INCOMPATIBLE AND COMPATIBLE PLANT PATHOGEN INTERACTIONS

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Dedication

To my beloved parents

Keshubhai and Jayaben

Abstract

Pathogens are one of the prevalent stresses to plants. Resistance mediated by the resistance genes is efficient mechanism for evading the pathogens. To understand the influence of various biotic and abiotic factors on resistance gene promoters, plants having N gene promoter fused with reporter genes were developed. Experiments with tobacco plants revealed that on tobacco mosaic virus infection, the N protein may increase in the cells. Also, extreme temperature may result in decrease in the N protein. The salicylic acid produced during the development of systemic acquired resistance does not hinder the N promoter function. Hence, it can be concluded that the promoter region of resistance genes can be influenced by many biotic and abiotic factors. In the tobacco plants lacking the N gene, infection with tobacco mosaic virus leads to generation of systemic recombination signal. Experiments suggest that this signal can lead to better tolerance of the pathogen in next generation. Also, in the plants which received systemic recombination signal, the resistance gene loci are hypermethylated and the frequency of rearrangement in these loci increases. Hence, the signal results in higher tolerance to pathogen and increased genetic variability in resistance genes.

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List of abbreviations

35S::GUS	35S gene promoter fused with ß-glucuronidase gene
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Avr	Avirulence
BSA	Bovine serum albumin
C-terminal	Carboxyl terminal
CaMV	Cauliflower mosaic virus
CC	Coiled coil
CCD	Charged coupled device
cDNA	Complementary Deoxyribonucleic acid
CDPK	Calcium dependent protein kinase
COBRA	Combined bisulfite restriction analysis
CP	Coat protein
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
DSB	Double strand break
GTP	Guanosine triphosphate
GUS	ß-glucuronidase
HR	Homologous recombination
IAA	Indole acetic acid

IM	Infiltration medium
LRR	Leucine-rich repeats
LUC	Luciferase
MP	Movement protein
MS	Murashige and Skoog media
MUG	4-Methyl umbelliferyl glucuronide
N-terminal	Amino terminal
NAA	Naphthalic acetic acid
NBS	Nucleotide binding site
N::GUS	N gene promoter fused with ß-glucuronidase
NHEJ	Non-homologous end joining
N::LUC	N gene promoter fused with luciferase
NO	Nitric oxide
ORF	Open reading frame
PCD	Programmed cell death
PCR	Polymerase chain reaction
PofC	Progeny of control
PofI	Progeny of infected
PR protein	Pathogenesis related protein
R gene	Resistance gene
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species

rRNA	Ribosomal ribonucleic acid
SA	Salicylic acid
SAR	Systemic acquired resistance
SRS	Systemic recombination signal
TIR	Toll/interleukin-1 like receptor
TMV	Tobacco mosaic virus
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

1.0 Introduction

Plants, as sessile organisms, are under continuous exposure to biotic stresses, and thus have evolved sophisticated mechanisms of protection. Some of these mechanisms are efficient against a broad range of pathogens, while other are limited to specific pathogens. Specific resistance results from the interaction of plant proteins, coded by resistance (*R*) genes, with pathogen proteins coded by avirulence (*Avr*) genes. The recognition of an *Avr* "factor" by an *R* "factor" results in the localization of pathogen infection. The *R* gene mediated response is a very efficient way to prevent pathogen growth and, therefore its spread and disease symptoms.

Tobacco mosaic virus (TMV) and its interaction with the product(s) of the N gene (*R* gene) is one of the most popular models in the study of *R* genes. The TMV-N gene interaction results in the hypersensitive response leading to the formation of necrotic lesions (Holmes, 1938). Two interesting phenomena occur that may help us understand N gene mediated resistance. Firstly, the N gene undergoes alternative splicing, which results in generation of two RNA molecules of different size, both being important for resistance (Dinesh-Kumar and Baker, 2000). It is not clear, however, whether the level of transcription influences the activity of alternative splicing. Secondly, tobacco plants lose the ability to restrict TMV infection when grown at temperature higher than 28^{o}C (Samuel, 1931). This could be due to a change in the activity of the N gene. Loss of the response to TMV could occur either at the level of transcription (inactivation of the promoter), posttranscriptionally (high RNA turn-over), at the level of translation or post translationaly (protein degradation). Since tobacco, as a plants can comfortably grow at 28-30^{o}C, it is highly unlikely that this temperature could have a strong influence on RNA/protein degradation/modification. Thus, down-regulation of the N gene could be one of the most important factors in loosing the resistance to TMV.

This information allows us to assume that the analysis of the N gene promoter is vital for further understanding the resistance mechanisms against TMV. Since, the TMV-N gene interaction leads to development of systemic acquired resistance (SAR), marked by the accumulation of salicylic acid (SA) (Ryals *et al.* 1996), it would be interesting to see whether the SA accumulation would have any effect on the N gene transcription.

For characterization of the N gene promoter region, a 4.1 kb region located upstream of the N gene was cloned and linked to either of two reporter genes coding for the enzymes, ß-glucuronidase (GUS) and luciferase (LUC). SR1 and Havana tobacco plants were transformed with these reporter systems and homozygous plants with a single locus insertion were selected.

N-GUS plants were used for analysis of the endogenous activity of the N gene promoter, whereas N-LUC plants were used for analysis of the influence of various biotic and abiotic factors. The following treatment groups were used: i) TMV infection; ii) changes in temperature; iii) SA treatment. Changes in the level of gene expression were analyzed with aid of reporter gene assays.

All plant species contain large numbers of *R* genes, which are generally present in clusters in the genome. It has been observed that *R* genes clusters may have developed from gene duplication events possibly promoted by exposure to pathogens (Baumgarten *et al.,* 2003). Many of the R gene alleles are not capable of imparting resistance to any particular pathogen (Hulbert *et al.,* 2001). Genes may acquire mutations that result in generation of a new function (neomorph) (Li and Graur, 1991). Hence, it can be concluded that increasing the rearrangements in the functionless *R* genes may promote the development of resistance to a virulent (compatible) pathogen.

