glycerol	15% v/v

Adjust pH to 5.8 with acetic acid

TFB II

MOPS	10 mM
Calcium chloride	75 mM
Rubidium chloride	10 mM
Glycerol	15% v/v

Adjust pH to 6.5 with NaOH

Psi broth l liter

Yeast extracts	5.0 g
Tryptone	20 g
Magnesium sulphate * H_2O	5.0 g

pH 7.6 with potassium hydroxide

2.4.6.3 Electro-competent A. tumefaciens cells

A single colony was grown overnight in 8 ml LB medium at 37°C and 220 rpm on a rotary shaker. 500 ml LB medium were inoculated with 5 ml of the overnight culture, incubated again for 3-4 h at 37°C with shaking 220 rpm up to an OD_{600} 0.5- 0.6. The flasks containing bacterial cultures were incubated on ice for 30 min, and then the cultures were divided into Pre-cooled Falcon tubes and centrifuged for 10 min at 4000 rpm. Supernatant was discarded, the pellet was suspended in 5 ml ice-cold H_2O, the suspension was exceeded to 250 ml, mixed and centrifuged for 20 min at 4200 rpm. The pellet was suspended in 5 ml ice-cold H_2O, the mixture was transferred to new tube, exceeded to 250 ml, mixed and centrifuged for 20 min at 4200 rpm. The supernatant was carefully discarded, the pellet was dissolved in 40 ml containing 10 % glycerol (ice-cold), mixed and centrifuged again for 20 min at 4200 rpm. The supernatant was carefully discarded; the pellet was dissolved in 500 µl containing 10 % glycerol (ice-cold). 50 µl aliquots were frozen in liquid nitrogen and stored at -70°C.

2.4.7 Transformation of competent bacterial cells

2.4.7.1 Transformation of E. coli by heat shock method

A 100 µl aliquot of chemically competent cells (DH5-α, invα, JM110) were moved from -70°C and thawed on ice. 5 µl of ligation mixture which contain plasmid DNA (Sambrook *et al*., 1989), 20 µl KCM (method 1) and 75 µl H_2O were mixed with the competent cells and incubated on ice for 30 minutes. The mixture was exposed to heat shock at 42°C for 1 minute; then immediately placed on ice for 2 minutes. 800 µl of SOC or liquid LB media were added and incubated at 37°C for 1 h at 220 rpm in a shaking incubator. 100 µl and 400 µl aliquots were plated on 25 ml LB agar plates containing appropriate antibiotics and incubated overnight at 37°C. Only upon the first cloning (PCR product) 40 µl of 20 mg/ml X-gal stock solution either with or without 1.5 µM IPTG were separated on the surface of the LB agar plates 30 minutes prior to adding the mixture of transformation. Retransformation of positive clone can be carried out in order to confirm that this clone is stable and absolutely recombinant and to obtain purified and higher amount DNA by using NucleoSpin plasmid kit; Macherey- Nagel.

SOC medium

Tryptone/peptone	20.0g
Yeast extracts	5.0 g
NaCl	0.2 g
KCl	0.2 g
$MgSO_4.7H_2O$	2.0 g

In 1 liter distilled H_2O

Adjust pH to 7.0 and autoclaved

20 ml of filtered 1M Glucose were added to the autoclaved solution and stored at -20°C.

X-Gal stock solution (5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside)

20 mg/ml dissolved in N, N Dimethylformamide (DMF), filtered, and stored at -20°C.

IPTG stock solution (isopropyl-1-thio- β-D-glactopyranoside)

100 mM in distilled H_2O, filtered, and stored at -20°C

5X KCM 100 ml

KCl	3.73 g (0.5 M)
$CaCl_2$	2.21 g (0.15 M)
$MgCl_2$	5.08 g (0.25 M)

Sterile filtration

5-bromo-4-chloro-3-inodlyl-β-D-glactopyranoside (X-Gal stock solution)

20 mg/ml dissolved in N, N Dimethylformamide (DMF), filtered, and stored at -20°C.

Isopropyl-1-thio-β-D-glactopyranoside (IPTG stock solution)

100 mM in distilled H_2O, filtered, and stored at -20°C.

2.4.7.2 Transformation of A. tumefaciens of by electroporation method

A 50 µl aliquot was thawed on ice and 1 µl of plasmid DNA was added into the cells. The cells were then transferred to a chilled electroporation cuvette, which was placed in the electroporator after capacitancing the apparatus at 25µF KV, charging voltage at 2.5 KV and resistance at 200-400 ohm. After the application of the electro-pulse 450 µl of SOC or LB media were added. The suspension was transferred to a 2 ml reaction tube and incubated at 28°C for 1 h. After incubation, 200 µl of the bacterial culture were plated on solid LB media supplemented with appropriate antibiotics, and incubated at 28°C for 1 h.

2.4.8 Mini-preparation of *E. coli* plasmid DNA

2.4.8.1 Alkaline lysis method

The plasmid DNA minipreps was carried out by using 2 ml of bacterial overnight cultures. To collect the cells, the cultures were centrifuged for 4 min at 13000 rpm at RT. The pellet was suspended in 100 µl of solution I, vortexed for 1 min and incubated for 5 min at RT. 200 µl of solution II was added, gently mixed by inverting 6-8 times and incubated for 5 min at RT. This mixture breaks the lipid components of the cell envelope and the cellular proteins, and denatures the chromosomal and plasmid DNA into single strands. 150 µl of solution III were added, incubated on ice for 5 min and centrifuged at 13000 rpm for 5 min at RT. This mixture

allows the DNA strands to renature by bringing the pH to neutral, it collapses the chromosomal DNA into a partially hybridize tangle, and precipitates the SDS from the cell suspension with the associated proteins and lipids. Only smaller plasmid DNA, fragments of chromosomal DNA and RNA molecules will be remain in the solution. The supernatant was transferred to fresh centrifuge tube, 800 µl of 100% cold ethanol were added, good mixed, incubated for 2 min at RT, centrifuged at 13000 for 5 min at RT and the supernatant was discarded. To wash the pellet from the remaining salts and SDS from the preparation, 70% ethanol was added, vortexed and centrifuged at 13000 rpm for 5 min at RT. The supernatant was discarded, the pellet was left to air dry; dissolved either in 20 µl TE buffer (solution IV) for long storing or in water for direct digestion with suitable restriction enzymes. EDTA protects the DNA from degradation by RNase by binding the divalent caution (especially Mg++) that is necessary for the RNase activity.

Solution: I

Glucose	50 mM
EDTA pH 8.0	10 mM
Tris-Hcl pH 8.0	25 mM

Autoclaved and stored at 4°C until use.

Solution: II

SDS	2%
NaOH	0.4 M

Solution: III

NaAc, or NH4-Ac, or K-Ac	3M
pH 5.2 with glacial acetic acid	

Solution: IV

Tris (H_2O free)	40 mM
EDTA	4 mM

2.4.8.2 With NucleoSpin plasmid kit (Macherey-Nagel)

To Prepare DNA for cloning and sequencing the *E. coli* cells (5 ml) with the desired plasmid were grown overnight. 2 ml of this culture were harvested by centrifuging at 13000 rpm. The plasmid DNA was isolated by the principle of SDS/alkaline lysis; according to the manufacturer's instruction.

2.4.9 Mini-preparation of *A. tumefaciens* plasmid DNA

The bacterial culture was submitted for plasmid DNA mini-preparation using the Wizard *plus* DNA purification system. Isolation was performed according to the instruction of the manufacturer.

2.4.10 Midi-preparation of plasmid DNA

A single colony was inoculated in 100 ml liquid LB medium supplemented with an appropriate antibiotic. The culture was incubated at 37°C overnight on a shaker with 220 rpm shaking. The culture was equally divided into 50 ml Falcon tubes. The cells were then harvested by centrifuging at 5000 rpm for 15 min at 4°C. The midiprep were performed by using JETstar plasmid midiprep kit according to the manufacturer's recommended procedure.

2.4.11 Purification of DNA

2.4.11.1 Purification of DNA by phenol/ chloroform extraction

Purification of digested plasmid DNA in a solution containing restriction enzymes; it was carried out on account of fit this plasmid for dephosphorylation by stopping and inactivating the restriction enzymes and their buffers. It was performed as following procedures:
Solution of 8- 20 µl containing ~1-2 µg DNA was brought up to a volume of 200 µl with TE buffer. The mixture was short vortexed, the same volume of phenol/ chloroform/ isoamylalcohol (25:24:1) pH 7.5 was added, vortexed, and centrifuged at full speed for 15 min at RT. The upper phase about 195 µl carefully transferred to new tube, the same volume of chloroform/ isoamylalcohol 24:1 was added and centrifuged again at full speed for 5 min at

RT. The upper aqueous was genteelly transferred to new reaction tube; the DNA could be then precipitated by ethanol. Precipitation with ethanol was used to concentrate nucleic acids where it causes the nucleic acids come out of the solution. Therefore, 3 M sodium acetate pH 5.2 to a final concentration of 0.3 mM (1/10 volume) and 2.5 volumes of cold ethanol were added to the DNA solution. After mixing, the ethanolic solution was placed for 2 h or overnight in –20C° or for 30 min in –70°C to allow precipitation of DNA. Afterwards, DNA was recovered by centrifuging for 30 min at maximum speed at RT. The supernatant was discarded; the pellet was washed once by centrifuging with 1 ml of 70% ethanol for 5 min at maximum speed at RT. The pellet was left to air dry, dissolved in a desired volume of Nuclease free water or TE buffer (pH 8.0). An aliquot was loaded in a gel to determine presence of DNA fragment.

2.4.11.2 Purification of DNA from agarose gel

DNA was loaded onto a 1-2% TAE agarose gel with suitable DNA marker, just electrophoresis was done, the agarose gel was placed on a UV transmittable plastic sheet and the fragment of interest was excised with a sterile scalpel. Then the DNA was extracted from the gel pieces either by using the QIAquick gel extraction kit (Qiagen) or by NucleoSpin extract (Macherey-Nagel) or by placing the gel piece into a 0.5 ml holed tube containing synthetic filter floss (JBL GmbH & Co.KG). The tube with gel piece was placed into a 1.5 centrifuge tube and centrifuged for 10 sec at 13000 rpm at RT.

2.4.12 Determination of plasmid DNA concentration and purity

Concentration of nucleic acid in a solution was determined in order to assess the purity and to know the amount of nucleic acids is available. The DNA quantity was performed by measuring the absorbency of spectrophotometer at 260 and 280nm. DNA and RNA absorb light of 260nm wavelength, proteins (aromatic amino acids) absorb light of 260nm wavelength too, but absorption is much stronger at 280nm. The ratio A_{260}/A_{280} gives an estimation of nucleic acids purity. If the ratio for DNA is 1.8 and for RNA is 2.0, the absorption is probably due to nucleic acids. A ratio less than 1.8 indicates that there may be protein contamination. A ratio higher than 2.0 indicates the samples may be contaminated

with chloroform or phenol. The unknown DNA concentration in µg/µl can then be calculated by the following formula:

DNA concentration (µg/µl) =OD_{260} * dilution factor * 50 µg/ml.

Where, A_{260} of 1 corresponds to 50 µg/ml of dsDNA, and to 40 µg/ml of ssDNA or RNA (Sambrook *et al*., 1989) in a 1 cm quartz cuvette. An Eppendorf spectrophotometer was used in which the samples were automatically measured, so we only dilute the samples with a suitable amount of distilled water, read the absorption and multiply by dilution factor.

2.4.13 Restriction analysis of plasmid DNA

For restriction enzyme analysis, a single or double digests of plasmid DNA were carried out with 5-10 units per 1µg DNA and 1X of a suitable enzyme buffer in a total reaction of 20-50 µl in one to three hour. Then an appropriate volume was loaded in electrophoresis gel as previously described in **2.3.2.**

2.4.14 Dephosphorylation of DNA

Shrimp Alkaline Phosphatase (SAP) or Calf Intestine Alkaline Phosphatase (CIAP) was used here to prevent self ligation of plasmid DNA during ligation procedure in vitro, when the ends of digested plasmid were compatible with each other. Therefore, treatment the plasmid with Phosphatase enzyme was essential to remove the 5´-phosphate residues from both termini of the plasmid DNA. The dephosphorylation reaction was as follows: 10-40 picomoles of DNA termini in 10-40 µl water, 5 µl 10X reaction buffer, 1u Phosphatase enzyme, and watered to 50 µl. The reaction was incubated at 37° C for 30 min, and the enzyme was inactivated at 65° C for SAP and at 85 ° C for CIAP for 15 min.

2.4.15 Ligation with T4 DNA ligase

The T4 DNA ligase was used to catalyze the formation of a phosphodiester bond between the 5`phosphate of one strand of DNA and the `3 hydroxyl group of the other. For its catalytic activity the enzyme requires the presence of ATP and Mg++.

The linearized, purified and dephosphorylated (when it is necessary) DNA vector and desired DNA fragment with compatible sticky ends or PCR fragment were ligated in a 10 µl reaction

mixture 50-200 ng containing DNA. A molar ratio between insert and vector of 3:1 was usually chosen. The Mixture of ligation reaction containing the following components: 1 µl 10X ligation buffer, 1u T4 DNA ligase, insert, vector and up to 10 µl with sterile H_2O. The mixture was incubated at RT for 4 h or at 16°C overnight.

2.5 Analysis of transgenic tissues

2.5.1 Fast genomic DNA extraction in a mini scale

Genomic DNA extraction was performed according to a method described by Edwards *et al*., (1991) as follows: Small part of leaf (~20 mg) of putative transformed plants was placed in 1.5 E-cup centrifuge tube and ground by using plastic pistil and mortar. 400 µl of extraction buffer were added and incubated at 65°C for 30 min. The same volume of chloroform was added, vortexed for 5 sec, and centrifuged at 13000 rpm for 5 min. The upper phase ~ 300 µl was transferred to new tube, the same volume of cooled isopropanol was added, carefully mixed, incubated 2 min at RT and centrifuged at 10000 rpm for 5 min. Pellet was washed in 500 µl of 70% ethanol by centrifuging at 13000 rpm for 5 min, The supernatant was discarded and the pellet was left for air dry. Then the pellet was resuspended in 200 µl of TE buffer pH 8.0 and incubated at 65°C until dissolved (1-4 h), then incubated at 4°C for continued dissolution. To check gel control 5 µl were loaded on 1% TAE electrophoresis gel and 1 µl was used as template for PCR reaction.

Extraction buffer

Final conc.	***Conc. of stock solution***	***Volume of stock solution***
200 mM Tris/HCl pH 7.5	1 M Tris/HCl pH 7.5	40 ml
250 mM NaCl	5 M NaCl	10 ml
25 mM EDTA	0.5 M EDTA	10 ml
0.5% SDS	10% SDS	10 ml up to 200 ml sterile H_2O

2.5.2 Genomic DNA extraction in a large scale

Genomic DNA was extracted from leaves of *Nicotiana bentamiana* plants using CTAB method as described by Roger and Bendich, (1985). Plant materials (2-2.5 g) were shock-frozen in liquid nitrogen and homogenized with homogenizer (Retsch, type MM 200 Germany) for 45 second. The fine powder was transferred into a 100ml Erlenmeyer flask containing 10ml extraction buffer, 100µl of 10X proteinase K, 20µl of 10X RNase A, and incubated at 65° C in water bath with shaking for 1 h.. The mixture was transferred into 50ml falcon tube, put on ice, one volume of chloroform/octanol (24:1) was added, mixed by inversion 4-5 times, and centrifuged at 6000 rpm for 15 min at 4° C. The aqueous phase was transferred to a new 50 ml falcon tube, 1/10 volume of pre-heated CTAB/NaCl at 65° C was added, one volume chloroform/octanol was added, centrifuged at 6000 rpm for 15 min at 4° C and the supernatant was transferred to a new tube. To precipitate the DNA, one volume of precipitation buffer was added, incubated at 65° C in water bath with shaking for 30 min, 1/10 volume of precipitation buffer was added and incubated at 37° C overnight. The pellet was collected by centrifugation at 6000 rpm for 15 min at 4° C, resuspended in 1 ml high salt TE buffer and incubated at 65° C to completely dissolve the DNA pellet if required. The DNA suspension was transferred to a 2 ml Eppendorf tube, precipitated with 0.6 volume of isopropanol, inverted 3-4 times and centrifuged at 5000 rpm for 15 min at 4° C. The supernatant was removed, 70% ethanol was added for washing, centrifuged at 5000 rpm for 5 min. The supernatant was discarded, DNA pellet was allowed to dry at RT, finally resuspended in 200µl of TE` and incubated at 65° C for 1-4 h. if required.

Extraction buffer:	2% CTAB		20 g
	100 mM Tris-HCl (pH 8.0)	1.0 M	100 ml
	20 mM EDTA (pH 8.0)	0.5 M	40 ml
	1.4 M NaCl	5.0 M	280 ml
	Up to 1 liter with sterile H_2O		

Precipitation buffer:	1% CTAB		10 g
	50 mM Tris-HCl (pH 8.0)	1.0 M	100 ml
	10 mM EDTA (pH 8.0)	0.5 M	40 ml
	Up to 1 liter with sterile H_2O		

CTAB/NaCl: 4.1 g NaCl dissolved in 80 ml H2O, 10 g of CTAB slowly were added under heating and stirring, and up to 100 ml with sterile H_2O.

TE` buffer:	10 mM Tris-HCl	(pH 8.0)	1.0 M	2.5 ml
	0.1 mM EDTA	(pH 8.0)	0.5 M	50 µl
	Up to 250 ml with sterile H_2O			

High salt TE:	10 mM Tris-HCl	(pH 8.0)	1.0 M	2.5 ml
	0.1 mM EDTA	(pH 8.0)	0.5 M	50 ml
	1 M NaCl		5.0 M	50 ml
	Up to 250 ml with sterile H_2O			

2.5.3 Histochemical Gus assay for squash embrogenic calli

Histochemical Gus assay was performed as described in 2.3.2.

2.5.4 Multiplex PCR

Multiplex PCR is a normal PCR procedure but with more than one primer pairs, which will amplify more than one fragment in the same DNA or in different DNA in the reaction mixture. It was used here to amplify two fragments in the transgene sequences. The PCR mixture components and condition were as follows: 1 µl DNA, 2.5 µl 10X Taq buffer, 1µl MgAc 25 mM, 0.5 µl dNPTs Mix 10 mM, 0.5 µl 5´-primer 1 (12pmol), 0.5 µl 3´-primer 1 (12pmol), 0.5 µl 5´-primer 2 (12pmol), 0.5 µl 3´-primer 2 (12pmol), 17.75 µl bidest H_2O, 0.5 µl Taq polymerase (5u/µl).