One of the major mechanisms responsible for genomic rearrangements is the homologous recombination (HR). This mechanism is one of the two major pathways for the repair of double strand breaks (DSB) in somatic tissues (Critchlow and Jackson 1998; Cromie *et al.,* 2001). Similar mechanism is behind the crossing-over events in meiotic cells. It was previously proposed that HR could be one of the several mechanisms responsible for R gene evolution (Tian et al., 2002; van der Hoorn et al., 2002). Involvement in somatic and meiotic recombination events allows HR to be a one of the key mechanisms generating diversity. It is possible that regulation of HR frequency is one of the responses to infection with compatible pathogen. Indeed, previous study has shown that infection by a virulent pathogen leads to the spread of a systemic recombination signal (SRS), which travels faster than the virus and promotes an increase in the frequency of HR in the non-infected tissue (Kovalchuk *et al.,* 2003). Although the purpose of this reduced genome stability is still unclear, it is possible that SRS might be a signal which can induce changes that can lead to better tolerance against the incoming pathogen.

To understand the effect of SRS, the changes in SR1 tobacco plants lacking the N gene upon the infection with compatible pathogen were characterized. The seeds of infected plants were collected and the progeny plants were analyzed for rearrangements and methylation patterns at *R* gene loci. Possible changes in the viral tolerance in progeny plants were studied by assaying the virus titer in plants at 6, 9 and 12 days after infection.

Further work in this area has shown that reduced genome stability can be specifically directed towards *R* gene loci, possibly allowing more freedom for rearrangements. Consequently, as early somatic events in meristematic cells can be inherited and transmitted to progeny (Walbot, 1985), novel *R* genes providing resistance to the pathogen might be generated.

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2.0 Literature Review

2.1 Introduction

One of the objectives of plant biotechnology is to maximize the production efficiency of field crops (Li *et al.,* 2001). Plants, as a source of nutrition, have a great impact on the economical and social structure of any country. These impacts become more significant coupled with our growing global population.

Recent advances in agricultural biotechnology have opened new prospects in crop improvement and alternative use of technologies. New crop varieties, which have improved traits, are being developed with the aid of molecular technologies (Serageldin, 1999). Resistance to drought, salinity, insects, and pathogens are a few of the traits that are highly desired. The ability to isolate and express transgenes provides new opportunities that could not be achieved through conventional breeding techniques. For example, insect resistance has been engineered by the transgenic expression of *Bacillus thuringiensis* toxins against Lepidopteran insects in various crops (van Rie, 2000).

Plants are constantly being attacked by various pathogens. Some of the major pathogens are various fungi and viruses that have a wide host range. As such, plants have evolved sophisticated mechanisms to resist or tolerate infection. These natural mechanisms can be manipulated to engineer more resistant plant varieties (Wilson, 1993). Consequently, it has become of the utmost importance to thoroughly understand innate resistance mechanisms in order to use them more efficiently.

2.2 Plant resistance mechanisms

Many microorganisms have evolved symbiotic relations with plants. This results either in a beneficial, mutualistic, or harmful, parasitic interaction for the plant. Organisms like nitrogen-fixing bacteria and mycorrhizae have mutualistic relationships, while most viruses, viroids, fungi and mycoplasma are parasitic and considered pathogenic.

If a particular group of plants is able to prevent the process of pathogen infection, proliferation, or translocation, the plant is termed to be resistant to that particular pathogen. As each pathogen requires certain growth conditions provided by the host plant, most plants are resistant to most of the potential pathogens, as they are not the permissive host. Such resistance is termed as non-host resistance or basic resistance (Heath, 1981). Resistance due to induction of specific plant processes upon microbe recognition is a part of active resistance. This resistance is specific for the race of pathogen and host. If the plant is able to resist the pathogen and prevent infection, the interaction is termed incompatible. Conversely, if the pathogen is able to multiply and spread in the plant, the interaction is termed compatible (Hammond-Kosack and Jones, 1997).

2.2.1 Non-host resistance

In order to enter the plant and multiply, a pathogen has to overcome many barriers. Such factors include the structural and chemical composition of the plant cell wall. For rust fungi, the surface topography and the wax composition of the cell wall are very important for induction of growth and multiplication of appressorium (Hoch *et al.*, 1987; Tsuba *et al.,* 2002). The cell wall composition and presence of certain chemicals can also hinder the growth of a pathogen. Avenacin A-1, an antimicrobial saponin, is present in the roots of oat. *Gauemannomyces graminis* var. *tritici* can infect wheat, but is unable to attack oat as it cannot detoxify the saponin; hence, *G. graminis* var. *tritici* is a non-host pathogen for oats (Papadopoulou *et al.*, 1999).

Non-host resistance can also involve non-specific recognition of pathogens and the induction of general barriers mechanisms. This process is based on recognition of general elicitors, produced by pathogenic fungi, bacteria or viruses. As a result, the cell can recognize the presence of fungi or virus, but not the specific strain of pathogen. Hence, such recognition is termed non-specific recognition. Papillae formation on the site of fungi penetration is a well-studied example. The plant cells produce a structural barrier in the form of papillae on the inner surface of the cell wall upon the recognition of the general elicitors from fungal pathogen (Thordal-Christensen *et al.*, 2000; Zeyen *et al.,* 2002).

Non-host resistance can prevent infections of a large number of pathogens, which are non-host pathogens for particular plant. It also decreases the infection from host pathogens up to a certain level (Thordal-Christensen, 2003). But efficient resistance against host pathogen is provided by specific gene-to-gene interaction or active immunity.

2.2.2 Active resistance

Once a pathogen overcomes the general structural and chemical resistance mechanisms and enters the plant cell, resistance depends on the specific identification of the pathogen by the cell. Recognition of specific pathogen leads to activation of counter mechanisms to stop its growth. This process relies on an interaction between gene products from the pathogen and the host.

Pathogens produce certain proteins known as virulence factors, products of *Avr* genes, which are unique for each pathogen. They are required for penetration into host cells and are necessary to colonize the host and avoid its immune response (van der Beizen and Jones, 1998). The products of *Avr* genes are identified by receptors in host cell that are the products of *R* genes. Hence, this form of immunity entirely depends on the presence of both, *Avr* and *R* genes (Keen, 1990).