Tem.	**Time**	**Cycles**
94°C	2 min	1
94°C	30 sec	30
60°C	30 sec	
68°C	30 sec	

2.5.5 Southern blot Analysis

●Digestion of genomic DNA

Restriction endonuclease digestion was carried out with *Bam*H1 (200u) for 20µg DNA in a total reaction of 400µl overnight at 37°C. For complete digestion 50u of enzyme were added, the samples were incubated 3-4 h. at the same condition. To check the digested DNA, 10µl were separated on 0.9% TAE agarose gel. The rest of digested DNA was mixed with 1/10 volume of 3M NaAc (pH5.2) and 2.7 volume of 100% ethanol, vigorously shacked by hand, incubated at -20° C for 1-2 h., and centrifuged at 13000 rpm for 10 min at RT. The DNA pellet was washed with 70% ethanol, centrifuged for 5 min at 13000 rpm at RT, the supernatant was discarded and the pellet was resuspended in 60µl TE. DNA concentration was measured by fluorometer with the fluorescent DNA quantitation kit (Bio-Rad).

●Separation of digested DNA in an agarose gel electrophoresis

10 µg of digested DNA were mixed with SB-lading dye, incubated at 70° C for 10 min with opened coat to remove the cohesive ends of DNA fragments and the rest of ethanol, the DNA samples and a labelled DNA [DNA molecular weight marker VII (0.08 to 8.57 kbp in sizes)] as size markers were loaded in a 9% TAE agarose gel, the DNA separation was done overnight at 30 volt, and check photo was taken.

SB-loading dye (6 X): 0.25% bromphenole blue
0.25% xylene cyanol FF
0.40% saccharose in H_2O

●Depurination

The DNA was depurinated by immersing the gel in 0.25 M HCl solution with a gently shaking for about 10 min until the colour of loading dye (bromphenole blue) was changed to yellow, the buffer was changed with new one, shaken for another 10 min and the buffer was changed with sterile H_2O with continuous shaking for 10 min. Depurination is the chemical step where the Purine base (adenine and guanine) is removed from the nucleotide moiety.

● Denaturation

To denaturate the DNA, the gel was completely covered twice in 0.4 M NaOH with gentle shaking for 20 min each. Treatment of the gel with alkaline solution hydrolyzes DNA at depurination sites resulting in fragmentation of the DNA polymer and dsDNA is denatured into ssDNA by disrupting the hydrogen bonds that enables binding of the DNA to the positively-charged nylon membrane and to bind single stranded probe. If acid depuration was carried out, the bromphenole blue will return to its original colour during this period.

●Membrane, Whatman paper and transfer container preparation

While the gel was denaturating a one piece of positively charged nylon membrane (Hybond-N+/ Amersham Pharmacia) and three pieces of Whatman papers were prepared by cutting the same area of the gel, a transfer container containing inverted casting tray (support) was filled with transfer buffer (0.4 M NaOH/ 1 M NaCl), two lengths of Whatman paper was prepared wider than the gel and long enough to fit under the gel and reach to the bottom of the transfer container on either side, wetted with transfer buffer, air bubbles between the inverted casting tray and Whatman paper was removed by rolling a pipette several times back and forth over the surface. The membrane was equilibrated by wetting with little amount of sterile H_2O and the same amount of transfer buffer for 5 min.

●Setting up the blot and transfer of DNA from gel to nylon membrane

The gel was placed on the Whatman paper, air bubbles was removed by gently rolling the a 25 ml pipette on the gel, the membrane was carefully placed one time upon the gel, the air bubbles was removed again with a pipette. Then, the three Whatman papers on the previous paragraph, were covered the membrane, a plastic warp or Para film was placed over the edges of the gel so that the buffer moves only through the gel, then a stack of paper towels were placed on top of that to draw the liquid up through the gel and membrane. A glass or wood plate and book were placed on top of the paper towels and the DNA was allowed to transfer overnight.

The next day the pyramid was disassembled, the membrane was marked by cutting a small piece from the upper right corner, and the gel was photographed to insure that the DNA was transferred onto the membrane.

The membrane was placed in neutralization buffer (0.5 M Tris-HCl pH 7.2/1 M NaCl) for 20 min with gently shaking and allowed to dry on a piece of Whatman paper, To store the membrane, it was place between two sheets of Whatman papers in aluminium foil at 4° C until needed for hybridization.

●Synthesis and preparation of DIG-labelled probes

The DIG system uses digoxygenin, a steroid hapten in the form of DIG-11 dUTP, to label DNA, RNA or Oligonucleotide. The DIG-labelled probe was prepared by PCR using Taq DNA polymerase, incorporating DIG-11-dUTP and 1 and 2 primers described in multiplex PCR. The PCR reaction was as following:

34.25 µl	bidest H_2O
5 µl	10X Taq Eppendorf buffer
5µl	labelling Mix^{plus}
2µl	5`primer 12 pmol/µl
2µl	3`primer 12 pmol/µl
1µl	DNA 15 ng/µl
0.75	Taq Eppendorf

The PCR was carried out under the following conditions: 10 cycles of (10 seconds denaturating at 90° C, 30 seconds annealing at 55° C and 2 min elongation at 72° C), 20 cycles of (10 seconds denaturating at 95° C, 30 seconds annealing at 55° C and 2 min elongation at 72° C + 5 seconds extended elongation time per cycle) and 7 min last elongation at 72° C. An aliquot of the probe was run on an agarose gel in order to check the size of the fragment obtained. It must keep in mind that the product seems bigger than the non-labelled control, since it contains DIG-UTP. The rest of the probe (~45µl) was divided into two equal amounts in 1.5 ml centrifuge tubes, denaturated at 100° C for 1 min, cooled in ice for 2 min, transferred into two falcon tubes containing 10 ml of DIG Easy Hyb solution and stored at -20° C until use.

●Pre-hybridization and hybridization of membranes

The membrane was carefully rolled and placed in a hybridization roller tube with DNA side in; 10 ml of pre-hybridization solution (DIG Easy Hyb, Roche) was carefully added without dropping directly on the membrane. The hybridization roller tube was then placed hybridization oven for 2 hour at 42° C. The hybridization temperature was calculated according to the following formula:

$T_m = 49.82 + 0.41$ (% G + C) - 600/1

$T_{hyb} = T_m - (20 \text{ to } 25° C)$

Where:

(% G+ C) = % of G and C residues in probe sequence.

1 = length of the probe in base pairs.

At the time of pre-hybridization, the probe was slowly thawed, denaturated at 65° C for 15 min in water bath, then transferred into the hybridization oven for ~ 30 min. The pre-hybridization solution was removed (it can be reused for 3 time), the probe was added, and incubated in hybridization oven overnight at least 16 h. at 42° C. The probe was removed from the tube (it can be reused more time so do not discard it), the membrane was washed twice with washing solution for 20 min at RT (2X SSC/ 0.1% SDS). While the washing step is running, the hybridization oven and 0.5X wash solution (0.5X SSC/ 0.1% SDS) were pre-heated to 68° C, the wash solution was changed with 0.5 wash solution twice for 20 min at 68° C, the 0.5 wash solution was discarded and the membrane was equilibrated 2 time 2 min in DIG-wash solution (0.1 M maleic acid/ 0.15 M NaCl pH 7.5+0.3% tween20). During the 0.5X washing step is going, a 1-fold blocking solution was prepared by adding 1 ml of blocking solution to 9 ml of maleic acid and to prepare antibody solution, an anti-dig-alkaline phosphate was first centrifuged at 13000 rpm for 2 min; then 1 µl from surface was added to 10 ml of 1-fold blocking solution, mixed well and placed at 4° C.

●Chemiluminescent detection with anti-DIG-AP and CSPD or CDP-star

To detect the bands that had hybridized with the DIG-labelled probe, an alkaline Phosphatase-conjugated anti-digoxigenin antibody and a chemiluminescent substrate were employed. Chemiluminescent substrates can be visualized by exposure to X-ray film. Following the equilibaration with DIG-wash solution, the blocking step was done by adding 1-fold of the

blocking solution in the hybridization tube to block non-specific binding of antibody to the membrane; the blocking was done for 1 h at RT in hybridization oven. The blocking solution was removed and the diluted anti-dig-AP 1: 10000 in blocking solution was added, incubated at RT in hybridization oven for 45 min. The solution was discarded, and the membrane was washed with DIG-wash solution for 5 min, then 2 times 30 min with the same buffer at RT. The membrane was washed twice 2 min with detection buffer 0.1 M Tris-HCl/ 0.1 M NaCl (pH 9.5) at RT. 20µl of substrate (CDP-Star) or (CSPD) were added in 2 ml detection buffer and vortexed. The membrane was placed between two sheets of transparency film warp with 2 ml of substrate-detection buffer was added onto the membrane; the fourth side was welded, carefully rubbed with the finger for 10 min, slowly placed in a new foil to avoid the air bubbles, foil' s borders were welded and the small air bubbles were removed.

●Expositing to X-ray film

The membrane was transferred in a hypercassette with DNA side to upon and adhered with a sticker to expand it. In a dark room and in the presence of a red light a sheet of an X-ray film was fixed on the membrane by a piece of sticker at one of its side; the cassette was closed and the membrane was exposed to the X-ray film for 1 h. In the presence of red light, the cassette was opened, and the probe-target hybrids were detected by incubating the X-ray film for 1-2 min in a tray containing a developing solution (diluted 1 : 5 in H_2O of Kodak GBX developer/ replenisher), washed in H_2O for 1-2 min, immersed in fixing solution (diluted 1 : 5 in H_2O of Kodak GBX fixer/ replenisher), and finally allowed to dry in RT. Multiple exposures from a single blot can be obtained for up to 2 days after the addition of the substrate.

●Stripping the membrane for re-probing (re-hybridization)

The membrane can be stripped and re-hybridized if the results was not ok, for this purpose, the membrane was washed with sterile H_2O by gentile agitating for 5 min, then washed with 0.2 M NaOH/ 0.1 % SDS solution twice by slowly shaking for 3 min each. The membrane was equilibrated in 2X SSC for 2 min, and then the pre-hybridization step was immediately followed.

At all time the experiment the membrane was handled with gloved hand by the corners and edges.

10X blocking stock solution:

Maleic acid buffer: 100 mM maleic acid
150 mM NaCl
pH to 7.5 with solid or concentrated NaOH

Blocking reagent was dissolved in maleic acid buffer to a final conc. of 10% (W/V) with shaking and heating. Autoclave and store at 4° C.

2.5.6 Segregation analysis of transgenic plants on kanamycin

To obtain seeds from T_0 plants, the transgenic lines were cover with plastic bags to be self fertilized. T_1 seeds obtained from self fertilized of T_0 transgenic *N. benthamiana* plants were surface sterilized in 6% sodium hypochlorite solution on a shaker for 10 min and washed five times with sterile water. After the last wash, seeds were allowed to dry by laying on a sterilized filter paper.

Sterilized seeds were placed on MS medium (25 seeds/plate) containing 50 mg/l kanamycin. Two plates per line, they were placed in a growth chamber at 16/8 h. day/ night at 25° C for 4 weeks. Statistical analysis was performed by the counting T_1 on kanamycin-containing selective MS media. Distribution of T_1 was tested against the expected ratios using the chi-square ($\chi 2$) test, which was used to analyze segregation of the transgene in the progenies.

2.5.7 Agro-infiltration procedure

In this method, a different *Agrobacterium tumefaciens* ATHV strains harbouring one of the following recombinant binary plasmid: pPZPgfp-WMV and pPZPgfp-ZYMV (sensor constructs), P19 (virus suppressor) and pKBgfp (as GFP positive control) were used. The four recombinant agrobacteria were separately grown in LB medium containing 300 mg/l spectinomycin/streptomycin each and 15 mg/l rifampciline at 28°C overnight on a shaker. Bacterial cells were harvested by centrifugation at 4000 rpm for 10 min and resuspended in induction medium (10 mM MgSO4, 10 mM MES pH 5.6 and 100µM Acetosyringone) to a final concentration corresponding to an optical density (OD) of 1 at 600 nm. Cultures were incubated at room temperature for 2 h. before infiltration. Prior to infiltration procedure, the suspension of *Agrobacterium* that carries P19 as viral silencing suppressor in the binary plasmid, was mixed in 1:1 ratio with the bacterial suspension that carry one of the two sensor

constructs. Then they were six different bacterial suspensions as follows: 1) mixture of P19 with pPZPgfp-WMV, 2) mixture of P19 with pPZPgfp-ZYMV, 3) suspension of P19 alone, 4) suspension of pPZPgfp-WMV alone, 5) suspension of pPZPgfp-ZYMV alone, and 6) suspension of pKBgfp alone. The bacterial suspension was applied to transgenic and non-transgenic plants using a one millilitre needless syringe. The cell suspension was slowly pressed with the finger into the underside of the plat leaves until the suspension spread throughout almost of the whole leaf. Nine plants in nine lines were infiltrated, one leaf per plant. Three plants per line were infiltrated with pPZPgfp-WMV, pPZPgfp-ZYMV, P19+ pPZPgfp-WMV, P19+ pPZPgfp-ZYMV, P19 and pKBgfp. Three plants per line were infiltrated with pPZPgfp-WMV, P19+ pPZPgfp-WMV, P19 and pKBgfp. Three plants per line were infiltrated with pPZPgfp-ZYMV, P19+ pPZPgfp-ZYMV, P19 and pKBgfp. The infiltrated leaves were photographed at 4 days after agro-infiltration (Wydro *et al.*, 2006) with florescent microscope.

2.6 Virological Methods

2.6.1 Virus inoculation

Inoculation was produced by grinding infected squash leaves using autoclaved and Pre-cold mortar and pestle in Na-K phosphate buffer 1:3. Leaves of *Nicotiana bentamiana* plants were dusted with carborundum at approximately one month old 4-6 leaves, then the inoculum was applied to the leaves by gently rubbing them across the surface of each leaf five times back and forth by forefinger. The inoculated and control plants were maintained in insect-proof cages until ELISA test.

Na-K phosphate buffer

(**A).** KH2PO4 (0.066 M) 0.908 g/100 ml

(**B).** $NaHPO_4$ (0.066 M) 0.937 g/100 ml

39.2 ml of (**A)** + 60.8 ml of (**B)** were mixed and stored in 4°C.

2.6.2 Enzyme-linked immunosorbent assay (ELISA)

The double-antibody sandwich (DAS) method of ELISA described by Clark and Adams 1977 was performed 17d post-Inoculation to detect plant virus infection on plants inoculated with WMV and/or mix-Inoculated with WMV/ZYMV. A polyclonal antibodies (antisera) obtained from Loewe Biochemica GmbH (Sauerlach, Germany) were used in this test. The ELISA test was carried out in a microtitre (96-well) NUNC MaxiSorp^{TM} plates. Anti-virus IgG was diluted 1:200 in coating buffer; the microtitre plate was coated with 200 µ1 per well and incubated for 4 h at 37°C. During the incubation time, small leaf part of putative infected plants were collected in BIOREBA plastic bags, squeezed in the presence of 20 fold extraction (sample) buffer (1:20 w/v) using a homogenizer, and placed in ice or 4°C until use. The incubated microtitre was washed with 200 µl of washing buffer 4 times 5 min intervals. 200 µl of the extracted plant sap per well were added, incubated overnight at 4°C, washed again as mentioned above but here 5 times instead of 4. Anti-virus-IgG-AP-conjugate was diluted 1:200 in conjugate buffer, 200 µl were added into the wells, incubated for 4 h at 37°C and washed again as described in the second washing. 200µl of substrate solution were added into the wells, and incubated for 1 h in darkness at room temperature. Optical densities (OD) of ELISA reaction measured spectrophotometically at 405nm. All assays included positive (systemically infected plants) and negative (non-infected plant) controls. A sample was defined positive if the A 405nm value was greater three times than that of the negative control.

Washing buffer (1liter)

NaCl	2.0 g
$Na_2HPO_4.12H_2O$	0.2 g
KCl	0.2 g
Tween20	0.5 ml
pH	7.2-7.4

Sample (Extraction) or conjugate buffer (1 liter)

Polyvinyl pyrrolidone (PVP) K_{10}-K_{40} or K_{25}	20 g
Albumin, Bovine N 15.6 %	2.0 g
NaN_3	0.05 g
pH	7.2

Coating buffer (250 ml)

Na_2CO_3	0.278 g
$NaHCO_3$	0.434 g
pH	9.6

Substrate buffer (250ml)

Di-ethanolamine	24.25 ml
$MgCl_3.6H_2O$	0.05 g

Substrate solution

4-nitrophenyl phosphate di-Na-salt dissolved in substrate buffer 1mg/ml. It was prepared during the last washing step

3 Results

3.1 Induction of somatic embryogenesis in squash

The current study was carried out on three squash cultivars to obtain embryogenic callus which are a good source for *Agrobacterium* transformation. Consequently, it enables us to introduce virus resistance to squash plant. Different plant growth regulators concentrations were examined using four explants types.

3.1.1 Development of somatic embryogenesis and plant regeneration

Mature seed, cotyledon, leaf and shoot tip explants were enlarged on the induction medium at day 7-10, the green colour of the cotyledon and leaf explants became light green or light brown after 4 weeks, and green colour disappeared within 8 to 10 weeks. All explants became brownish after 8 weeks. Formation of somatic embryos were observed on mature seed explants derived from cultivar Dundoo and CX3005 after 17 weeks, and for cultivar CX3006 after 20 weeks. Somatic embryos were observed on the shoot tip explants derived from Dundoo and CX3005 after 15 weeks and 13 weeks for CX3006. Leaf and cotyledon explants of CX3006 produced somatic embryos after 13 and 18 weeks, respectively. Somatic embryos in globular stage were well matured in dark on plant growth regulator free medium containing activated charcoal for four weeks. Somatic embryos converted into plantlets within two to three weeks under light/dark condition (16/8) and developed into entire plants within three to four weeks for Dundoo and CX3006, respectively (figure 7). 66 embryos were accounted from embryogenic callus (500 mg) derived from Dundoo, 46 embryos were normally developed into entire plants and 20 embryos showed abnormal development. From CX3006, 55 embryos were accounted from one embryogenic callus (500 mg), 25 embryos developed normally into whole plants and 30 embryos showed abnormal developments.

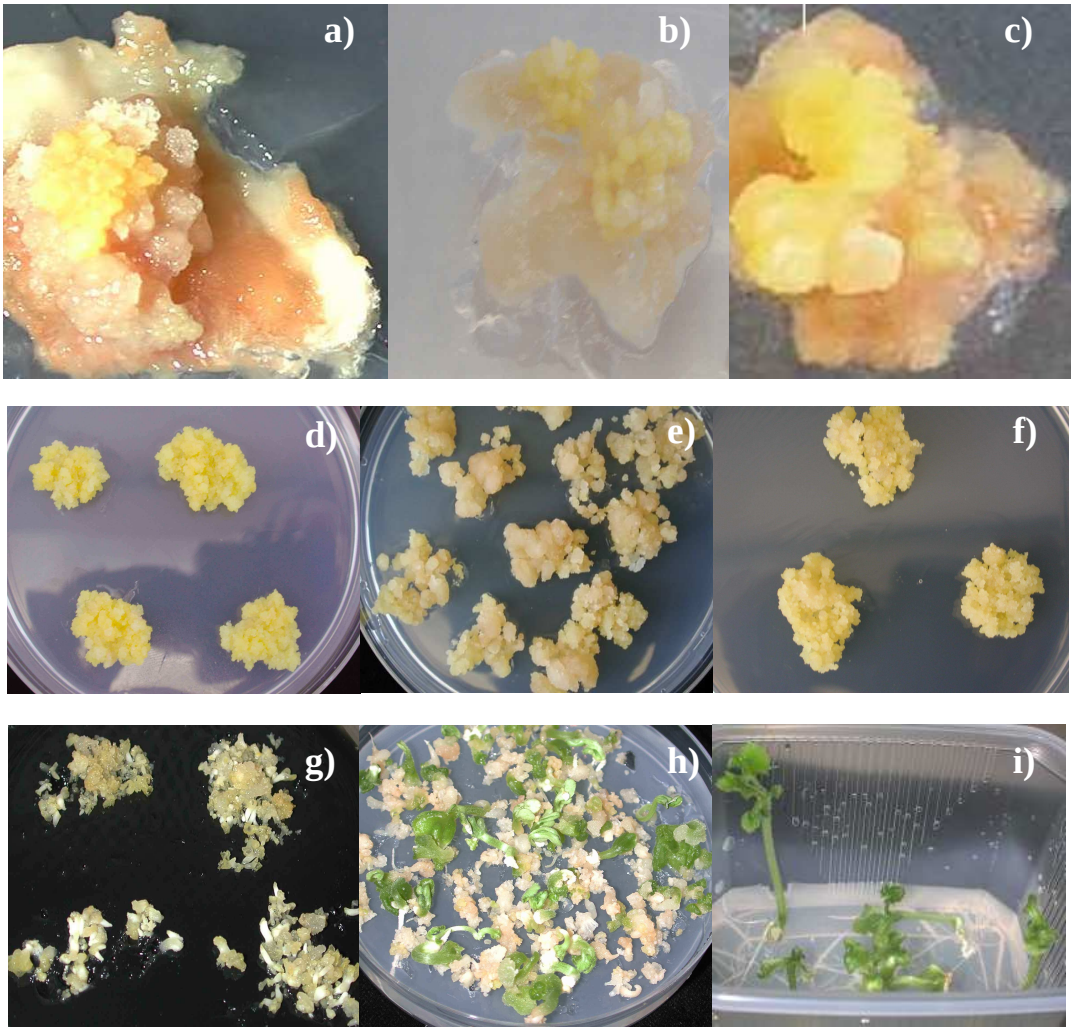


Figure 7: Development of somatic embryos and plant regeneration: a), b), and c) formation of somatic embryos on different types of squash explants; on mature seed explants, on leaf explants and on shoot tip explants, respectively. d), e) and f) propagation of somatic embryos from different genotypes; from Dundoo, from CX3006 and from CX3005, respectively. g); maturation of somatic embryos on MS hormone free medium containing activated charcoal. h); conversion of somatic embryos after exposing to light/dark 16/8 for two weeks. i): germination of somatic embryos into entire plants after 3 weeks.

3.1.2 Effect of different growth regulator regimes

In mature seed explants derived from CX3006, different 2,4-D concentrations were tested alone or in combination with kinetin. The results in table 3 indicated that 5 mg/l 2,4-D was the best concentration giving the highest percentage of embryogenesis (50 %). No effect was observed when the kinetin was combined with 5 mg/l 2,4-D. Reducing 2,4-D concentration to 2 mg/l in the presence or in the absence of the kinetin resulted in decreasing of embryogenesis induction. No embryogenic calli were observed with the highest 2,4-D concentration (10 mg/l) in the presence or in the absence of the kinetin. In regard to the seed explants derived from Dundoo in table 4 the highest percentage of embryogenesis was obtained with a combination of 5 mg/l 2,4-D and 3 mg/1 kinetin. Reducing 2,4-D concentration in the presence or in the absence of the kinetin resulted in low percentage of the embryogenesis. In mature seed explants of CX3005, somatic embryos were obtained with a combination of 1.2 mg/l 2,4,5-T, 0.8 mg/l BAP and 0.1 mg/l NAA (table 5).

In shoot tip explants derived from CX3006 (table 3), it was found that the highest percentage of embryogenesis (100 %) was obtained with 2 mg/l 2,4-D. Adding of kinetin in combination with 2 mg/l or 10 mg/l 2,4-D led to decreasing the percentage of embryogenesis induction. Hundred percent of embryogenesis was also obtained with 2 mg/l of 2,4-D from shoot tip explants derived from Dundoo (table 4).

In cotyledon and leaf explants derived from CX3006 (table 3), The increase of 2,4-D concentration from 5 to 10 mg/l did not effect the embryogenesis induction. Sixteen percent embryogenesis was observed in both mentioned concentrations in the cotyledon explants. Effect of 2 mg/l 2,4-D was almost similar to the effect of 10 mg /l 2,4-D in leaf explants; 44 and 50 %, respectively.

Table 3: Effect of plant growth regulators on somatic embryo induction in different types of explants of squash (*Cucurbita pepo L*) cultivar CX3006

Growth regulators (mg/l)		Mature seed			Shoot tip			Cotyledon			Leaf		
2,4-D	Kinetin	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%	No. of explants	No. of embryogenic callus	%	No. of explants	No.of embryogenic callus	%
2	0	30	10	33	4	4	100	-	-	-	9	4	44
5	0	28	14	50	-	-	-	12	2	16	-	-	-
10	0	30	0	0	-	-	-	12	2	16	8	4	50
2	1.5	38	2	5	9	1	11	-	-	-	-	-	-
5	1.5	22	10	45	-	-	-	-	-	-	-	-	-
10	1.5	15	0	0	10	5	50	-	-	-	-	-	-

Table 4: Effect of plant growth regulators on somatic embryo induction in squash (*Cucurbita pepo L*) from different types of explants of cultivar Dundoo

Growth regulators (mg/l)		Mature seed			Shoot tip		
2,4-D	Kinetin	No. of explants	No. embryogenic callus	%	No. of explants	No. embryogenic callus	%
2	0	10	1	10	4	4	100
2	1.5	12	1	8	-	-	-
5	3	17	6	35	-	-	-

Table 5: Effect of plant growth regulators on somatic embryo induction in squash (*Cucurbita pepo L*) from mature seed explants of cultivar CX3005

Growth regulators (mg/l)			Embryo induction		
2,4,5-T	BAP	NAA	No. of explants	No. of embryogenic calli	%
1.2	0.8	0.1	45	3	7

3.2 Establishment of *Agrobacterium*-mediated transformation of squash embryogenic callus

3.2.1 Optimization of parameters influencing *Agrobacterium* transformation

Measurement of transient expression of GUS gene in transformed cells was calculated as a number of blue spots expessing cells.

● Effect of *Agrobacterium* strains

Four *Agrobacterium* strains were examined to estimate their effects on transformation efficiency. The results on figure 8a showed the highest number of blue spots (22 and 20) with *Agrobacterium* strains ATHV and AGL1, respectively. Low number of blue spots (3 and 2) was observed with *Agrobacterium* strains LBA4404 and GV3101.

● Washing before *Agrobacterium* infection

The results in figure 8b indicated that washing of embryogenic callus with liquid MS medium before inoculation with the *Agrobacterium* gave a higher percentage of blue spots in all genotypes tested (Dundoo, CX3005 and CX3006) which were 399, 346 and 531 % respectively, than in non washed callus which was speculated as 100%.

●Effect of *Agrobacterium* concentration

Different optical densities (OD) of bacterial culture (0.1, 0.5 and 0.85) were examined. The results presented on figure 8c showed the highest percentage of blue spots with an OD 0.85 for all genotypes (Dundoo, CX3005 and CX3006), with 351, 374 and 268 %, respectively.

● Effect of sub-culture length

The embryogenic callus was sub-cultured 1, 5, 9, 13 and 21 days prior co-culturing with *Agrobacterium*. The results in figure 8d showed that the highest number of blue spots was

obtained at 5 and 9 days after sub-culturing (231.3 and 179 spots), respectively. No blue spots were observed after 21 days from sub-culture period on the medium.

● Effect of acetosyringone

The acetosyringone was added to *Agrobacterium* in induction medium at 1 mM in the presence of the embryogenic callus for 1 hour and without acetosyringone as control treatment. Acetosyringone treated and untreated callus was transferred onto solid MS medium (co-culture medium) containing different acetosyringone concentrations (0, 0.1, 0.2, 0.5 and 1mM) for 3 days. The results in figure 8e indicated that the addition of acetosyringone at 1 mM for 1 hour in induction medium and transferring the callus onto solid MS acetosyringone-free medium was the best treatment giving the highest number of blue spots (42 spots), while, the addition of the acetosyringone at 1 mM for 1 hour in induction medium prior transferring the callus onto solid MS acetosyringone-containing medium inhibited transformation efficiency at all concentrations.

In the case of the control treatment in figure 8f, where the acetosyringone was not added to induction medium, the highest number of blue spots (42 spots) with acetosyringone concentration of 0.2 mM was obtained, whereas, no blue spots were observed in acetosyringone-free medium. The lowest number of blue spots was observed with high acetosyringone concentration (0.5 and 1 mM) in co-culture medium with treated and untreated callus in induction medium (figure 8e and 8f).

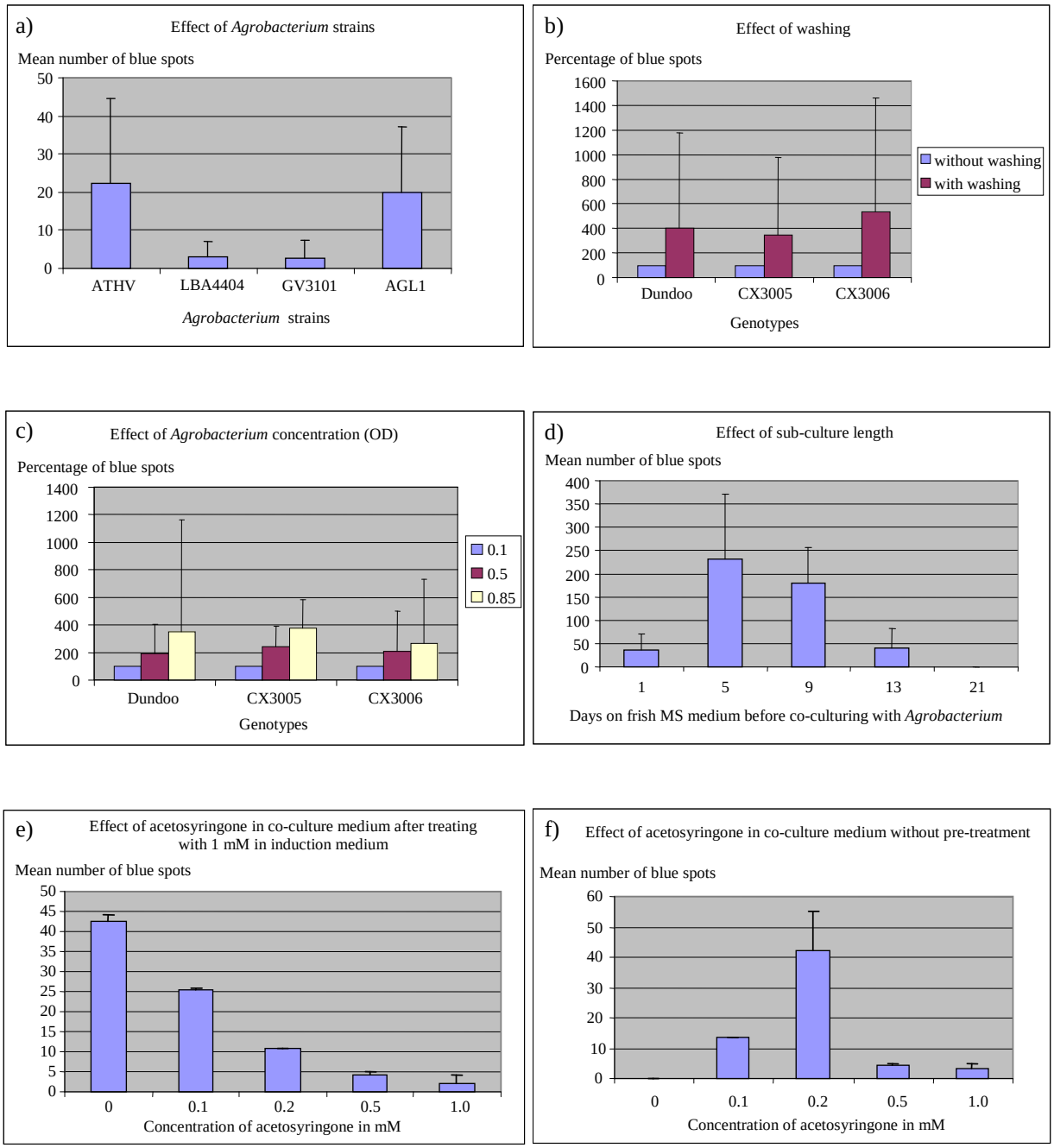


Figure 8: Assessment of different parameters enhancing the *Agrobacterium* mediated transformation efficiency of squash embrogenic calli. a: four *Agrobacterium* strains in the genotype Dundoo, b: washing and without washing with liquid MS medium for the genotypes Dundoo, CX3005 and CX3006, c: different bacterial densities for the genotypes Dundoo, CX3005 and CX3006, d: different sub-culture length prior to co-culture with *Agrobacterium* for genotype Dundoo, e: different acetosyringone concentration in co-culture medium for the genotype CX3005, f: addition of 1 mM acetosyringone in induction medium prior to co-culture on medium containing different acetosyringone concentration for the genotype CX3005.

3.2.2 Selection system

For the stable *Agrobacterium* transformation, the GFP reporter gene was used to monitor transgenic cells. In this experiment, GFP was quantified as a number of fluorescent areas originated from an independent transformation event on callus of CX3006 and Dundoo as shown in table 6. The effect of selection agents on embryogenic callus showed in figure 9, and the expression of green fluorescence of the GFP gene showed in figure 10. The results observed for CX3006 revealed that the expression of GFP in transformed cells increased by increasing kanamycin concentration. The highest number of GFP fluorescent areas (240) was observed with the highest kanamycin concentration (300 mg/l) for CX3006. Effect of kanamycin concentrations (100 and 200 mg/l) on the number of GFP-expression areas in Dundoo was similar. No fluorescent areas were observed in Dundoo with lowest (50 mg/l) and highest (300 mg/l) kanamycin concentations, respectively. By using paromomycin as a selective agent, the highest number of GFP fluorescent areas (103 and 215) was observed with concentration 150 and 200 mg/l in Dundoo and CX3006, respectively. GFP fluorescent areas were the highest (29 and 300) by using kanamycin/Paromomycin cobmination (150/150 mg/l) in Dundoo and CX3006, respectively. In the case of Phosphinothricin, the concentration of 12.5 mg/l resulted in the highest number of GFP fluorescent areas (14 and 160) in Dundoo and CX3006, respectively.

Table 6: Quantification of GFP fluorescence areas on embryonic calli of CX6 and Dundoo varieties of squash plant (*Cucurbita pepo)* after incubation for four months on MS medium supplemented with different concentrations of selection agents and 5 mg/l 2, 4-D. Selection agents were added five days after inoculation of embryonic callus with *Agrobacterium tumefaciens* and subcultured monthly on the same medium.

Kind of selection agents	Concentration of selection agents (mg/1)	No. of fluorescent areas on callus of CX3006	No. of fluorescent areas on callus of Dundoo
Kanamycin (Km)	50	32	0
	100	100	16
	150	155	10
	200	165	16
	300	240	0
Paromomycin (Pm)	50	70	0
	100	1	20
	150	215	53
	200	1	103
	300	36	25
Km/Pm	25/25	0	7
	50/50	120	0
	75/75	16	0
	100/100	245	2
	150/150	300	29
Phosphinothricin (PPT)	2.5	6	0
	5	27	1
	7.5	6	0
	12.5	160	14

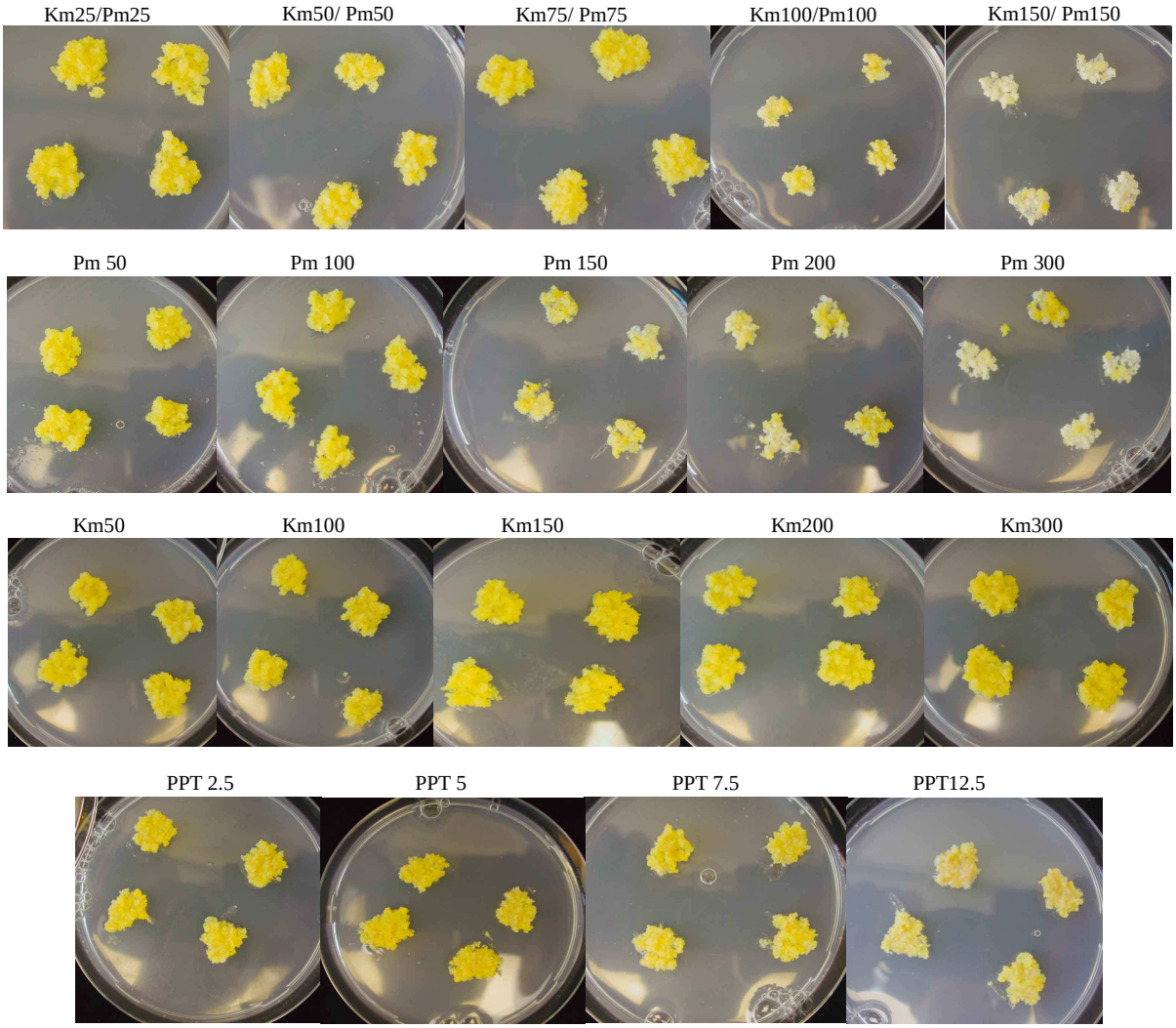


Figure 9: Effect of different concentration of selection agents on embryogenic callus of cv. Dundoo three weeks post co-cultivation.