2.2.2.1 The Structure of *R* genes

The genetic structure of *R* genes as defined by its DNA sequence, is a key to their function. Although there is much variation in the origin and function, *R* genes share a significant homology in DNA and hence, in their protein sequence. Based on the structure, plant *R* genes are divided into five major classes (Jones *et al.*, 1993; Hammond-Kosack and Jones 1997).

The first group is the serine/ threonine protein kinases which are cytoplasmic proteins. The best-known example is the *R* gene *Pto* from tomato. *Pto* provides resistance against the *AvrPto* gene from *Pseudomonas syringae* pv. *tomato* (Keen *et al.,* 1990; Martin *et al.,* 1993).

R proteins with amino (N) terminal coiled coil (CC), nucleotide binding site (NBS), and carboxyl (C) terminal leucine-rich repeats (LRR) domains represent the second class of *R* genes. The Rx protein from potato, which confers resistance to the potato virus X, belongs to this class of intracellular proteins (Bendahamane *et al.,* 1999).

NBS and LRR domains are also present in the third class of *R* proteins, but instead of N terminal CC domains, they contain a Toll/interleukin-1 like receptor (TIR), similar to those found in drosophila and mammals, respectively. They are predicted to be cytoplasmic proteins. This class includes the N gene from tobacco, which confers resistance against TMV (Whitham *et al.,* 1994, 1996).

The fourth class of *R* proteins possesses an extracellular domain. The Cf protein family from tomato has an extracellular N-terminal LRR region, which is attached to a transmembrane region, followed by short, C-terminal cytoplasmic tail. The Cf protein family provides resistance against *Cladosporium fulvum* (van Den Ackerveken *et al.,* 1992; Dixon *et al.,* 1996).

The last class of *R* proteins contains an extracellular, N-terminal LRR regions, a transmembrane region, and an intracellular C-terminal serine threonine kinase domain. *Xa21* from rice best represents this class of R genes (Liu *et al.,* 2002)

2.2.2.2 The function of *R* genes

The primary function of *R* genes is to recognize the pathogen and trigger a rapid resistance response. Currently, two different models describing the highly specific *Avr* and *R* gene interaction have been suggested.

Initially, it was proposed that there is a one-on-one interaction between the *R* and *Avr* proteins. Flor (1971) first proposed the receptor-ligand model of interaction. It suggested that the *R* gene product directly interacted with the *Avr* gene product, with no other gene needed for recognition. In contradiction, evidence from many sources indicates that this kind of interaction is rare (Schneider 2002; van der Hoorn *et al.,* 2002). Alternatively, the guard model, proposed by van der Biezen and Jones (1998), explains that *R* proteins monitor some of the cellular proteins (guardees) that are affected by the presence of pathogen. Any change in the confirmation of a guardee results in recognition of a pathogen. Hence, *R* proteins are not directly associated with the pathogen, rather they monitor other cellular components that are a substrate for pathogen virulence factors or those involved in early pathogen response (van der Beizen and Jones, 1998).

Each domain in the structure of the *R* gene products is responsible for a specific role during its function. Most of the *R* genes classes contain NBS and LRR domains. NBS domains share homology with mammalian cell death proteins. They are involved in the binding and hydrolysis of adenosine triphosphate (ATP) and guanosine triphophate (GTP); hence, these domains could activate kinases (Traut, 1994). This indicates their possible role in signal transduction. The LRR domain is a key player in the specificity of recognition of avirulence factors (Dangl and Jones, 2001). Various alleles of the resistance gene L from flax contain differences only in their LRR regions. These alleles give resistance to different strains of the flax rust pathogen *Melampsora lini* (Ellis *et al.,* 1999). Further analysis indicated that the TIR region was also involved (Luck *et al.,* 2000). Additionally, the analysis of mutations in the LRR region of the *Arabidopsis R* gene, *RPS2* (CC-NBS-LRR class), has revealed that these mutations lead to suppression in the function of multiple *R* genes. This suggests that the LRR region is involved not only in signal perception, but also in downstream signal transduction (Warren *et al.,* 1998).

The CC region may be involved in the homodimerization or heterodimerization of *R* proteins with other proteins (Landschulz *et al.,* 1988). The TIR domain could play a role in initiating the defense response and may be required for production of reactive oxygen species (ROS) (Hammond-Kosack and Jones, 1996). The serine/threonine kinase domain of many R proteins can function in down stream signaling. The kinase domain of Pto *R* gene product is shown to induce phosphorylation of many other genes. This in turn activates the pathogen resistance response (Sessa and Martin, 2000).

When *R* proteins recognize a pathogen, the activation of many downstream responses can occur. Programmed cell death (PCD), as depicted by localized death of the infected cell or hypersensitive response, is the most common response to pathogen infection (Greenberg and Vinatzen, 2003; Nimchuk *et al.,* 2003). The death of the infected and surrounding cells results in the formation of necrotic lesions. This prevents the further spread of the pathogen into the host.

Many other responses may also be triggered, many of which aid in hindering the spread of the pathogen. This includes the generation of ROS, changes in ion flux, activation of signaling pathways, alteration in transcription profile, and nitric oxide (NO) production (Gozzo, 2003)

Changes in ion flux result in the activation of many signaling pathways. Calcium binding can trigger phosphorylation of many calcium dependent protein kinases (CDPK), which are involved in pathogen response (Rudd and Franklin-Tong, 2001). The production of ROS initiates programmed cell death. It is also associated with the induction of defense related genes and could increase crosslinking in the cell wall (Vranova *et al.,* 2002). The function of NO is similar to that of ROS. It is required in PCD, as well as the induction of defense related genes (del Rio *et al.,* 2004).

2.2.2.3 Systemic acquired resistance (SAR)

Following PCD induced by an incompatible interaction, distal tissues of the plant become more resistant to secondary infection. This spread of resistance from a local site of infection to all plant tissues is termed SAR (Ross, 1961). A signal generated from a cell that undergoes PCD activates the SAR, with the induced resistance that can last for the rest of the plant's life. An efficient broad-spectrum resistance is given by SAR against a large number of fungal, viral and bacterial pathogens (Ryals *et al.,* 1996; Sticher *et al.,* 1997).