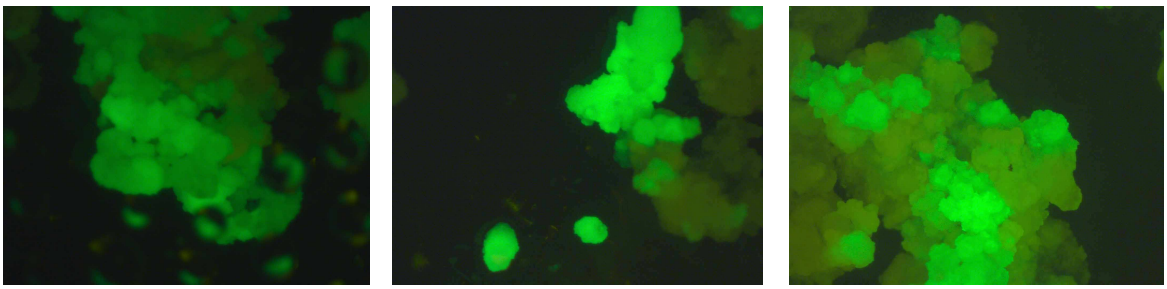


Figure 10: GFP expressing callus 8 weeks post co-cultivation

3.3 Identification of local WMV-II and ZYMV strains in Palatinate

3.3.1 Screening of field samples

Total RNA was extracted from virus infected squash harvested on fields in Palatinate. High amount of RNA was isolated from the collected samples, suitable for RT-PCR, and typical RNA pattern was found with different sizes of rRNA shown in figure11.

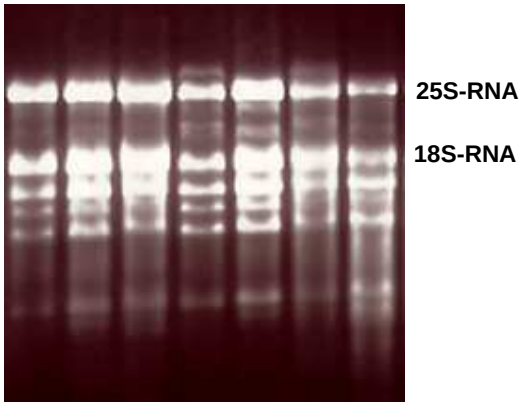


Figure 11: Total RNA extraction from infected squash leaves.

Two primer pairs were designed from available sequences of the isolates of ZYMV and WMV in the gene bank. These primers were designed to amplify 1000 and 700 bps in length in the coat protein genes (the highly conserved region) of ZYMV and WMV, respectively. 48 RNA squash samples were tested for the presence of the targeted viruses. Only gels which have some positive samples are showed in the figure 12. Samples 29, 30 and 31 on figure 12a have positive bands at 700bps for WMV and 8, 9, 10, 12 and 15 on the figure 12b have positive bands at 1 kb. The results indicated that the designed primer pairs were succecessful to amplify the expected fragments in both viruses. The amplified fragments of both viruses were sequenced and the sequencing data were aligned compared to the sequences of the world isolates in the gene bank. The compared results of the sequences shared 99 % identity, indicating that our isolates are actually related strains of ZYMV and WMV-2.

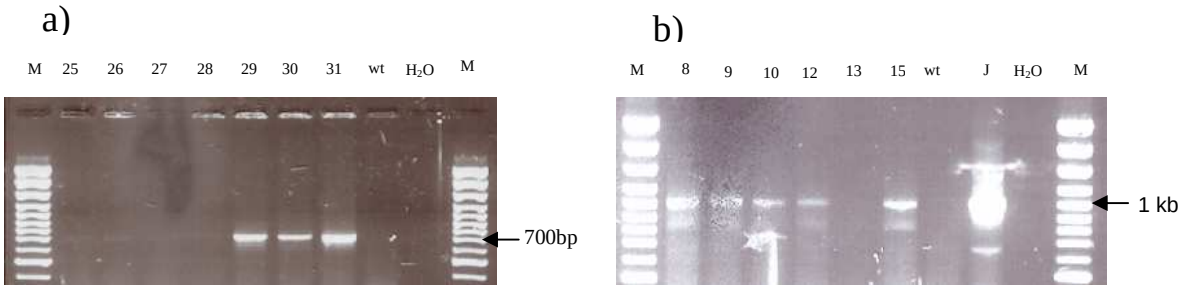
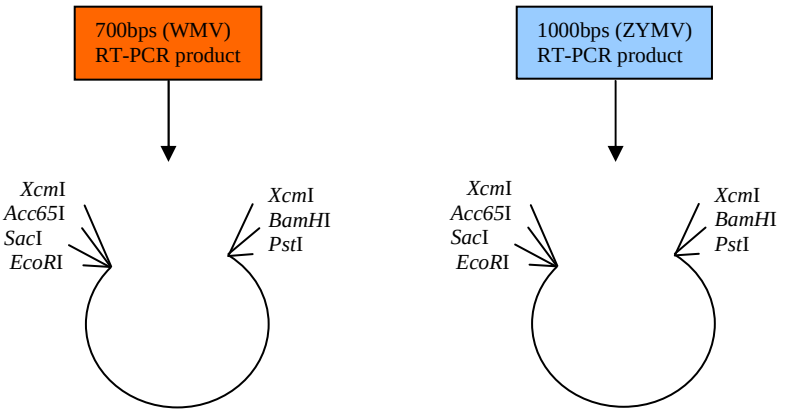


Figure 12: a) RT-PCR amplification of 700bp in the coat protein gene of WMV-2 in lanes 29, 30 and 31. M = 100bp DNA Ladder, wt = negative control. b) RT-PCR amplification of 1000bp in the coat protein gene of ZYMV in lanes 8, 9, 10, 12 and 15. J = positive sample from Jordan. M = 100bp DNA Ladder, wt = negative control.

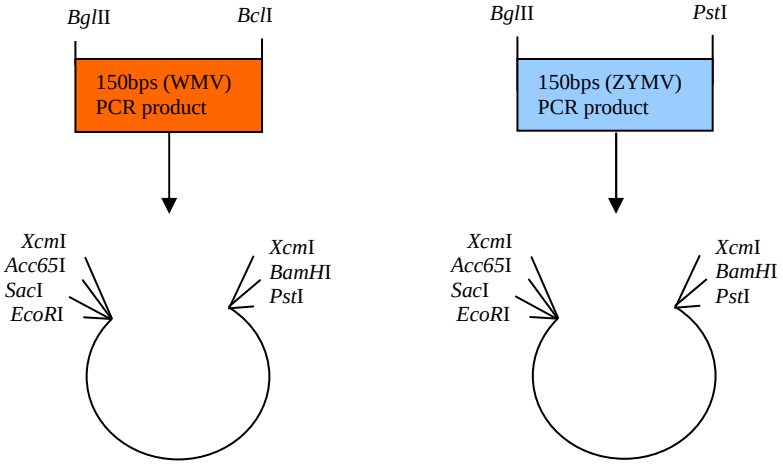
3.4 Cloning of inverted repeat constructs derived from WMV-II and ZYMV

Cloning of an inverted repeat construct derived from WMV-2 and ZYMV was obtained in several steps as outlined in figure 13 as following: 1) Long cDNA fragments were generated from the core of the coat protein genes of WMV-2 and ZYMV (700 and 1000bps) respectively by RT-PCR, they were cloned into a pTPCR cloning vector linearized with the restriction enzyme *Xcm*I. The recombinant plasmid was then sequenced. 2) A highly conserved region was amplified in the previous sequences to produce short DNA fragments (150bps) with primer pairs containing *Bcl*I and *Bgl*II restriction enzyme sites for cloning in the case of WMV-2 and *Bgl*II and *Pst*I in the case of ZYMV. Then the resulting fragments were separately cloned into the pTPCR vector. 3) To bind both fragments of WMV-2 and ZYMV together, the plasmid contains WMV-2 fragment was linearized at the 3´end of this fragment with *Bcl*I and *Pst*I restriction enzymes respectively. Then the ZYMV fragment was subcloned with *Bgl*II and *Pst*I restriction enzymes respectively. 4) To ligate WMV2-ZYMV fragment to an Intron, the plasmid pBluescript+/- contains Intron-C (509 bp) of the NiR gene from *Phaseolus vulgaris* (Johansen, 1996) was linearized at 3´end of the Intron with *Nsi*I and *Acc65*I restriction enzymes and the WMV-ZYMV fragment was inversely subcloned with *Acc65*I and *Pst*I restriction enzymes in this position. 5) In this step, the pBluescript+/- contains Intron-ZYMV-WMV was linearized with *Sac*I and *Pst*I restriction enzymes at 5´of the Intron and the fragment of WMV-ZYMV in step 3 was subcloned in this position with

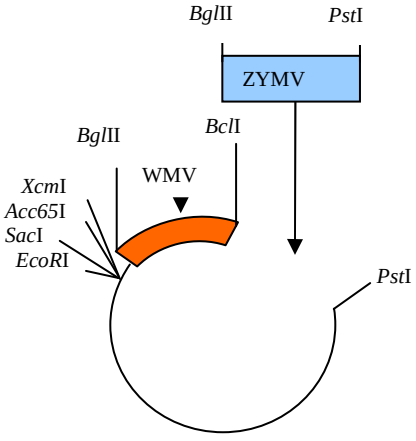
*Sac*I and *Pst*I restriction enzymes. 6) The accomplished inverted repeat construct was then subcloned between the35S promoter and pA35S terminator with *Bgl*II restriction enzyme in the cloning vector pRT101, which was first linearized with *Bam*HI restriction enzyme. The orientation of the insert was checked by digesting the plasmid either with *Pst*I alone or with *Pst*I/*Eco*RV restriction enzymes and sequencing was analysed. 7) The complete cassette was subcloned into binary vectors pPZP family (Hajukiewicz *et al*., 1994) pPZP200, pPZPnpt and pPZPbar at the *Hind*III restriction enzyme site. The positive clones were transferred into *Agrobacterium tumefaciens* ATHV strain by electroporation. The orientation of the constructs was checked, for pPZPnpt by digesting the plasmid with *Eco*RV alone and with *Hind*III alone, for pPZPbar by double digestion with *Eco*RV/*Xho*I and with *Hind*III alone.



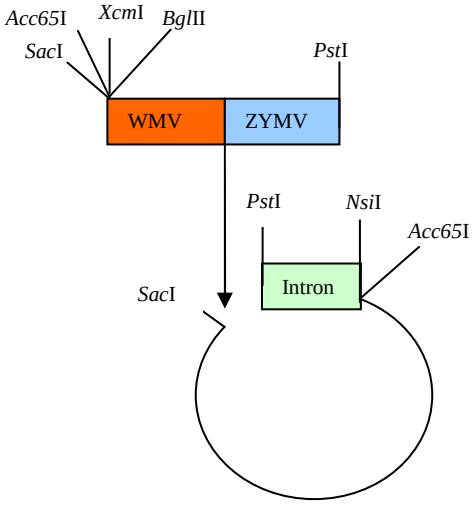
Step 1) cloning of 800 and 1000bps derived by RT-PCR from WMV and ZYMV respectively in the pTPCR plasmid vector.



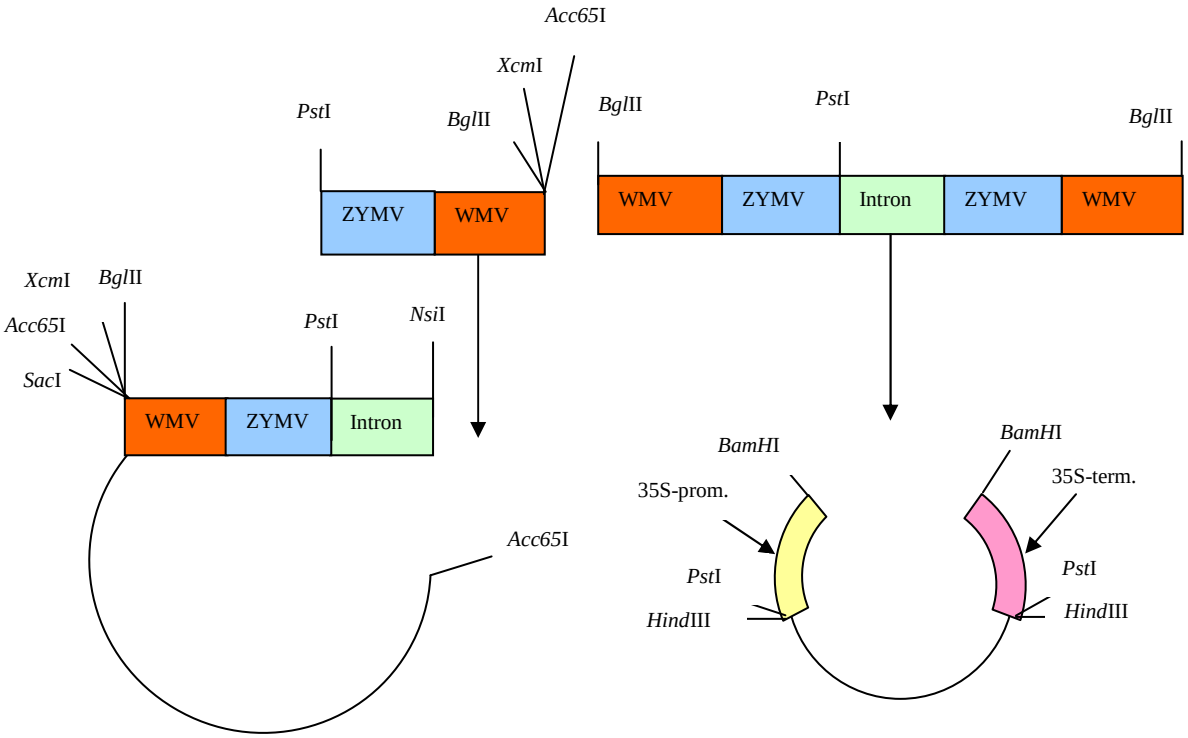
Step 2) Cloning of 150bps derived by PCR from step 1) of WMV and ZYMV in the pTPCR plasmid vector, respectively



Step 3) Ligation of ZYMV to WMV into the pTPCR plasmid vector

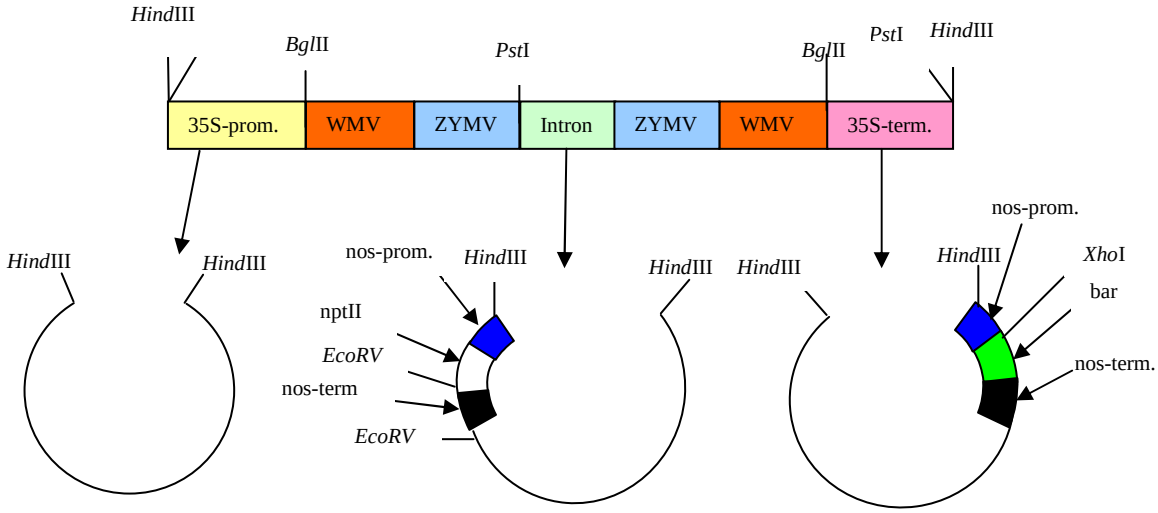


Step 4) Cloning of WMV-ZYMV at the left of the Intron into the pBluescript II SK (+/-) plasmid vector.



Step 5) Cloning of ZYMV-WMV at the right of the Intron into the pBluescript II SK (+/-) plasmid vector.

Step 6) Cloning of the inverted repeat construct separated by the Intron into the pRT101 plasmid vector to ligate it to 35S promoter.

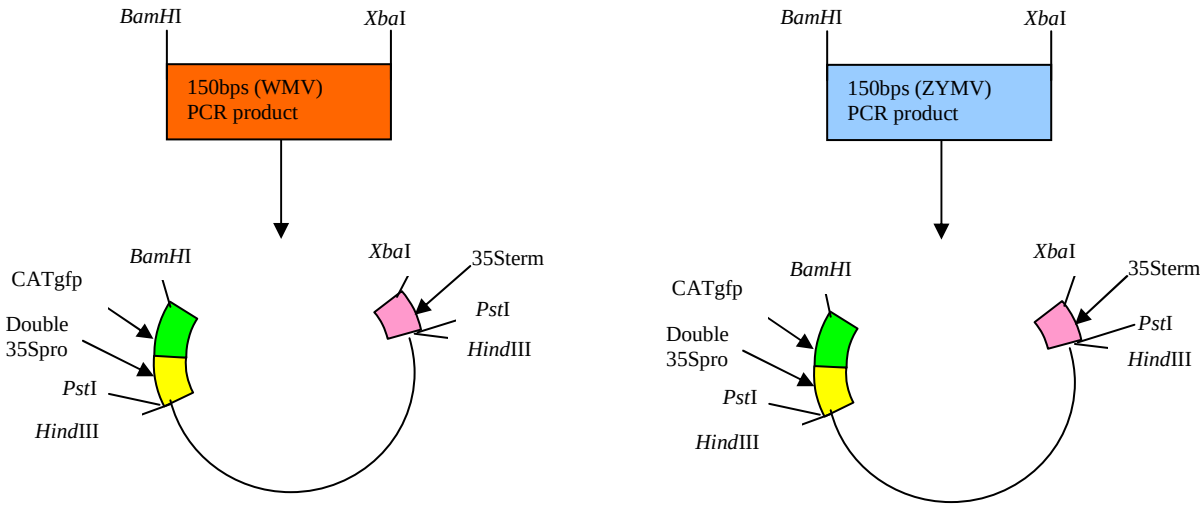


Step 7) cloning of the complete cassette into pPZP plasmid binary vector contains nptII or bar genes as selectable marker or without as shown in the figure.

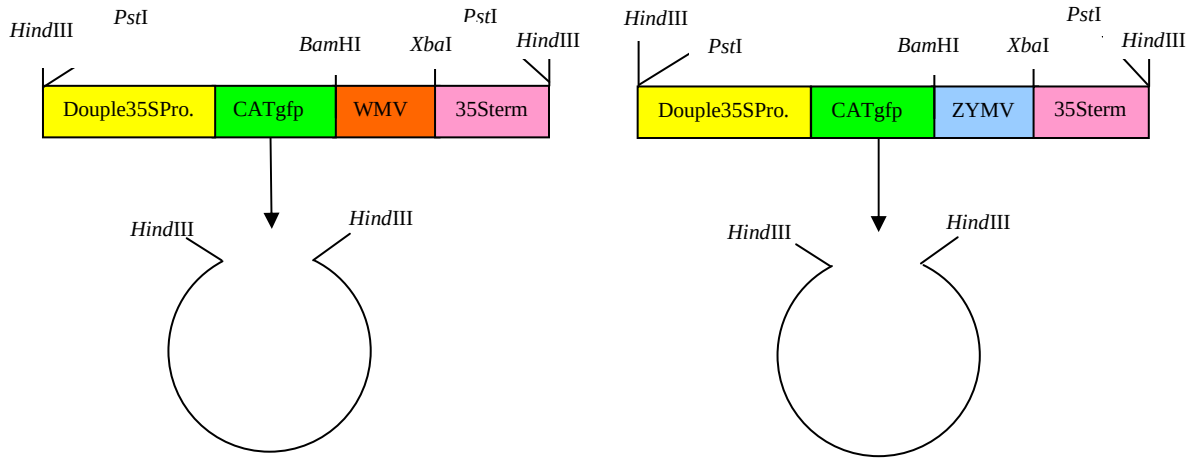
Figure 13: Schematic representation of the cloning strategy from step 1-7 of the inverted repeat construct derived from WMV and ZYMV.

3.5 Cloning of sensor constructs of WMV-2 and ZYMV for infiltration

The sensor constructs were obtained in two steps as outlined in figure 14. First, the generated sequences of the second step in the inverted repeat constructs of WMV-II and ZYMV in figure 13 were amplified with new primer pairs contain *Bam*HI and *Xba*I restriction enzyme sites each.



Step 1) Cloning of 150bps derived by PCR from step 2) of the cloning strategy of WMV and ZYMV in the pCATgfp plasmid the GFP gene under control of double 35S promoter.



Step 2) cloning of the complete cassette into pPZP plasmid binary vector

Figure 14: Schematic representation of cloning strategy of sensor constructs of WMV-II and ZYMV.

The purified fragments were digested with *Bam*HI and *Xba*I restriction enzymes and re-purified again to inactivate the restriction enzymes. The purified fragments of both viruses were separately cloned between *Bam*HI and *Xba*I restriction enzyme sites by the 3´end of a GFP gene. The GFP gene is located in the pCATgfp cloning vector under control of double 35S promoter. Before cloning, the host plasmid pCATgfp was first linearized with the same restriction enzymes as indicated above, restriction enzymes were inactivated at 80°C for 15 min, plasmids were purified by phenol/chloroform and dephosphorylated with Calf Intestine Alkaline Phosphatase as previously described. Second, the complete cassettes were separately subcloned into binary vectors of pPZP200 at the *Hind*III restriction enzyme site and then transferred into *Agrobacterium* ATHV strain by electroporation.

3.6 Evaluation of the function of the inverted repeat construct in *N. benthamiana*

To evaluate the function of the inverted repeat construct of WMV- ZYMV the inverted repeat construct was transferred into *Nicotiana benthamiana* plants.

3.6.1 Screening of putative transgenic plants by multiplex PCR analysis

DNA was extracted from 13 putative transgenic plants (lines) grown *in vitro* on kanamycin containing medium, a non-transgenic plant as negative control, and the plasmid carrying the transgene as positive control were used as templates for PCR amplification (figure 15a). The lane numbers in figure 15a are also indicated the number of lines. The presence of bands at 534 and 807 bp in the lanes 1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 13 and in the lane P (positive control) confirmed the integration of the transgene. These bands were not observed in lane N (negative control). False amplification in lane 9 was observed. Binding sites of primers are presented on figure 15b.

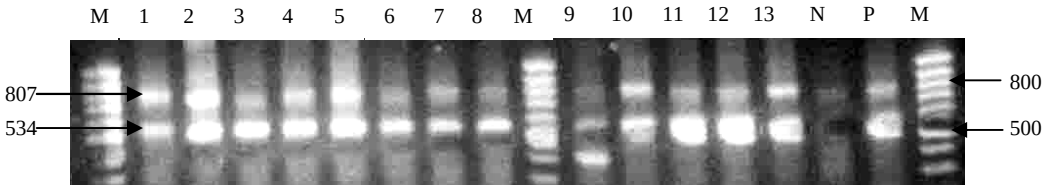


Figure 15a: PCR analysis of transgenic T_0 plant transformed with inverted repeat construct, M (O' GeneRuler™ 100bp DNA Ladder, ready-to-use), N (negative control wild type plant) and P (plasmid DNA positive control).

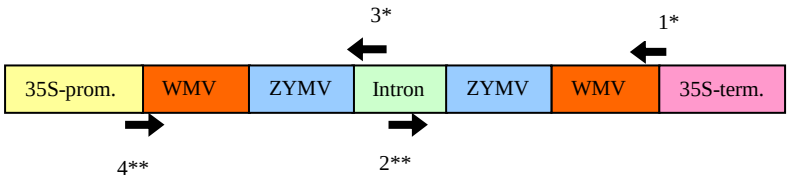


Figure 15b: Inverted repeat construct of WMV and ZYMV and multiplex PCR primers binding, the arrows indicted at the position of primer binding, 1 and 2 amplified fragments at 534 bp in length, 3 and 4 amplified fragments at 807 bp in length, * and ** = forward and reverse primers, respectively.

3.6.2 Segregation of *N. benthamiana* T_1 plants

T_1 seeds derived from self pollination of the regenerated T_0 plants were harvested and allowed to germinate on MS medium containing kanamycin. Kanamycin resistant seedlings develop normal plantlets as they were well rooted on kanamycin medium. Sensitive individuals can not form true leaves; their leaves become white, they can not develop roots, and the seedlings finally died on kanamycin medium. The segregation data obtained (Table 7) were compared with the expected values in a Chi-square test. The results were not significantly different from a 3: 1 segregation in all lines tested except for line 5. These data indicated that the T-DNA, in which the NPT-II gene and the gene of interest were inherited as a single locus. A ratio of 3: 1 segregation correlates with the Mendelian segregation of a single dominant gene.

Table 7: Segregation data of NPT-II activity of T1 progeny derived from 13 transformed T_0 lines

Line No.	Km^r	Km^s	total	Segregation ratio	χ^2
WT	0	50	50	-	-
1	36	14	50	2.6: 1	0.24
2	43	7	50	6.1: 1	3.23
3	27	13	40	2.1: 1	1.2
4	42	8	50	5.3: 1	2.16
5	49	1	50	49: 1	14.11
6	38	12	50	3.2: 1	0.03
7	18	7	25	2.6: 1	0.12
8	35	15	50	2.3: 1	0.67
9	41	9	50	4.6: 1	1.31
10	38	12	50	3.2: 1	0.03
11	37	13	50	2.8: 1	0.03
12	33	10	43	3.3: 1	0.07
13	34	15	49	2.3: 1	0.82

3.6.3 Southern blot analysis of transgenic *N. benthamiana* plants

Southern blot was analysed (figure 16) after *Bam*HI digesting genomic DNA was extracted from T_0 transgenic plants, non-transgenic plant (negative control), and plasmid contained the inverted repeat construct of WMV and ZYMV sequences (positive control). Hybridization of the probe derived from the transgene (inverted repeat construct) with the DNA isolated from transgenic lines was detected in lines 1, 6, 7, 8, 10 and 13. No hybridization was detected in lines 4 and 11 as well as in non-transgenic plant (negative control). Plasmid DNA (positive control) generated hybridization signal at the expected size. The copy number in transgenic lines ranged from 1 to 2 based on the number of the bands present in each lane. Two copies in lines 8 and 10 and one in the other were detected.

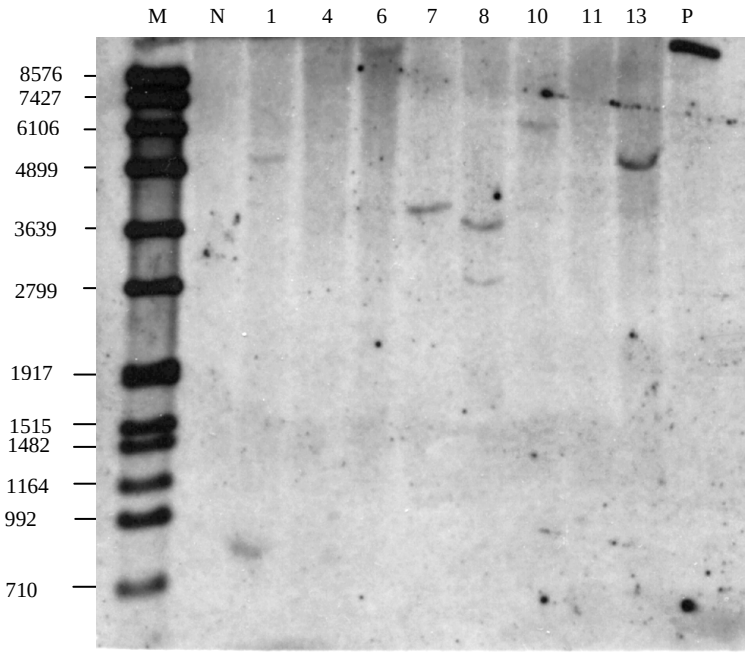


Figure 16: Southern blot analysis of genomic DNA extracted from T_0 transgenic lines, restricted with *Bam*H1, blotted on a 0.9 % agarose gel and transferred to a nylon memberane and hybredized with with probe from the inverted repeat construct. Lane M: DNA molecular weight marker VII, lane N: non-transgenic plant (negative control), lane P: plasmid DNA (positive control), lanes 1, 4, 6,7,8,10,11 and 13 are the analyzed lines.

3.6.4 Evaluation of gene silencing in transgenic *N. benthamiana* plant by *Agrobacterium* infiltration method

This experiment was done to evaluate the efficiency of silencing induction of the inverted repeat construct in transgenic plants. The sensor constructs GFP-WMV and GFP-ZYMV developed in this study consisting of the same sequences of the transgene as well as the GFP gene under control of the double 35S promoter were used in the infiltration experiments. The P19 silencing suppressor was separately co-infiltrated with these constructs in leaves of transgenic and non-transgenic plants. The results on the figure 17 showed no expression was visually observed with both sensor constructs (GFP-ZYMV) and (GFP-WMV) (A) and (B), respectively in transgenic plants. In contrast to wild type (control) plants which reflect GFP expression with both constructs in (D) and (E) respectively. No GFP expression was observed on the plants infiltrated with P19 in leaves of transgenic or non-transgenic plants as shown in (C) and (F) respectively, while GFP expression in the leaves of transgenic plants was

presented bright green fluorescence when P19 was co-infiltrated with sensor constructs ZYMV or WMW (G) and (H) respectively. Also GFP gene was expressed in the leaves of both transgenic and non-transgenic plants with pKBgfp as positive control (I) and (G).

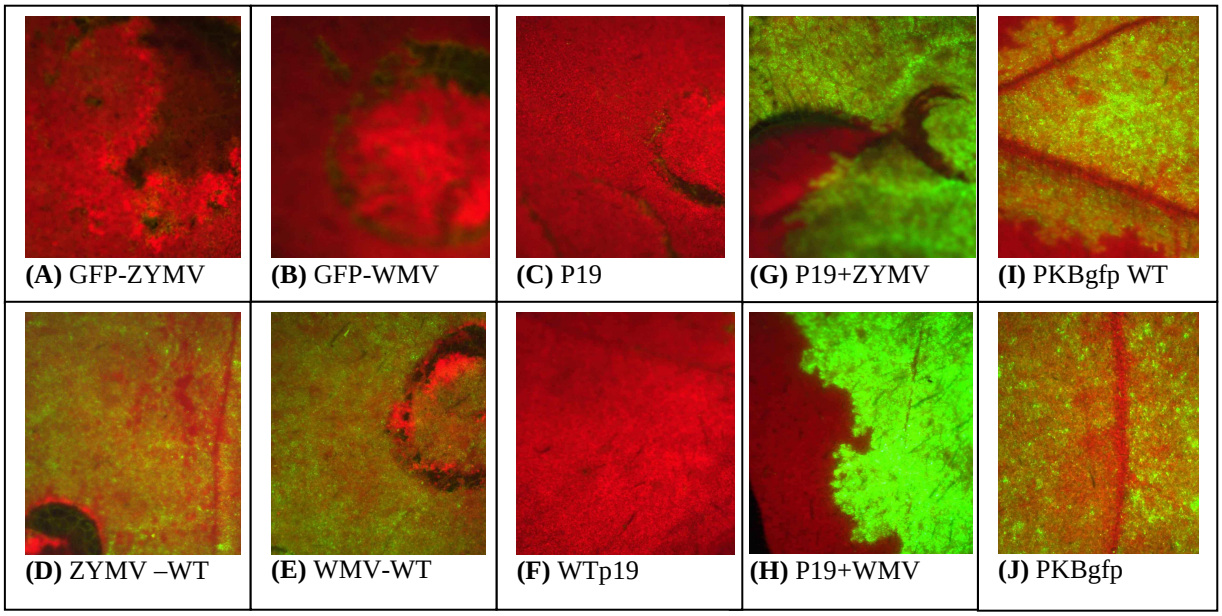


Figure 17: Infiltration of viral silencing suppressor (p19) and sensor constructs in leaves of transgenic and non-transgenic *N. benthamiana*. (A), infiltration of GFP-ZYMV sensor construct in transgenic plant. (B), infiltration of GFP-WMV sensor construct in transgenic plant. (C), infiltration of p19 construct in transgenic plant. (D), infiltration of GFP-ZYMV sensor construct in wild type plant. (E), infiltration of GFP-WMV sensor construct in wild type plant. (F), infiltration of p19 construct in wild type plant. (G), infiltration of mixture of p19 and GFP-ZYMV sensor construct in transgenic plant. (H), infiltration of mixture of p19 and GFP-WMV sensor construct in transgenic plant. (I) and (J), infiltration of PKBgfp as a GFP control in non-transgenic and transgenic plants, respectively.

4 Discussion

Viral diseases of cucurbits are an important limitation in the crop production, therefore virus-resistant transgenic crops is an essential requirement when resistant cultivars are not available. The discovery that double-stranded RNA (dsRNA) is a more effective inducer of post-transcriptional gene silencing (PTGS) than either sense or antisense RNA in plants and animals (Fire *et al*., 1998; Kennerdell and Carthew, 1998; Sanchez-Alvarado and Newmark, 1999; Waterhause *et al*., 1998) has prompted the development of improved gene-silencing methodology. The strategy of resistance through PTGS mechanism in this study is depending on the short virus sequences derived from the invaded viruses (ZYMV and WMV-2) were constructed through several cloning steps as an inverted repeat construct followed by transferring process into the plant cells using *Agrobacterium*-mediated transformation. The transformation method requires the delivery of DNA into the nucleus of cells and its insertion into the genome and regeneration of transformed plant which take place through somatic embryogenesis.

4.1 Induction of somatic embryogenesis and plant regeneration from young and old somatic embryos

The first reports on somatic embryogenesis were published by Reinert (1958) and Steward *et al.* (1958). Regeneration of plants *in vitro* via somatic embryogenesis has some distinct features such as single-cell origin, the consequent low frequency of chimeras and the production of high number of regenerates (Ammirato, 1983; Sato *et al*., 1993). For these reasons we decided to choose somatic embryogenesis as a regeneration method in the current study.

Using explants derived from mature seeds, cotyledons, shoot tips and leaves of three summer squash (*Cucurbita pepo* L.) cultivars, efficient plant regeneration via somatic embryogenesis was established. The length of induction period of somatic embryogenesis ranged from 13 to 20 weeks depending on the explant type and genotype used. Formation of somatic embryos was noted on leaf explants 13 weeks, on mature seed explants 17 to 20 weeks and on

cotyledon explants 18 weeks after starting of the induction. In a study by Kintzios *et al*., 2002 somatic embryogenesis was observed 4 weeks after initiation of induction from leaf explant derived from squash and melon. However it has taken only 9 weeks by a method described by Chee (1992) who used mature seeds as source of explants in squash (*Cucurbita pepo* L. cv.YC60), whereas in another study on Styrin pumpkin by the method of Urbanek *et al.* (2004) it lasts 20 weeks. These results may be referred to the different genotypes and explants used.

For plant regeneration, only two reports have been published on regeneration of summer squash (*Cucurbita pepo* L.) through somatic embryogenesis (Chee, 1991, Chee, 1992; Gonsalves *et al*., 1995). In this study, 110 and 132 embryos were converted into plantlets per gram of embryogenic callus from CX3006 and Dundoo cultivars, respectively. 50 % and 70 % of the converted embryos were normal looking plants from CX3006 and Dundoo cultivars, respectively. On the other hand, the regeneration was not possible after transforming the callus with the invereted repeat construct when material was maintained *in vitro* on culture medium for over two years. The mentioned (70 %) normal looking plants are nearly similar to results obtained by Juretic and Jelaska (1991) who obtained 77 % normal plants from pumpkin and the 50 % are in agreement with results obtained by Chee (1992) in squash.