One of the signatures of SAR is the induced expression of pathogenesis related (PR) proteins. The genes encoding for these proteins are induced both in the local area of infection as well as in distant tissues. When tobacco plants were infected with TMV, various novel proteins were detected, which revealed the existence of pathogenesis related (PR) genes (van Loon and van Kammen, 1970; van Loon and van Strien, 1999). The PR-1 group was the first to be identified, and many experiments have been conducted on PR-1a, -1b and -1c proteins from tobacco. These proteins have more than 90% homology between each other and with PR-1 proteins from other plant species (Nasser *et al.,* 1988; Nassuth and Stinger, 1986). The PR genes act against the infection of the pathogens. It is proposed that PR-1 genes have indirect antifungal activity (Niderman *et al.,* 1993), while other groups of PR genes have shown direct antimicrobial activity. For example, the PR proteins that belong to a chitinase group (PR-2 and PR-3) have the ability to digest the cell wall of pathogenic fungi (Boller *et al.,* 1983; Pegg and Young, 1982). The function of all the PR proteins is still unknown and needs to be characterized. It is thought that resistance due to SAR is not an outcome of a single PR protein, but is the result of a combined effect of genes encoding many PR proteins.

In tobacco, SAR can be induced by external application of SA, aspirin, and benzoic acid, leading to the hypothesis that SA is the signaling molecule, which systemically spreads SAR from the infected site (White, 1979). This hypothesis was supported by the observations that the concentration of endogenous SA rises in local and systemic tissue as well as in the phloem in response to pathogens (Métraux *et al.,* 1990; Malamy *et al.,* 1990). The infected leaf produces 70% of the SA present in the systemic tissue (Shulaev *et al.,* 1995). These experiments indicate that SA is central in the induction of SAR. However, the function of SA is still debated. Some researchers have challenged SA being the systemic signal. Experiments were conducted using the bacterial *nahG* gene, which codes for the enzyme salicylate hydroxylase. The salicylate hydroxylase converts SA to catechol, hence the level of SA is reduced in the plant (Gaffney *et al.*, 1993; Delaney *et al.*, 1994). Experiments revealed that the plants that were not able to accumulate SA were able to produce and translocate the signal to other tissue (Vernooij *et al.,* 1994). Moreover, it is proposed that the signal might be lipid-based. Plants with a mutation in *Dir1*, which has sequence similarity to lipid transfer proteins (PR-14), are not able to develop SAR or induce PR proteins (Maldonado *et al.,* 2002). While the molecule for the signal of SAR is under review, the function of SAR in resistance is well understood.

2.2.2.4 The Evolution of *R* genes

The list of *R* genes is growing day by day, and their striking specificities have been revealed. One of the lingering questions from many of these studies is how did *R* genes evolve to such a high degree of specificity.

Genomic organization of *R* genes is very complex; they are generally found in clusters and genes within the cluster often belong to the same gene family. *Xa21*, an *R* gene from rice, is a member of a gene family of at least eight other members (Song *et al.,* 1995). The majority of these members are present on chromosome 11 within a region of ~230 kb. At this locus, nine other *R* genes, which are not related to the *Xa* gene family, are also present (Ronald *et al.,* 1992; Williams *et al.,* 1996). Members of the same family can also be distributed in different, but closely associated loci. Two members of *Cf-x R* gene family from tomato, *Cf-9* and *Cf-4* are present on the short arm of chromosome 1, while two other closely related members, *Cf-2* and *Cf-5*, are present on chromosome 6 (Jones *et al.,* 1993). Hence, it can be interpreted that the majority of *R* genes are present in linked loci of related or unrelated *R* genes.

It has been proposed that duplication events, which are followed by diversification, may be the reason behind the evolution of *R* gene families (Whitham *et al.,* 1994; Lawrence *et al.,* 1995). A molecular analysis of the *Cf-x* gene family has shown that gene duplication by unequal crossing over played an important role in their evolution. Due to the use of unequally aligned sequences for recombination, the number and cluster composition of gene family members can change. This results in an increase in the variation present in the population (Parniske *et al.,* 1997).

The pattern of nucleotide substitution has been studied for different domains of *R* genes. A nucleotide substitution that leads to a change in the amino acid coded is known as a nonsynonymous mutation, whereas if there is no change in the primary structure of protein by the nucleotide substitution, it is known as a synonymous substitution. If the ratio of nonsynonymous to synonymous mutation is higher than one, it provides evidence for positive selection at that locus (Stahl and Bishop, 2000). This ratio has been found to be high in the LRR domain of Cf family *R* genes from tomato. They code for an extracellular LRR domain. In contrast, in the same genes, the ratio was less than one in the sequences that do not code for the LRR domain (Parniske *et al.,* 1997). A comparison of LRR regions between the family members *Xa21* and *Xa21D* has revealed that the rate of nonsynonymous substitution is significantly higher than that of synonymous substitutions. The ratio was less than one when substitution rates for the entire genes were calculated (Wang *et al.,* 1998). In the genes, which are not under selection pressure, like pseudogenes, it was observed that this ratio of nonsynonymous to synonymous substitution is less than one (Hughes, 1995). This indicated that the rate of evolution of R protein in the LRR domain could be higher than in the other R protein domains.

2.3 The roles of homologous recombination

DSBs are a major threat to the organism as even a single unrepaired break can result in cell death (Bennet *et al.,* 1993; Bennet *et al.,* 1996). Hence, it becomes very important to efficiently repair a DSB. There are two basic pathways involved in the processing of DSBs, non-homologous end joining (NHEJ) and homologous recombination. The NHEJ pathway is simpler, and requires little or no homology for repair. This kind of repair is more inaccurate and very often results in the deletion or addition of DNA nucleotides (Krogh and Symington, 2004; Lees-Miller and Meek, 2003; Meek *et al.,* 2004).

Homologous recombination serves as a basic mechanism of DNA repair and gene rearrangement. One of the primary roles of HR is to maintain genome stability by the correction of DNA damages in the form of DSBs. HR is a more complex and accurate pathway for repair. In HR, the sequence information is given a higher priority. Repair of DSBs is done using homologous sequences; hence, there is no loss of genetic information (Puchta, 2005). However, in HR, the potential for unequal crossing over and gene duplication is larger. Thus, repair via both, NHEJ and HR pathway can result in increased genetic variation (Brown, 1999). Furthermore, HR can recognize homologous sequences from adjacent genes and this may result in a newly recombined gene.