Phenotypic abnormality of embryos was observed on cucumber (Ziv and Gadasi, 1986) on pumpkin (Juretic and Jelaska, 1991), and on squash (Chee, 1992). These abnormalities may refer to somaclonal variation occurred in tissue culture in response to the effect of components of culture media. Somaclonal variation events generated through tissue culture may induce unwanted mutation (Venkatachalam *et al*., 2000). On the other hand, somaclonal variation can produce desirable agronomic characteristics such as increases in salt tolerance, resistance to herbicide, diseases, extreme temperatures, desiccation, or can yield interesting biochemical or ornamental mutations (Maluszynsky *et al*., 1995). For example, cell selection and regeneration via somatic embryogenesis have been used to improve salt tolerance and disease resistance in Citrus (Litz *et al*., 1985), virus resistance in sugarcane (Oropeza and de Gracia, 1996), germination at low temperature in melon (Ezura *et al*., 1995) and resistance to phytotoxins in coffee (Nyange et al., 1995).

Our results showed that the highest number of embryos obtained from various explant types and different genotypes were induced on medium supplemented with 2,4-D alone. These

results indicated that stress induction through high auxin concentration, especially 2,4-D is required for induction of somatic embryogenesis in squash. The effect of auxin on induction of somatic embryogenesis was investigated in several cucurbitaceous species. Previous investigations have demonstrated that the induction of somatic embryogenesis among cucurbitaceous species can be controlled by the ratio of auxin and cytokinin in the culture medium. Oridate and Oosawa (1986) found that 0.26 µM BA was the most suitable concentration for inducing embryo formation in melon. The induction of somatic embryogenesis in the same species was favored at high levels (105-525 µM) of indole-3-acetic acid (IAA) but at relatively low concentration of 2,4-D (4.5-9 µM) (Tabei *et al.*, 1991). 2,4-D alone or in combination with other auxin is usually required for the induction of somatic embryogenesis (Debeaujon and Branchard 1993; Nadolska-Orczyk and Malepszy, 1987; Rajasekaran *et al*., 1983; Tabei *et al*., 1991). Somatic embryogenesis was achieved in squash on medium contained 2,4-D alone (Chee, 1992; Gonsalves *et al*., 1995) or in combination with Kinetin (Kintzios *et al*., 2002).

The maturation of somatic embryos was commonly conducted on media without growth regulators and in some cases by the addition of activated charcoal. Its addition to culture medium may promote or inhibit *in vitro* growth depending on the species and tissue used. The effect of activated charcoal may attributed to establishing a darkened environment, adsorption of undesirable/inhibitory substances, adsorption of growth regulators and other organic compounds or the release of growth promoting substances present in or adsorbed by activated charcoal (Pan and Staden, 1998). In *Cucumis sativus* L differentiation of embryos was enhanced by washing the suspension culture cells with MS medium containing 0.5% activated charcoal. 60 to 70% of the embryos pre-washed with activated charcoal germinated into plantlets with normal morphology (Chee and Tricoli, 1988).

By using the old embryogenic tissue (two years old, maintained under regular *in vitro* culture conditions) for transformation upon regeneration time, it was impossible to obtain transgenic plantlet. In this case, long term maintaining of embryogenic callus may have caused loss of regeneration competency. It may partially attributed to long term application of plant growth regulators in culture medium since the auxin was known to prevent the development of embryos from embryogenic cell clusters and cause unorganised growth. It was reported that regeneration capacity from *in vitro* tissue culture are basically influenced by the genotype and physiological status of the donor plant, the plant organ used as an explant, the culture medium

and the interactions between them (Mathias and Simpson, 1986; Lazer *et al*., 1984; Bregitzer, 1992; Henry *et al*., 1994).
Lai and Chen (2002) found that regeneration frequency of transgenic explants has also been linked to the cell cycle phase at the time of transformation. There was a strong positive correlation observed between the frequency of S and G2 nuclei and regeneration ability in *Petunia hybrida* and a strong negative correlation between frequency of G0 and G1 nuclei and percentage of regeneration after using *Agrobacterium* transformation.

The problem of regeneration of old material introduced here may be solved by the following suggesting solution: 1) Permanent initiation of new-induction experiments to always have fresh material. 2) Regeneration through organogenesis. 3) Cryopreservation of the embryogenic callus 4) Avoiding tissue culture or regeneration procedure by transforming tissues that give rise to gametes.

For the first strategy, the frequent initiation of new embryogenic cell cultures can reduce the adverse effects on genetic fidelity and regeneration potential but it is expensive and time consuming. Moreover, the initiation of new cultures could be particularly difficult in some species in which the establishment of embryogenic cell cultures have proven to be complicated or in which the embryogenic explants are only available during short periods of the year (Vicient and Martinez, 1998). For the second strategy, regeneration of summer squash (*Cucurbita pepo* L.) through organogenesis has only been published by Ananthakrishnan *et al*., 2003. However, we have found that the organogenic method was hard to produce squash shoot in our laboratory, perhaps due to the genotypes used or batch differences. In the third strategy, the embryogenic callus once it has been obtained, it can be stored for long-term at -196°C in liquid nitrogen. However, maintenance of cell cultures by subculture is time consuming and expensive and involves the risk of loss of valuable materials through contaminations or human technical errors. Additional problems of long-term cell cultures include the risk of genetic changes by somaclonal variation and decreases in the plant regeneration capacity and in the viability of the regenerated plants (Fitch and Moore, 1993). Cryopreservation (storage at -196°C in liquid nitrogen) of embryogenic cell cultures, explants or somatic embryos is a good alternative to cell culture maintenance (Kartha, 1987). Cryopreservation minimizes the necessity of the establishment and maintenance of the embryogenic cell cultures, reducing manipulations, genetic variation and risks of loss. The possibility of combining the preservation potential of cryopreservation with the propagation

potential of somatic embryogenesis makes this technique very interesting for germplasm conservation institutes. Moreover, in some cases the embryogenic potential and/ or regeneration capacity of the embryogenic cell culture can be improved after cryopreservation (Bercetche *et al*, 1990; Lynch *et al.*, 1994). This phenomenon may be explained by a selective survival of the embryogenic cells. The fourth strategy involved in development of plant transformation methods that avoid tissue culture or regeneration procedures. In many cases these have targeted meristems or other tissues that will ultimately give rise to gametes (Chee and Slighton, 1995; Brich, 1997). Feldmann and Marks (1987) applied *Agrobacterium* to Arabidopsis seeds, grew plants to maturity in the absence of any selection and then germinated progeny seeds on antibiotic-containing media to identify transformed plants. Desfeux *et al*., (2000) obtained transformants by floral-dip of Arabidopsis plants in an *Agrobacterium* solution 5 days prior anthesis.

4.2 *Agrobacterium*-mediated transformation

The efficiency of transformation is greatly influenced by the compatibility between plant and bacteria. One of the most important factors influencing *Agrobacterium*-mediated transformation is susceptibility of the host genotype to the specific *Agrobacterium* strain. Some strains of *Agrobacterium* are more virulent than other on a particular plant species and, conversely, some plant species or genotypes are more or less sensitive to particular strains of *Agrobacterium* (De Cleene and De Ley, 1976; Anderson and Moore, 1979). Such variation may be partly due to differences in the ability of the bacteria to attach to plant cells or differences in either bacterial- or plant- encoded T-DNA transfer machinery (Lippincott *et al*., 1977; Yanofsky *et al*., 1985; Nam *et al*., 1997). Therefore, evaluation of strains-cultivar compatibility is an important step in the establishment of a transformation protocol.

In this study a compatibility test between squash embryogenic calli and four commonly used disarmed *Agrobacterium* strains revealed genotype-dependent response on the efficiency of the T-DNA transfer as determined by expression of the *uid A* (Gus) marker gene. As shown in the results the highest transient expression was observed with two *Agrobacterium* strains ATHV, which is an EHA 101 derivation and AGL1. On the other hand, a low transient expression was observed with *Agrobacterium* strains LBA4404 and GV3101. This can be attributed to the fact that ATHV and AGL1 strains are known as supervirulent. Nadolska-

Orczyk and Orczyk, (2000) have compared the virulence of three *Agrobacterium* strains (LBA4404, C58C1 and EHA105) and they reported that transformation efficiency was highest with strain EHA105. Cao *et al.* (1998) have reported similar results when comparing LBA4404 and EHA105. Strains EHA101 and EHA105 are more effective than LBA4404 since both are derived from supervirulent wild-type strain A281 (Hood *et al.*, 1986, 1993), whereas LBA4404 was derived from less virulent strain Ach5 (Hoekema *et al.,* 1983).
Transformation of squash is reported only in patents (Gaba *et al*., 2004). AGL1 strain carrying the binary vector pCambia3301 containing Bar and Gus genes was used to produce transgenic bottle groud (*Lagenaria siceraria* Standl) (Han *et al.,* 2005), and LBA4404 strain carrying the binary vector pCGN783 containing NPT-II gene was used for cucumber transformation (*Cucumis sativus* L.) (Sarmento *et al.,* 1992). Although, there are no reviewed publications on the transformation of Cucurbita spp. of such transgenic crops, the first commercial transgenic cucurbit product was *C. pepo.*

A positive effect of phenolic compounds such as acetosyringone on *Agrobacterium*-mediated transformation has also been demonstrated in many *Cucurbitaceae* species such as *Cucumis sativus* L. (Ganapathi and Perl-Treves 2000; Nishibayashi *et al*., 1996) and *Cucumis melo* (Bordas *et al*., 1997). Our results indicated that the presence of higher acetosyringone concentration (1mM) in the induction medium for 1 hour or lower acetosyringone concentration (0.2 mM) in co-cultivation medium for three days resulted in the highest transient expression. However, higher acetosyringone concentrations for three days in co-cultivation medium or lower acetosyringone concentration (1h.) did not enhance *Agrobacterium* transformation. These observations indicated that the amount and time of acetosyringone treatment is a critical factor for *Agrobacterium* transformation of squash. The lower acetosyringone concentration was probably not enough to induce *Agrobacterium vir* genes efficiently and higher acetosyringone for three days may have toxic effects on plant cells or are inhibitory to *Agrobacterium*. De Clercq *et al*., (2002) investigated whether acetosyringone is needed in the co-cultivation medium to obtain *Agrobacterium*-mediated gene transfer to *Phaseolus acutifolius* calli and whether the efficiency can be improved, by adding acetosyringone at different concentration (0.02, 0.2 and 2 mM) to the co-cultivation medium. They found an increasing number of *uid* A-expressing zones with increasing acetosyringone concentration and recorded a maximum of 0.2 mM acetosyringone. When 2 mM acetosyringone was used, less blue spots were observed and the callus showed necrotic zones. More than 10% of the explants did not survive under such co-culture contitions.

Acetosyringone is required for the activation of the *Agrobacterium* virulence machinery (Stachel *et al.*, 1985; Hiei *et al.*, 1994). Acetosyringone as a phenolic signal is transduced through a receptor *vir*A protein in the inner membrane of the bacterial cell (*vir* A senses the presence of wound-induced phenolic compounds). The *vir*A protein then transfers a phosphate group to an inactive *vir*G protein, thereby activating the *vir*G protein. Activated *vir*G protein is a transcriptional regulator that binds to DNA sequences ("vir box" sequences) preceding each *vir* operon, thus activating the expression of these genes. The expression of these genes triggers the transfer of the T-DNA from the Ti- plasmid to plant cell and its integration into the plant nuclear DNA.

The increasing of transient expression by washing of squash embryogenic callus may refer to removing of compounds or substances produced by the plant cells in response to stress of the exogenous hormones added in the culture medium. For instance, ethylene can be induced by variety of external factors including the application of auxins or wounding. The ethylene production is generally higher in tissue containing auxins or when these are applied to tissues (Larue and Gamborg, 1971). These compounds may inhibit *Agrobacterium* to bind to the plant cells. Excluding these compounds by washing may allow *Agrobacterium* to be better attached to plant cells and transfer its T-DNA. It has been suggested that auxin may stimulate ethylene production by inducing the enzymes involved in the formation of the gas (Kang *et al.*, 1971).

The effect of ethylene on gene transfer mediated by an *Agrobacterium tumefaciens* harbouring a binary vector with the β-glucuronidase (*uidA*) gene was investigated in melon, (*Cucumis melo* L.) (*Ezura et al.*, 2000). They found that the explants excised from melon cotyledons produced ethylene. The ethylene production increased by the addition of 1-aminocyclopropane-1-carboxylic acid (ACC, 20 or 200 μM), and was inhibited by the addition of aminoethoxy-vinylglycin (AVG, 10 μM). *Agrobacterium* inoculation of explants increased ethylene production, while application of AVG during inoculation reduced it. After 4 days of co-cultivation with *Agrobacterium*, gene transfer in the explants was assayed by transient *uidA* expression. Application of ACC to the co-cultivation medium reduced *Agrobacterium*-mediated gene transfer to explants and that of AVG increased it. These results suggest that ethylene affects the *A. tumefaciens*-mediated gene transfer to the explants excised from melon cotyledons, and the efficiency of *Agrobacterium*-mediated gene transfer can be improved by inhibiting ethylene production from the explants.

Attachment of the bacterium to the host plant is an initial step in the process of infection (Lippincott *et al*., 1977). Attachment can be affected by plant or tissue age, cell type, cell cycle stage and other physiological parameters (Graves *et al*., 1988). This work has showed that the highest transient expression was observed in explants sub-cultured on fresh medium for 5 or 9 days pre-cocultivation. This may be due to high cell division occurred when the callus was sub-cultured on the new medium. This cell division may reaches to maximum between 5 and 9 days resulted in high number of dividing cells. These dividing cells are highly competent to be transformed by *A. tumefaciens* (Binns, 1990). Transformation can be increased by manipulating either the explants and/or the bacteria, such as increasing the number of competent plant cells for transformation by pre-culturing explants (Mchughen *et al*., 1989; Sangwan *et al*., 1992).

Several studies have shown that the phase of the plant cell cycle at the time of transformation is a major determinant of transformation and regeneration efficiency. Transformation frequency is positively associated with cell division or dedifferentiation with higher transformation observed in cells with nuclei at the S and G2 phases of the cycle (Lai and Chen 2002; Pena *et al.*, 2004; Villemont *et al*., 1997). Using flow cytometry, Villemont *et al*. (1997) found that the highest stable transgene expression in *Petunia hybrida* takes place when cells are at S phase during transformation with *Agrobacterium*. No transformation occurred if cells were at G0–G1 (quiescent phase), and only transient transgene expression occurred when cells were at M phase (Villemont *et al*., 1997). Flow cytometric studies in citrus showed that a high frequency of actively dividing cells in S phase resulted in high rates of stable integration of DNA when co-cultivated with *Agrobacterium* in a medium rich in auxins (Pena *et al.*, 2004).

To allow recovery of sufficient number of transgenic plants from tissue, it is important to optimize the amount of the bacteria for inoculation the plant tissue (so as to minimize cell death while maintaining a sufficiently high level of T-DNA transfer). The presented work showed the highest percentage of blue spots at a bacteria density of OD 0.85. The increase may refer to the increasing of *Agrobacterium* cells in the co-cultivation medium which allows high number of *Agrobacterium* cells to attach to the plant cells resulting in high number of infected plant cells. The results are in agreement with Lin *et al*. (1994) who found an increasing of both, transient expression and stable transformation with the increasing number of *Agrobacterium* cells from 10^6 to 10^{10}. For the *Agrobacterium*-mediated transformation of

Arabidopsis thaliana, using high concentrations of *Agrobacterium* cells significantly increased the regeneration of green callus of the ecotype Landsberg as well as the ecotype Columbia. However, increasing the *Agrobacterium* concentration did not always increases the transformation rate. High *Agrobacterium* concentration may causes bacterial overgrowth which becomes problematic for the elimination of the *Agrobacterium* from the *in vitro* culture post-infection. Bacterial overgrowth can lead to the damage of the infected tissues or can result in tissue necrosis and interference with tissue regeneration.

Selection of stable transformed cells

The selective marker genes are introduced into plant genome along with the gene of interest in plant transformation process to give transformed cells a selective advantage allowing them to grow faster and better and to eliminate the non-transformed cells. These genes expressed a protein with, generally, an enzymatic activity allowing distinguishing transformed from non-transformed cells (Brasileiro and Dusi, 1999). The selective agents are generally used in the initial stages of transformation for an early selection of transgenic cells (Sawahel, 1994). The probability to recover transgenic plants in the presence of a selective agent is greater than in its absence, especially because the transformation rate is always low (10^{-3} to 10^{-6}). Moreover, only in rare cells the introduced gene is correctly integrated and expressed (Brasileiro and Aragao, 2001).

In this study several selection agents with different concentrations were used to select transformed callus derived from two squash cultivars in the presence of the GFP gene. In general the highest number of green areas was observed with a maximum concentration of the selection agents used. The squash calli can bear more than 300 mg/l from both antibiotics used which were kanamycin (Km), paromomycin (Pm), combination of both (Pm/Km) and PPT . The highest number of green areas was obtained in the genotype CX6 using Km (300 mg/l) Pm (150 mg/l), Pm/Km (150/150 mg/l) and PPT (12.5 mg/l) with 240, 215, 300 and 160, respectively. Using Km (100 mg/l) Pm (200 mg/l), Pm/Km (150/150 mg/l) and PPT (12.5 mg/l) resulted in the highest number of green areas in Dundoo with 16, 103, 29 and 14, respectively. From this result, transformed cells could be efficiently selected on moderate concentration of the examined selective agents, but it was not possible to regenerate planlets from these materials as indicated previously in 4.1.

Selection of transformants was often a problem as melon cotyledon was moderately resistant to Kanamycin, the most common antibiotic used for selection with the *NPT-II* gene (Dong *et al.*, 1991). Selection on Phosphinothricin using the bar gene for herbicide resistance was more efficient than selection with the *NPT-II* gene in the production of transformed shoots from cotyledons by organogenesis (Gaba *et al.*, 2004). The effect of various selection agents were used in other Cucurbitaceae species. Km (100 mg/l) was used for selection of transformed cotyledon tissue of melon (*Cucumis melo* L.) (Valles and Lasa, 1994; Cürük *et al.*, 2005); *Citrullus colocynthis* L. (Dabauza *et al*., 1997). PPT (2 mg/l) was used to select transformed cotyledon tissues of bottle gourd (*Lagenaria siceraria*) (Han *et al.*, 2005).

4.3 Evaluation of the functionality of the inverted repeat construct in *N. benthamiana*

To examine the functionality of the inverted repeat construct, it was transferred into the model plant (*N. benthamiana*). Regenerated plants were analyzed for the presence of the transgene by PCR, Southern blot and segregation of T1 on Kanamycin containing medium. As ZYMV does not infect *N. benthamiana* and inoculation experiments with WMV resulted in 50 % infected plants only, the silencing mechanism of the inverted repeat construct was examined by infiltration of the sensor construct (GFP-WMV and GFP-ZYMV) and a construct containing the viral silencing suppressor P19.