Homologous recombination also serves an important role in meiotic recombination (Schuermann *et al.*, 2005). It is responsible for the alignment and crossing over of homologous sequences as a result of synapsis. One of the prime requirements for the initiation of meiotic recombination is the creation of a double strand break. Subsequent to

DSB, HR is responsible for the alignment and crossing over of the homologous sequences as a result of synapsis. Recombination in meiotic cells is an important factor leading to increased genetic diversity present in a population (Schuermann et al., 2005). Hence, HR serves two functions; in somatic cells, it stabilizes the genome via damage repair, while in meiotic cells, it results in the formation of new allelic combinations and creation of additional genetic diversity.

2.3.1 Genome stability and evolution

Maintaining the stability of the genome is essential to any organism. Changes in the genome may lead to alterations in genes that are required for the normal functioning of the cell. Such changes may lead to an alteration in the phenotype of the organism. This alteration can be beneficial, neutral, or detrimental in nature. If the alteration is detrimental, the organism has to avoid this permanent change, as it can be lethal. On the other hand, such changes may result in characters that can ensure better survival of the organism during the adverse conditions. It can be suggested that every organism has to be able to balance the plasticity and flexibility of the genome in order to adjust to environmental conditions.

Adaptation to constantly changing environment requires constant micro- and macro-evolutionary processes in plants. New phenotypes may arise as a result of mutations including insertions, deletions and nucleotide substitutions. Duplication and rearrangement of entire genes as a result of recombination events are some of the causes of major phenotype changes (Brown, 1999). Many abiotic and biotic factors, such as radiation (Kovalchuk *et al.,* 2003c) and mutagenic chemicals (Kovalchuk *et al.,* 2003a) can lead to rearrangements in the genome. Hence, stress can have a direct influence on the genome stability of an organism. The increased rate of rearrangements can result in the creation of high phenotypic variability. This process can be mediated by HR and can lead to the increased probability of individuals having higher resistance to the stresses as a result of novel stress-related genes.

2.3.2 The role of homologous recombination in plant-pathogen interactions

As for many other stresses, pathogens can have a direct influence on genome stability. A report from Lucht et al. (2002), suggested a direct relation between pathogen stress and HR, as infection with *Peronospora parasitica* resulted in the increased frequency of HR.

Studies by Kovalchuk *et al.,* (2003b) supported this link between pathogen infection and HR. They reported a signal, which spreads ahead of the pathogen, termed the SRS. The SRS increases the frequency of HR in uninfected distal tissues and results in three fold increased somatic recombination frequency (Kovalchuk *et al.,* 2003b).

This signal may direct higher rearrangements in clustered *R* genes, which can lead to the creation of novel specificities in *R* gene recognition for the resistance to new pathogens. Hence, it can be predicted that plants can trigger the frequency of rearrangements through the HR mechanism resulting in the evolution of new *R* gene specificities.

2.4 TMV and its interaction with tobacco

TMV is the best-studied virus among the plant viruses. The research on TMV dates back 100 years, and has proven to be an ideal system for understanding virus particle structure and function. It is a rod shape virus, which is composed of a positive sense single stranded RNA, coated with proteins. In an intact virus particle, this RNA is 6.4 kb in size and is helically bound to 2160 subunits of coat proteins. At the 5' terminus, the RNA contains a 7-methyl guanosine cap and at the 3' terminus it forms a tRNA-like structure. The TMV genome consists of three open reading frames (ORF). Coding sequence from ORF1 codes for a 126-kDa replicase protein, which can also produce a 183-kDa protein by read through transcription. Coding sequence from ORF2 produces a 30-kDa-movement protein (MP), while coding sequence from ORF3 codes for a 17.5-kDa-coat protein (CP) (Lewandowski and Dawson, 2000).

The mode of entry of TMV into plant cells is thought to be passive, through damaged cells. The process of infection involves the attachment of the virus particle, entry into the cell, and the immediate uncoating of its RNA genome (Shaw, 1999). Once TMV enters the cell, it rapidly disassembles, followed by transcription leading to viral replication (Wu and Shaw, 1997). These processes occur at specific sites in the cell, leading researchers to believe that the virus particle, or its RNA alone, enters through ectodesma or via pinocytosis (Gaard and Zoeten, 1979; Hills *et al.,* 1987; Shaw, 1999). The disassembly of viral RNA results in production of MP. The RNA can now form a complex with its MP for cellular movement, or with CP for movement though the phloem and systemic infection (Deom *et al.,* 1992; Hilf and Dawson, 1993). The systemic infection by TMV in tobacco results in the appearance of mosaic-like symptoms characterized by light and dark blotches on the tissue.

Resistance genes conferring resistance to TMV have been identified in some tobacco cultivars. The N gene product from tobacco recognizes the TMV replicase protein, while the N'-gene recognizes the TMV CP. The N' gene has not been thoroughly studied, but due to its specificity to the CP, it is predicted that N' functions as an *R* gene (Erickson *et al.,* 1999; Padgett *et al.,* 1997).

2.5 N gene: Structure and function

The interaction between N gene product and TMV is one of the earliest examples of the study of plant pathogen interactions. Holmes (1938) carried out many of the earliest studies and described the interaction. The N gene was identified in *Nicotiana glutinosa* and was introduced into *Nicotiana tabacum* by reciprocal crosses (Holmes, 1938). The N gene is able to impart resistance against all TMV except type Ob (Culver *et al.,* 1991; Tobias *et al.,* 1982). Consistent with other *R* genes, the N gene also induces SAR (Ryals *et al.* 1996).

2.5.1 N gene structure

To predict the function of a protein, it is essential to know genetic structure of the gene. The total length of the N gene is 10.6 kb (Whitham *et al.,* 1994). It belongs to the NBS-LRR class of *R* proteins, and it contains a TIR domain. There are four introns and five exons in the coding sequence of the N gene. The total coding sequence is 3432 base pairs and codes for a protein of a predicted mass of 131.4-kDa. N gene also undergoes alternative splicing, forming a truncated version of the normal transcript. Alternative splicing takes place at the third intron, resulting in the truncation of exon four and five. The protein formed by the alternative transcript is predicted to be 75.3 kDa, and differs from the longer protein by 22 amino acids at the C terminal region corresponding to the LRR domain. Protein sequence prediction shows that neither transcripts contain any extracellular or transmembrane domain, suggesting it is localized to the cytosol (Whitham *et al.,* 1994; Erickson *et al.,* 1999).