In this study PCR analysis of 13 plants which were selected on Kanamycin containing medium has confirmed the presence of the inverted repeat construct in regenerated *N. benthamiana* plants. All analyzed plants showed the expected bands of the transgene. This result referred to an effective selection system with Kanamycin. The southern blot analysis also confirmed the stable integration of the transgene as well the copy number. 5 out of 6 positive analyzed lines (No.1, 6, 7, 10 and 13) showed single copy integration of the transgene.

The segregation test is useful for identifying transgenic plant with single-locus DNA integration, but it does not allow the determination of the copy number at the locus. Most segregation generation (T1) T-DNA was inherited in a typical Mendelian fashion with a ratio of about 3: 1 in the progenies derived from *Agrobacterium* transformed plants suggesting

single locus integration of the T-DNA. Except line no. 5 was inherited in a non-Mendelian manner indicating more than single locus integration of the T-DNA. T-DNA integration can occur in any chromosome (Ambros *et al*., 1986; Chyi *et al*., 1986; Robbins *et al*., 1995; Wallroth *et al*., 1986) and involves illegitimate recombination (Gheysen *et al*., 1991; Matsumoto *et al*., 1990; Mayerhofer *et al*., 1991). T-DNA integrates either at one locus or at several independent loci. T-DNA loci frequently consist of several T-DNA copies which can be linked to each other in complex T-DNA structures (Jorgensen *et al*., 1987; Koncz *et al*., 1994).

Suppression of Post-transcriptional gene silencing

Transient expression provides a rapid method for assaying the function of some types of genes; transgene can often be assayed within a few days of infiltration (Janssen and Gardner, 1989). The results herein showed that the co-infiltration of plasmid carrying viral silencing suppressor Protein (P19) with plasmid carried the corresponding transgene sequences and GFP gene clearly resulted in a stronger expression of GFP gene in infiltrated leaves. While infiltration of sensor constructs (GFP-WMV or GFP-ZYMV) alone into transgenic plants without P19 infiltration did not result in silencing suppression. However GFP was observed when these sensor constructs were infiltrated in wild type plants. These observations confirmed that the silencing of transgene in transgenic plants is establsihed. The enhancement of GFP expression in transgenic plants refers to the fact that P19 encoded by *Tomato bushy stunt virus* binds to siRNA triggered by the inverted repeat construct (transgene) and blocks siRNA interaction with its target (the specific viral RNA) (Baulcombe and Molnar, 2004). This obviously-GFP expression effects when the P19 was co-infiltrated with plasmid GFP-sensor constructs is in agreement with the observation of Voinnet *et al*. (2003).

RNA silencing is one of the major defence mechanisms against viruses invading plants (Dougherty and Parks, 1995; Lindbo *et al*., 1993; Smith *et al*., 1994; English *et al*., 1996; Baulcombe, 1996; Baulcombe, 2004; Al-Kaff *et al*., 1998; Tenllado *et al*., 2004). Specific viral proteins able to suppress the RNA silencing mechanism have been identified in many plant viruses (Roth *et al*., 2004). The plant viral suppressor proteins are expected to play important roles in the study of the mechanism of RNA silencing and as a tool for industrial production of proteins in plants.

5 Summary

Viral diseases of Cucurbits are an important limitation in the production of the crop. *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus*-2 (WMV-2) are the most important squash (*Cucurbita pepo L.*) infecting viruses. Mixed infections with these viruses are deleterious for cucurbitaceous plants leading annually to significant yield losses world wide. All varieties of economical importance are susceptible for these viruses and classical breeding did not yield resistance. Therefore, a transgenic approach was chosen to induce resistance against both viruses by post-transcriptional gene silencing (PTGS).

Highly conserved regions in the coat protein genes of ZYMV and WMV-2 were chosen to establish an inverted repeat construct. This construct was cloned into binary vector under control of the 35S promoter.

Embryogenic callus was induced from different organs of three squash cultivars as target tissues for *Agrobacterium* transformation. The embryogenic callus was developed within 13-20 weeks incubation on MS medium containing different plant growth regulator combinations of auxin and cytokinin. Induction of embryogenesis in different explants ranged from 5 to 100 % depending on the organ and genotype used. Efficiency of embryo maturation, conversion and germination into entire plants from squash embryogenic callus was found to be callus age depended. Regeneration with young (2 months old) material was efficient, whereas regeneration of material maintained under in vitro conditions for more than 2 years was not possible.

Agrobacterium-mediated transformation of squash embryogenic callus was established using transient GUS-gene expression. The highest transformation efficiency was obtained with the supervirulent ATHV strain, bacterial density of 0.85, washing procedure of the embryogenic material prior to *Agrobacterium* co-culture, application of 1 mM Acetosyringone in induction medium and sub-culturing of embryogenic callus on fresh MS medium 5-9 days prior co-culturing with the *Agrobacterium*.

Selection strategy was optimized using GFP as reporter gene. For the genotype CX3006 300 mg/l Kanamycin showed the highest number of green areas but most efficient selection agent was Paromomycin 150 mg/l. For the genotype Dundoo 200 mg/l Paromomycin was the

effective selection agent and showed the highest number of green areas. Selection of transformed calli could be efficient with the used selection agents but regeneration of transgenic plant was not possible because the old material was only one to be used for transformation experiments. It seems that these old materials may have lost their competency when they were maintained for long term in tissue culture. Therefore, the functionality of the inverted repeat construct was evaluated in *Nicotiana benthamiana* as a model plant. Transgenic lines were analyzed by PCR, Southern blot analysis and segregation analysis of T1 offspring.

The transgene-induced PTGS in transgenic lines was confirmed by infiltration of GFP-sensor constructs containing viral derived sequences as silencing target and /or a construct containing the p19 silencing suppressor. In all transgenic lines tested, GFP fluorescence in infiltrated leaves was extinguished three days post-infection with GFP-sensor constructs. In contrast, all transgenic lines showed GFP fluorescence in infiltrated leaves when GFP-sensor constructs were co-infiltrated with a binary vector containing the viral silencing suppressor p19.

With this work, tools have been developed to engineer virus resistance in squash. Using the optimized *Agrobacterium*-mediated transformation procedure together with the efficient RNA-silencing of the inverted inverted repeat construct and freshely induced embryogenic material it is quite possible to establish virus resistance in squash.

6 Zusammenfassung

Virusbefall verursacht beim Zucchini-Anbau weltweit einen großen wirtschaftlichen Schaden. Früchte von virusinfizierten Pflanzen weisen mosaikähnliche Verfärbungen und schwerwiegende Deformationen auf, Pflanzen sind gestaucht und Blätter deformiert. Von den verschiedenen Viren, die weltweit für den Befall von Zucchini und anderen Cucurbitaceaen verantwortlich sind, gehören ZYMV und WMV-2 zu den bedeutendsten. Die konventionelle Züchtung konnte zwar bereits virustolerante Sorten hervorbringen, diese können jedoch Mischinfektionen oder der Befall von aggressiven Stämmen der o. g. Viren auf dem Felde nicht standhalten. Im Rahmen dieser Arbeit wurde daher ein transgene Strategie gewählt, um mittels ‚Post Transkriptionales Gen Silencing' Resistenzen gegen ZYMV und WMV-2 in Zucchini zu induzieren.

Für die Herstellung eines ‚Inverted Repeat'-Konstrukts wurden hochkonservierte Sequenzbereiche aus den Hüllproteinen des ZYMV und des WMV-2 verwendet. Das Konstrukt wurde unter Kontrolle des 35S Promotors in einen binären Vektor kloniert.

Als Ausgangsmaterial für die *Agrobacterium*-vermittelte Transformation von Zucchini wurde somatisch embryogener Kallus ausgewählt. Nach 13 bis 20 Wochen Inkubation auf hormonhaltigem Medium konnte an verschiedenen Explantaten bei allen 3 verwendeten Zucchini Genotypen embryogener Kallus induziert werden. Je nach verwendetem Explantat und Genotypen wurde eine Effizienz der Embryogenese von 5 bis 100% festgestellt. Die Reifung der somatischen Embryonen und ihre Konversion zu Pflanzen zeigten sich als stark abhängig vom Alter des embryogenen Kallus. Die Reifung und Konversion von frisch induziertem embryogenem Material war sehr effizient. Nach einer 2 jährigen Erhaltungsphase unter *in vitro* –Bedingungen war eine Reifung und Konversion jedoch nicht mehr möglich.

Die *Agrobacterium*-vermittelte Transformation von embryogenem Kallus wurde mit Hilfe der transienten Expression des GUS-Gens etabliert. Die höchste transiente Expression konnte bei Anwendung folgender Parameter erreicht werden: Verwendung des supervirulenten *Agrobacterium*-Stamms ATHV, optische Dichte von 0,85 der Bakteriensuspension, Waschbehandlung des embryogenen Materials vor der Cokultur, die Applikation von 1 mM Acetosyringon während der Cokultur und einer Vorkulturphase von 5-9 Tagen vor der

Cokultur. Die Optimierung der Selektion von transformierten Zellen wurde mit Hilfe des GFP-Reportergens durchgeführt. Für den Genotypen CX3006 zeigte eine Kanamycin-Konzentration von 300 mg/l die größte Zahl von GFP-positiven Bereichen, jedoch war die Selektion mit Paramomycin am effizientesten, da nur eine Konzentration von 150 mg/l benötigt wurde. Für den Genotypen Dundoo erwies sich Paramomycin in einer Konzentration von 200 mg/l als effizientestes Selektionsmittel. Obwohl gezeigt werden konnte, dass die Selektion von transformierten embryogenen Zellen möglich ist, konnte während dieser Arbeit keine transgenen Zucchinipflanzen regeneriert werden, da für die Transformationsexperimente bedauerlicherweise nur auf altes embryogenes Kallusmaterial zurückgegriffen werden konnte, das während der langen Erhaltungskulturphase die Kompetenz zur Regeneration verloren hat.

Die Funktionalität des ‚Inverted Repeat'-Konstrukts wurde in der Modellpflanze *Nicotiana benthamiana* nachgewiesen. Transgene *N. benthamiana*-Pflanzen wurde mit PCR, Southern Blot Analyse untersucht und die Aufspaltung in der T1-Generation überprüft. Bei allen untersuchten Linien konnte PTGS in Infiltrationsexperimenten gezeigt werden. Hierbei wurden GFP-Sensorkonstrukte allein bzw-. in Kombination mit einem P19-Konstrukt infiltriert. In allen Linien konnte nach Infiltration der Sensorkonstrukte keine GFP-Fluoreszenz beobachtet werden, hingegen führte eine kombinierte Infiltration der Sensorkonstrukte mit dem P19 Supressor bei allen Linien zu einer starken GFP-Fluoreszenz.

Mit dieser Arbeit konnte die Vorraussetzung für die Herstellung von virusresistentem Zucchinimaterial geschaffen werden. Mit Hilfe des etablierten Protokoll für die *Agrobacterium*-vermittelte Transformation von embryogenen Zucchinizellen und dem funktionellen ‚Inverted Repeat'-Konstrukt besteht die Möglichkeit, in Zukunft ausgehend von frisch induziertem embryogenem Kallus transgene virusresistente Zucchinipflanzen erzeugt zu erzeugen.