2.5.2 N gene function

Tobacco plants without the N gene are sensitive to TMV. Upon inoculation with TMV in these plants, the virus spreads systemically to all tissues and forms mosaic like symptoms. Conversely, if the plant contains the N gene, inoculation of TMV results in hypersensitive response within 48 hours of infection (Holmes, 1934).

Many studies have focused on the identification of the avirulence factor in the TMV. It has been determined that the N protein recognizes the TMV replicase (Padgett and Beachy, 1993). More detailed experiments have shown that the expression of the C-terminal 50-kDa portion (p50) of the 126-kDa replicase protein is sufficient to induce a hypersensitive response (Abbink *et al.,* 1998; Erickson *et al.,* 1999).

Interaction between the N protein and TMV is apparently influenced by temperature. At temperatures above 28°C, the N protein fails to impart resistance. In this case, the pathogen is able to spread throughout the plant systemically despite the presence of the N gene. Interestingly, if this plant, having systemic spread of TMV, is brought back to temperatures below 28°C, the N protein regains its functionality. This leads to a hypersensitive response throughout the plant, known as the systemic hypersensitive response, and results in plant death (Samuel, 1931).

Structurally, the N gene belongs to TIR-NBS-LRR class of *R* genes and the importance of each domain of the N protein in its function has been analyzed. The study of effect of mutation at nine different amino acids in the TIR domain revealed that one mutation resulted in complete loss, six in partial loss, and two in no change in function of the N protein. Similarly, mutations in the NBS and LRR domains result in loss of function of N protein as well. Hence, mutations in any of the three domains may lead to complete or partial loss of the N protein function (Dinesh-Kumar *et al.,* 2000).

Like many other *R* genes of its class, N gene also undergoes alternative splicing. It is suggested that both transcripts, the full version (N_S) and truncated (N_L), are required for resistance. The expression of N_S cDNA or N_L cDNA alone, driven by a native N gene promoter, results in either the partial or complete loss of resistance (Dinesh-Kumar and Baker, 2000). The expression of both the cDNAs in the same plant under the N promoter also does not confer resistance. Based on these findings, it is proposed that the ratio of N_S to N_L is the critical factor in the resistance response. The ratio of N_S to N_L is 28:1 under regular conditions, but drastically changes to 1:23 six hours post-infection. The ratio returns to the initial state of 28:1 at ~9 hours after infection. There is no change in the rate of transcription of the N gene during these changes in the ratio (Dinesh-Kumar and Baker, 2000).

Interestingly, the expression of both transcripts under the control of the constitutive CaMV 35S promoter has shown contradicting results. Dinesh-Kumar and Baker (2000) showed that the constitutive expression of both transcripts resulted in no resistance response, while Mestre and Baulcombe (2006) showed it could impart resistance. These contradicting studies put the transcript ratio hypothesis in question. Another report by Levy *et al.,* (2004) analyzed the changes in the rate of N gene transcription. They found that in TMV inoculated leaves, there was a 4.1-fold increase in the accumulation of N gene transcript 24 hours after inoculation. At 72 hours after infection, a ~165-fold increase was observed. (Levy *et al.,* 2004). Hence, these results lead to uncertainty about the importance of promoter region of the N gene process of resistance and thus it is necessary to study its effect on transcriptional activity.

2.6 The role of promoter regions in gene regulation

The promoter governs the temporal and spatial expression of any gene. The region of the DNA that is upstream of the coding sequence and recognized by specific proteins involved in transcription is known as promoter (Buchanan *et al.,* 2000). Promoters have three common components, transcription start site, sequences bound by transcription regulators and TATA box. The transcription starts with binding of transcription regulators at various sequences on the promoter. The RNA polymerase II transcription complex is recruited to the promoter region by transcription activators, resulting in initiation of the transcription (Lee and Young, 2000). Several sequence elements upstream or downstream of this binding site that are recognized by the transcription regulators may also influence the start of transcription (Russell, 1996). These sequence specific transcription regulators give rise to the complexity of promoter function and fine-tuning of the expression pattern (Alberts *et al.,* 2002). Studies have revealed a large number of transcription factors in plants with functions linked to tissue specificity, response to light, and presence of particular cellular substrates, as well as in response to such stresses as pathogens. Hence, the sequence of the promoter is an important factor that affects the expression and function of the downstream gene.

There have been very few studies analyzing the factors governing the control of *R* gene promoters. A N gene from tobacco, which belongs to NBS-LRR class undergoes alternative splicing (Dinesh-Kumar and Baker, 2000). The level of expression of these transcripts during pathogen interaction is not fully understood. It is possible that the rate of *R* gene transcription changes during the pathogen encounter. This, coupled with alternative splicing, could result in an alteration in the amount and type of produced proteins. Such changes might lead to the activation of responses against the pathogen. This indicates the importance to study the transcription regulation by the promoter in order to understand the regulation of an *R* gene activity.

2.7 Reporter genes and their use in gene characterization

The analysis of the expression of genes requires the use of reporter genes. Gene sequences that are easily detected when they are expressed are reporter genes. These genes should not normally be present in the organism being studied. Reporter genes have been utilized for many purposes, one of which is use as a marker gene. In transformation experiments, marker gene expression can be utilized for identification of a transformation event. Another important use for the reporter genes, however, is for the analysis of the activity of various promoters.