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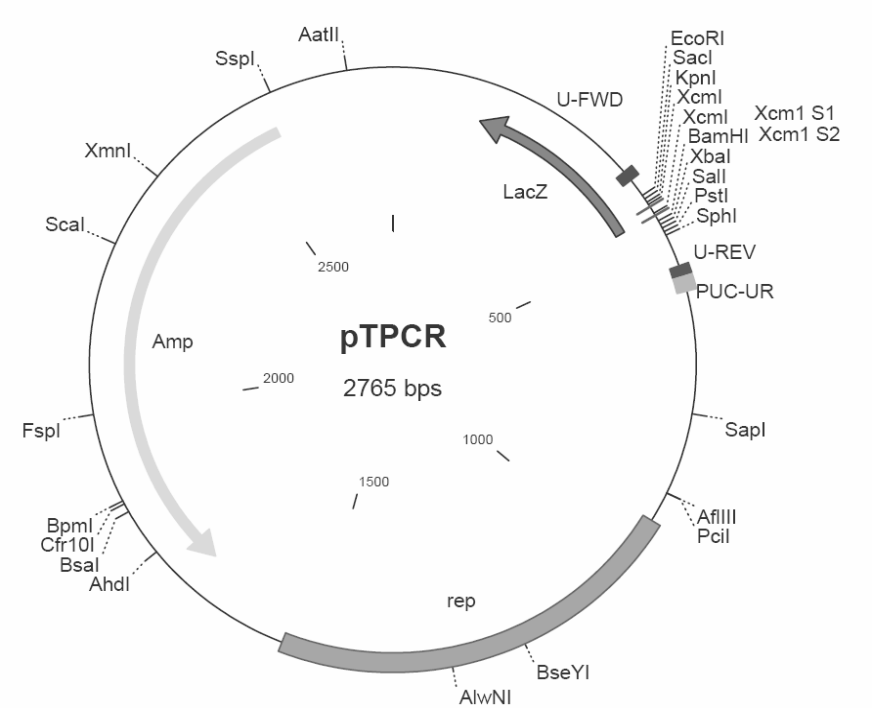
8 Appendix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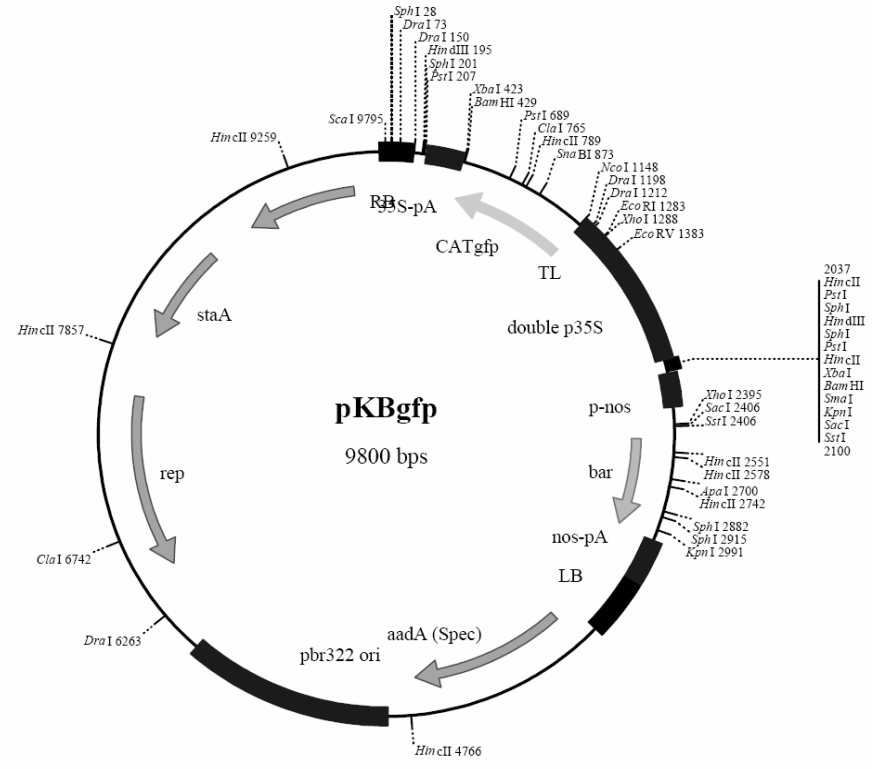
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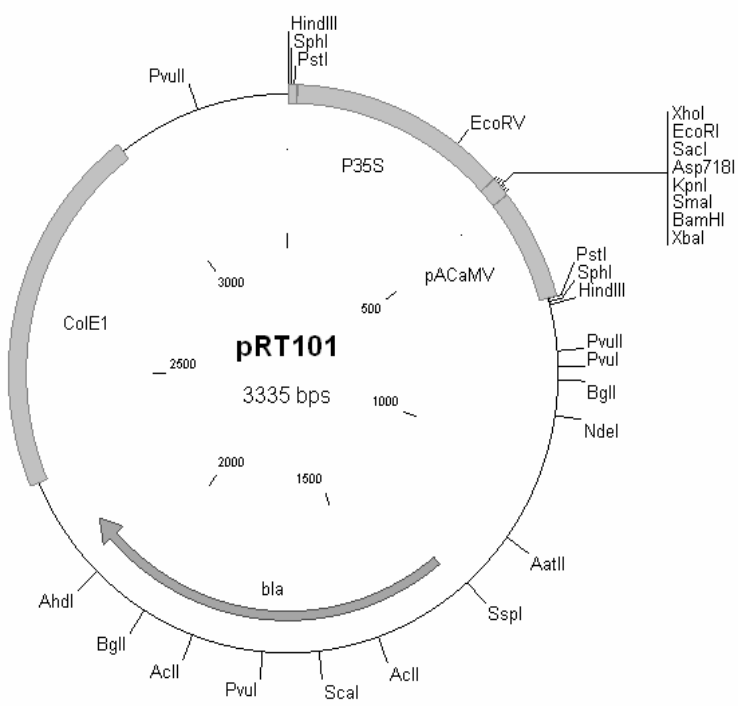
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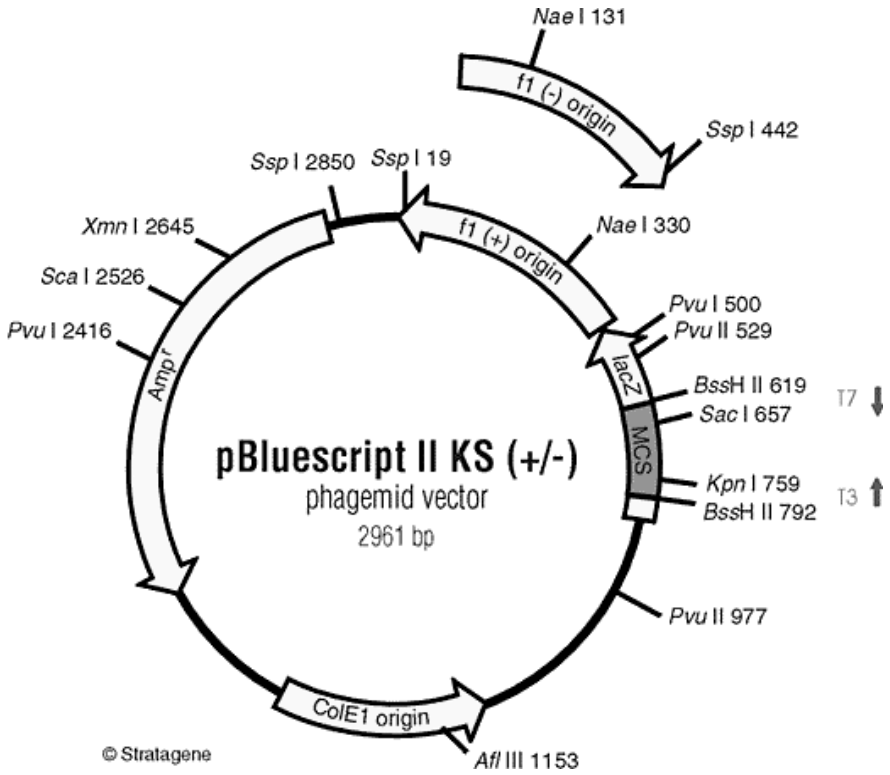
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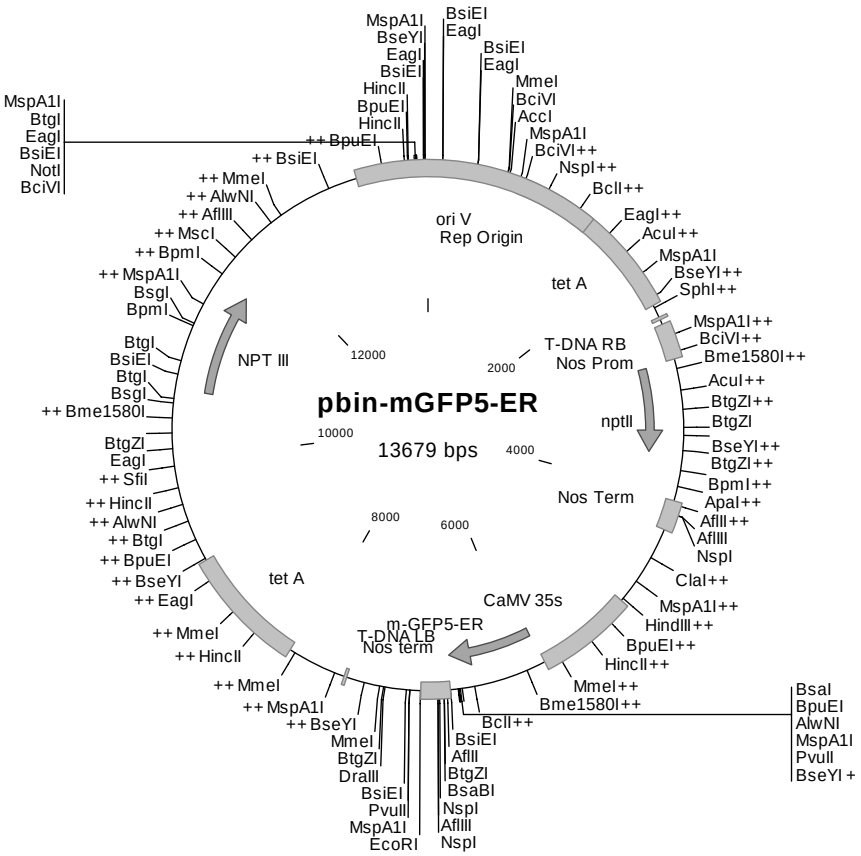
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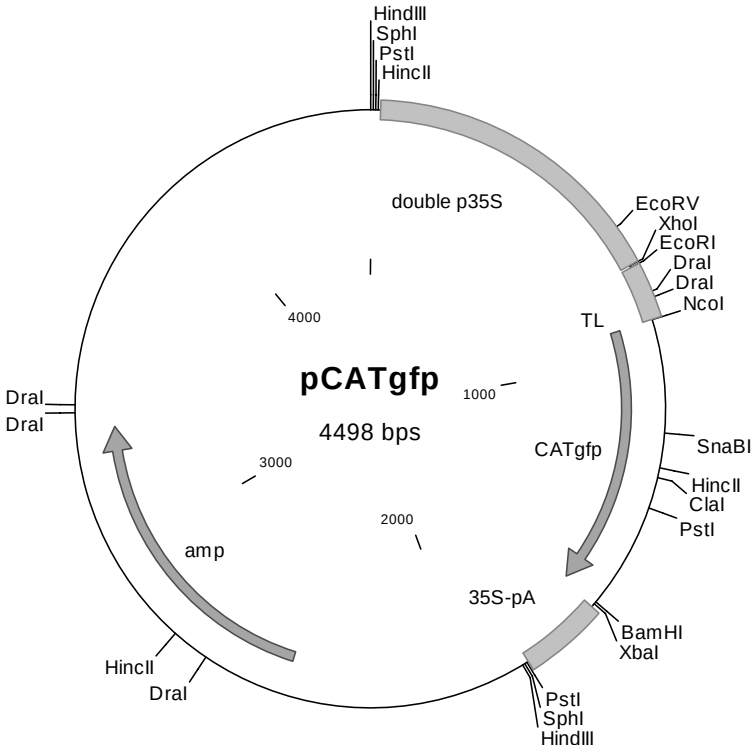
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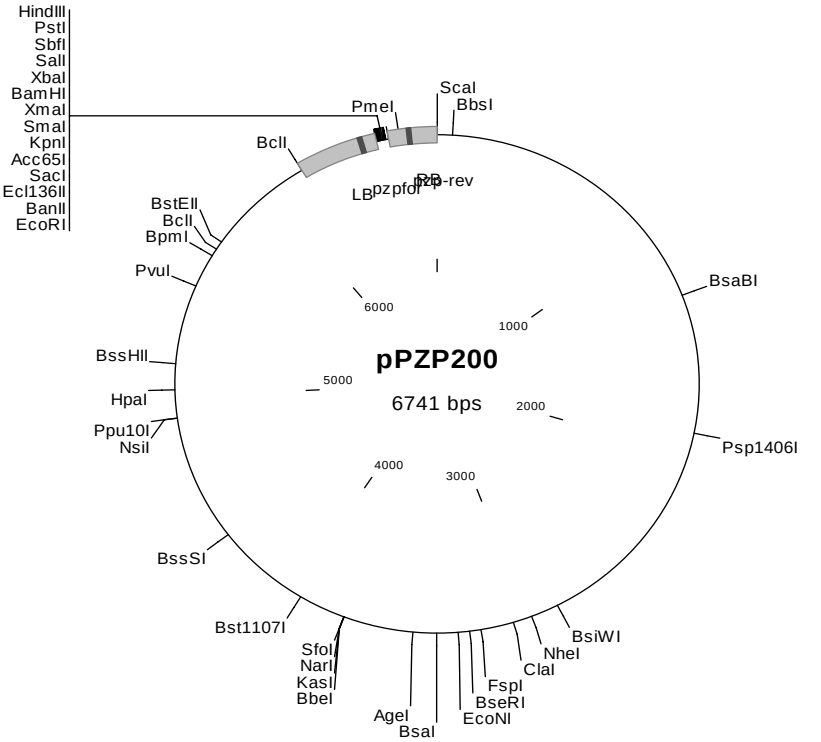
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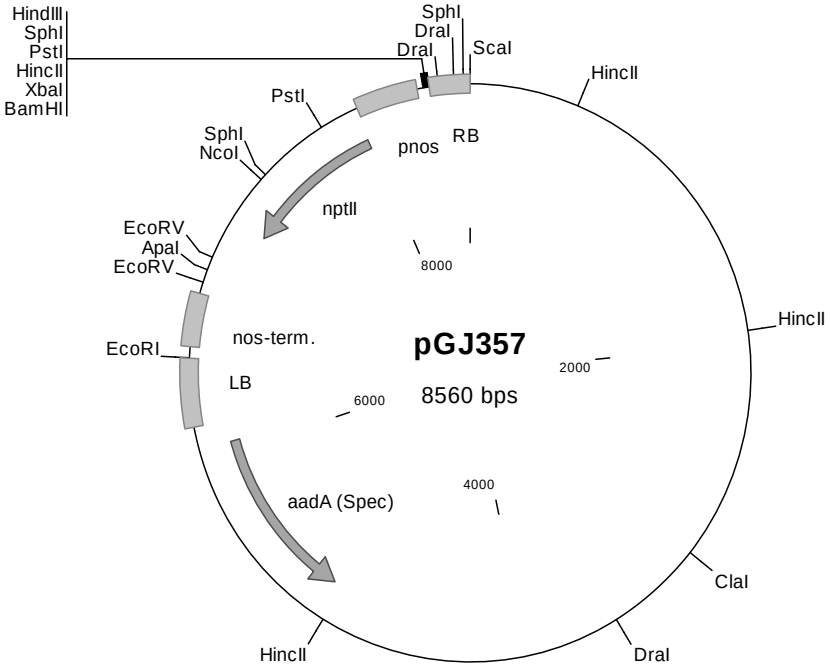
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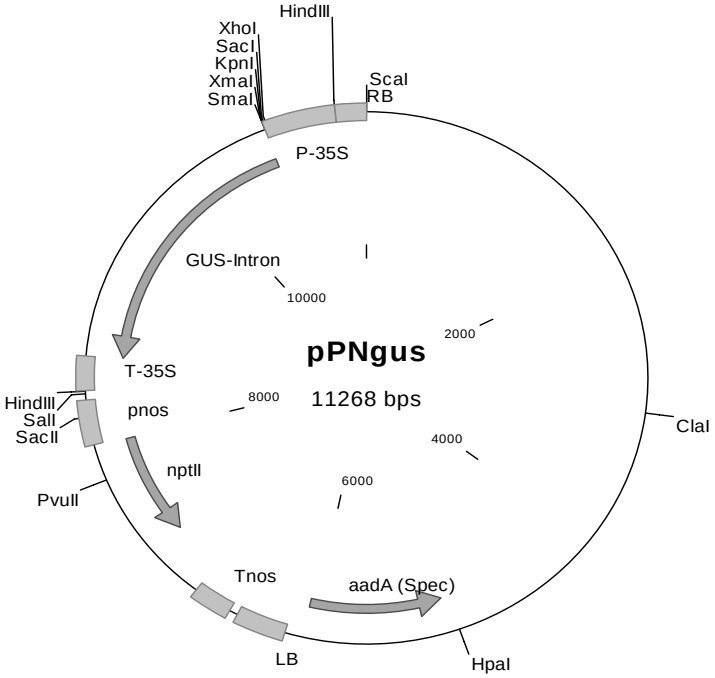
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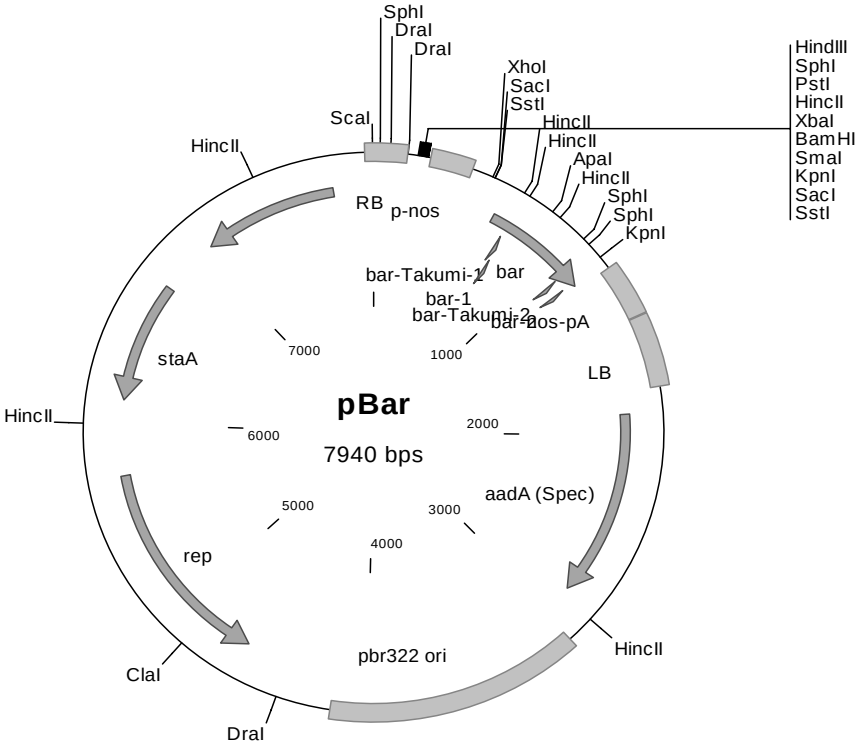
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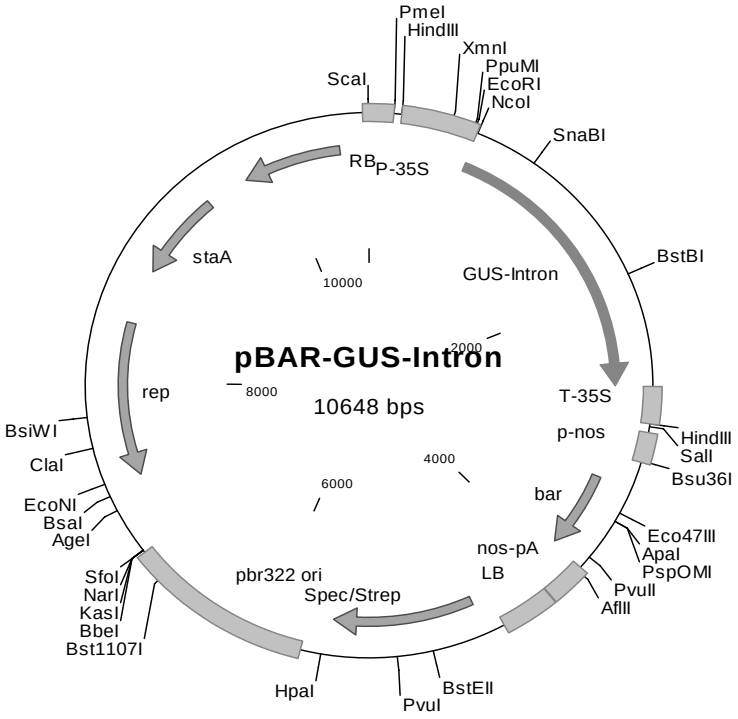
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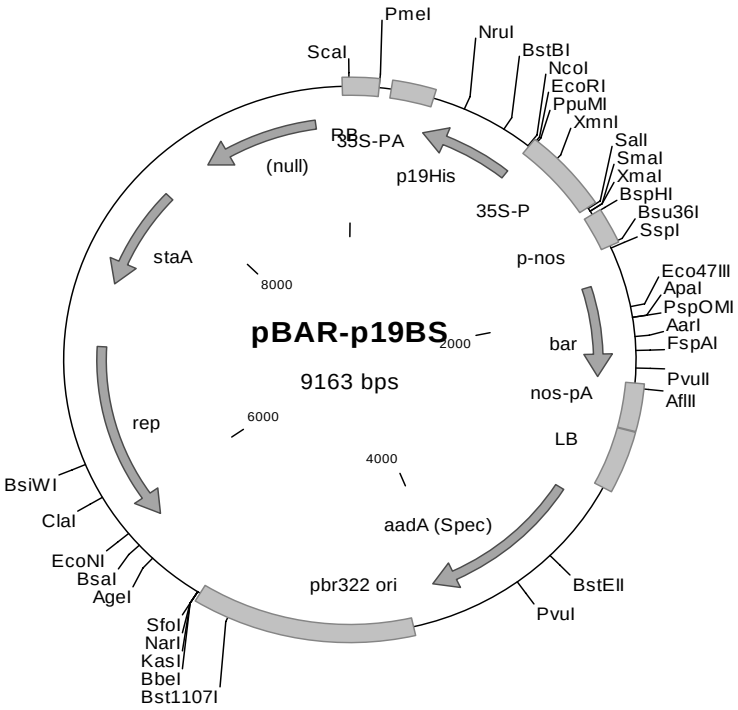
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Table 3: primer used for RT-PCR

Primer name	sequence	Tm (C°)
W2-3270as	5`-TCA CAC TAC AAA CAA CTC CG-`3	55.3
W2-2570s	5-AGC AAA TGG GTG TGA TTA TG-`3	53.2
Z-9440as	5`TTA GAA TTA CGT CGG CAG CG-`3	57.3
Z-8440s	5-AA(AG) GC(CT) CCA TAC ATA GCT G-`3	54.5

S= Forward, and as = Reverse

Table 4: Primers used for PCR

Primer name	sequence	Tm (C°)
Bgl2-ZYMV-s	5´-AGA TCT ATG AGC AGG TTG AGT ATC CT-`3	61.6
Pst1-ZYMV-as	5´-CTG CAG GAA GCA AAC CAT ACC TCG GCA-`3	68.0
Bcl1-WMV2-as	5`-TGA TCA CGT CCC TTG CAG TGT GCC TC-`3	68.0
BamH1-ZYMV-s	5´-ATG GAT CCA TGA GCA GGT TGA GTA TCC-´3	65.1
Xba1-ZYMV-as	5´-ATT CTA GAG AAG CAA ACC ATA CCT CGG CA-´3	65.3
BamH1-WMVs	5´-ATG GAT CCA GGT TAC TTC CAA AAC ACC-´3	63.4
Xba1-WMV-as	5´-ATT CTA GAC GTC CCT TGC AGT GTG CCT-´3	68.0
35sPA-1*	5´-GAC TGG TGA TTT TTG CGG ACT CT-´3	65.0
IntronC-2**	5´-CCT AAA CAA CCA AAT CAC AAT CTT-´3	65.0
ItronC-3*	5´-TGC ATT AGT TTT TGT TTA GAT TGA-´3	65.0
35sProm-4**	5´-AAA GGA CAG TAG AAA AGG AAG ATG-´3	65.0

S or *= Forward, and as or ** = Reverse

Table 5: Primers used for sequencing

Primer name	sequence	Tm (C°)
U-FWD*	5`-GTA AAA CGA CGG CCA GT-`3	52.0
U-REV**	5`AAC AGC TAT GAC CAT G-`3	54.0
35 Tas-Pcass*	5`-ATG CTC AAC ACA TGA GCG`3	54.0
CassEV**	5`-AAA GCA AGT GGT TTG ATG TGA TAT C-´3	64.0
35Sprom106-low*	5`-GAA AAG TCT CAA TAG CCC TCT GG-`3	56.0
PZP-Rev**	5`-TCT CTT AGG TTT ACC CGC C-`3	58.0
PZP-For*	5`-AAA CAA ATT GAC GCT TAG AC-`3	54.0
PZP-Rev**	5`-TCT CTT AGG TTT ACC CGC C-`3	58.0
pCatgfp-wmw (35SbA568-low)	5' -CCT TAT CTG GGA ACT ACT CAC A- 3'	54.0
pCatgfp-zymv (35SbA568-low)	5' -CCT TAT CTG GGA ACT ACT CAC A- 3'	54.0

* = Forward, and ** = Reverse

Erklärung:

Ich versichere, dass ich die vorliegende Dissertation selbständig und ohne fremde Hilfe angefertigt, nur die angegebenen Hilfsmittel verwendet und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Neustadt, 16. 01. 2007 Yehia Khidr

Curriculum vitae

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Scientific activity

Khidr Y, Wallbraun M, Wetzel T, Krczal G and Reustle GM (2005) Agrobacterium-mediated transformation of squash plant (Cucurbita pepo L.) to induce virus resistance by gene silencing. Workshop “Genexpression – aktuelle Trends – neue Herausforderungen” 28-29-Juli, 2005.

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