2.7.1 The ß-glucuronidase reporter gene

The *uidA* (GUS) gene, which codes for the enzyme ß-glucuronidase, was isolated from *Escherichia coli*. The GUS gene is widely used reporter genes in plants, and can act on two specific substrates. ß-glucuronidase converts 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) to a blue chromophore, which can be used for histological localization. Alternatively, 4-methyl umbelliferyl glucuronide (MUG) is converted to a fluorescent product by the ß-glucuronidase and is utilized for measuring the specific activity of the enzyme (Jefferson *et al.,* 1987). The GUS gene is widely used in plants due to the lack of background GUS activity, frequently observed in microorganisms, vertebrates and invertebrates (Gilissen *et al.,* 1998). In histochemical localization, expression of this gene can be detected at the subcellular level (Daniell *et al.,* 1991). The GUS gene is ideal for gene fusions and has been used for many gene-tagging experiments that led to the study of many genetic elements and regulatory elements (Fobert *et al.,* 1994; Foster *et al.,* 1999). The only disadvantage with the GUS reporter system is that the assay used to analyze gene expression is destructive to plants. Hence, it cannot be utilized for *in vivo* reporter gene function analysis.

2.7.2 The luciferase reporter gene

Luciferase is present in many unicellular and multicellular organisms. The luciferase from the firefly (*Photinus pyrelis*) is widely accepted form of the luciferases. This enzyme acts through an ATP dependent oxidative decarboxylation of the substrate luciferin, which produces a reaction accompanied with emission of photons. Sensitive apparatus capable of detecting these photons can be utilized to measure LUC activity. This assay is very sensitive and can be utilized for *in vivo* expression analyses, as it does not require destructive methods for analysis (Millar *et al.,* 1992; Ow *et al.,* 1986). This system has been proven to be very efficient for the analysis of gene expression during various developmental stages in potato tubers (Verhees *et al.,* 2002). The half-life of LUC *in vivo* is very short, and it does not accumulate over time. Half life of LUC mRNA is 45 minutes, while the half life of LUC protein is 155 minutes and with the presence of luciferin it is 15.3 minutes (van Leeuwen *et al.,* 2000). Hence, it is the most efficient and accurate reporter gene to assay indirect transcriptional activity based on gene expression analysis (van Leeuwen *et al.,* 2000; Millar *et al.,* 1992).

2.8 Conclusion

Pathogens are one of the most prevalent stresses encountered by plants. Resistance response mediated by plant R proteins is one of the most efficient mechanisms to counteract the pathogens. In tobacco, N protein imparts resistance against TMV as marked by hypersensitive response. It is uncertain whether alternative splicing or transcription level or both are required for N function on pathogen encounter. Also, the N gene mediated response is compromised at higher temperatures. The role of transcription activity in this inefficient response is not known. The N gene response to TMV is followed by development of SAR and accumulation of systemic SA. This accumulation may influence the transcription activity. Hence, it becomes important to analyze the regulation of the pathogen response via transcriptional activation of the N gene promoter.

During compatible interaction, the pathogen infection results in generation of SRS. This signal spreads systemically in plants and is faster than spread of the pathogen. Plants that receive the SRS exhibit a higher rate of meiotic as well as somatic recombination. The increase in the recombination is associated with reduced genome stability in plants. The fact that SRS is capable of spreading to non-infected tissue suggests that production of SRS may be a part of plant mechanism to counteract the pathogen. Hence, detail study of N gene promoter and SRS is important for understanding the plant and pathogen interactions.

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3.0 Effect of external factors on reporter gene expression fused with N gene promoter

3.1 Abstract

The role of *R* genes in plant pathogen resistance has long been studied due to its economical impact on agriculture. Interaction between TMV and the N protein from tobacco is one of the most widely used models to understand various aspects of this resistance. The transcription activity as governed by N gene promoter is one of the least understood elements of the model. In this study, the N gene promoter was cloned and fused with two different reporter genes, β-glucuronidase (N::GUS) and luciferase (N::LUC). Tobacco and *Arabidopsis* plants transformed with the N-reporter construct were screened for homozygosity and stable expression. Expression analysis using N::GUS tobacco plants revealed that the expression is organ specific and developmentally regulated. During early stages of growth, GUS expression was limited to midveins with no root expression. As the plant matured, expression spread into the leaf lamella. Using N::LUC tobacco plants, experiments to analyze the effect of various biotic and abiotic factors on the reporter gene activity were carried out. Results indicate that there is an up regulation of LUC activity following TMV infection. Also, an initial induction and then subsequent down regulation in reporter gene activity was noted when plants are subjected to high and low temperatures. At the same time, the SA didn't show any significant increase or decrease in the LUC activity.

3.2 Introduction

The survival of an organism depends on its ability to counteract the environmental influence or stress. Plants, being different in that they are unable to escape from their niche, have evolved in the in ability to resist many of these stresses. Among others, pathogen infection is one of the most significant stresses due to its direct impact on agriculture. Plants inherit mechanisms to recognize and express resistance against specific pathogens. This results from the molecular interactions between the pathogen and the host plant. The products of plant *R* genes can recognize pathogen derived elicitors or avirulence factors (Nimchuk *et al.,* 2003; van der Beizen and Jones, 1998). Upon recognition, *R* proteins trigger a signal, which results in the localized cell death and formation of necrotic lesions, a reaction termed hypersensitive response (Nimchuk *et al.,* 2003). Such an interaction is known as an incompatible interaction. If the *R* gene is not present, the pathogen can infect and multiply in the host due to lack of resistance. Such an interaction is termed a compatible interaction (Keen *et al.*, 1990).

The majority of *R* genes code for a NBS domain and a C-terminal LRR domain. The LRR domain is well known to play a role in the higher specificity molecular interactions that are responsible for the recognition of a pathogen (Dangl and Jones, 2001). At the N terminus, either a TIR domain or a coiled coil (CC) domain is present. Very often these R genes are members of multigene families (Song *et al.,* 1995).

Much research has been conducted on TMV as a model to understand the interaction of pathogen with the host plants. TMV is a rod shaped virus with a positive, single stranded RNA genome. During a compatible interaction with tobacco, it causes mosaic-like symptoms. As for many other plant pathogens, there is a specific *R* gene for TMV in tobacco (*Nicotiana glutinosa*), the N gene, which imparts complete resistance against infection (Holmes, 1938). The N gene was named after the necrotic lesions on the leaf surface that are produced due to the hypersensitive response (Whitham *et al.,* 1994; Erickson *et al.,* 1999; Ryals *et al.*, 1996).

N gene belongs to the NBS-LRR class of *R* genes and contains an N terminus that is similar to Toll and interleukin-1 receptors (Hashimoto *et al.,* 1988; Sims *et al.,* 1989; Baker *et al.,* 1997). All the three domains, TIR, NBS and LRR are important for the N protein function (Dinesh-Kumar *et al.,* 2000). It is well known that the replicase protein is the Avirulence gene product recognized by the N gene (Dawson, 1992). It is predicted that N protein forms a receptor complex and as soon as there is elicitor binding, a signal is transduced by the complex. This signal results in hypersensitive response against the TMV. There is a rapid burst of reactive oxygen species within ten minutes after TMV infection (Doke and Ohashi, 1988).

The structure of the N gene coding sequence consists of five exons that code for a 131.4 kDa protein. Like several other *R* genes, N gene produces two transcripts as a result of alternative splicing at the third exon (Dinesh-Kumar and Baker, 2000). This frameshift in transcription results in a truncated protein of 75.3 kDa mass. There are few previous reports revealing the function of the alternative splicing in imparting resistance. However, it has been suggested that, not only both transcripts are required for resistance, but also the specific ratio of shorter to longer transcript is important for the normal N function. This ratio, 28:1 (short:long) at the time of infection, changes to 1:23 six hours after infection, and returns to 28:1 nine hours after infection. It is important to mention that previous work suggests that this change in the ratio occurs without a change in the transcription rate (Dinesh-Kumar and Baker, 2000).

Temperature plays a very important role in the interaction between TMV and the N protein. N protein is not able to trigger HR when the plant is grown at temperatures above 28°C, and at this temperature TMV can systemically infect the plant. If a temperature-sensitized plant is brought back to a temperature below 28°C after infection, the N gene triggers a global HR resulting in death of the plant. The factors that influence the efficiency of N protein above certain temperatures are still unclear (Samuel, 1931).

The role of transcription activity of N gene in the resistance development is one of the least understood aspects. N gene produces two transcripts by alternative splicing. Earlier reports suggested that plants expressing both long and short versions of transcripts under the regulation of the N gene promoter are able to resist TMV. However, if both the transcripts are expressed under the control of a 35S constitutive promoter, the interaction was compatible (Dinesh-Kumar and Baker, 2000). This suggests the importance of the N gene promoter in resistance development. In contrast, a different report showed that during a transient assay, the expression of both transcripts under the constitutive 35S promoter resulted in functional resistance to TMV (Mestre and Baulcombe, 2006). This further emphasizes the need to study how the promoter regulates the expression of the N gene.

To understand the function of the N gene promoter region in more details, promoter::reporter gene fusions were used. The 4.1 kb region corresponding to the promoter of the N gene was fused to the LUC and GUS reporter genes. Plants of tobacco, SR1 (no functional N gene) and Havana (homozygous for a functional N gene) cultivars as well as *Arabidopsis* plants were transformed using *A. tumefaciens*-mediated gene delivery system. Stable homozygous plants were identified by selective screening and reporter gene expression analysis. N::GUS and N::LUC tobacco SR1 plants were used for further experiments. In N::GUS plants, the expression of GUS driven by the N gene promoter was compared to plants expressing GUS constitutively (35S::GUS plants). Experiments to characterize the effect of virus infection, temperature extremes and SA on reporter gene activity were also carried out.

3.3 Materials and Methods

3.3.1 Plant growth conditions

3.3.1.1 Growing plants in soil

Seeds of tobacco plants (*Nicotiana tabaccum*) were grown on potting soil in individual pots in a growth chamber (Enconair ecological chambers Inc., Winnipeg, MB, Canada) at 24°C, with 80% humidity and light intensity of 200 $\mu Mm^{-2}s^{-1}$. The chamber was set at 16:8 hours of light:dark conditions. Plants that were grown in these conditions unless mentioned were referred to as grown at normal conditions. *Arabidopsis* plants were grown in soil in growth chamber with at 22°C, with 65% humidity and light intensity of 175 $\mu Mm^{-2}s^{-1}$.

3.3.1.2 Growing plants on sterile media

Seeds were surface sterilized by washing them in 1% sodium hypochlorite solution for 5 min followed by three subsequent rinses with sterile, distilled water. Sterilized seeds were sown on 100 mm diameter Petri dishes with basic Murashige and Skoog (MS) media (Murashige and Skoog, 1962).

After sowing seeds, the petri dishes were transferred to growth chamber. Seeds were allowed to germinate and grow at aforementioned conditions. *Arabidopsis* seeds were incubated at 4°C for two days for breaking seed dormacy before being placed into growth chamber.

3.3.2 Vector description

The transformation vectors were developed from the pCAMBIA1300 binary vector system that contained an 8.9 kb backbone sequence. It is a minimal selection vector with sequences required for plant transformation and subsequent selection of transformants. The vector sequence contains the kanamycin resistance gene for selection. The T-DNA contains a hygromycin resistance gene under the control of the 35S promoter at the 5' and the 35S terminator sequence at the 3' end. pUC18 polylinker has been used in this vector (Hajdukiewicz *et al.,* 1994).

3.3.2.1 pCAMBIA N::LUC vector

The total size of the pCAMBIA N::LUC vector was 14.4 kb. The T-DNA included two ORFs. For plant selection, the hygromycin herbicide resistance gene (*hptII*) was present in the T-DNA. Near the T-DNA right border, the luciferase cDNA was cloned downstream of the N gene promoter. At the 3' end of luciferase cDNA, a Nos gene terminator sequence was placed. (Fig. 3.1).

3.3.2.2 pCAMBIA N::GUS vector

The pCAMBIA N::GUS vector was similar to the luciferase vector. The total size of the vector was 15.3 kb. In the T-DNA region, the *hptII* gene was present near the left T-DNA border. GUS cDNA was under the control of the N gene promoter (Fig. 3.2).

3.3.3 Plant transformation

The binary vectors, pCAMBIA-N::GUS and pCAMBIA-N::LUC were transferred to *Agrobacterium tumefaciens* (strain GV3101) via electroporation. Single colonies were obtained from yeast extract-peptone (YEP) medium plates. YEP consisted of 10 g Peptone (EM Sciences, Gibbston, New Jersey), 10 g Yeast extract (EMD Chemicals Inc. Gibbston, New Jersey), 5 g NaCl and 10 mM $MgSO_4$ in 1 L H_20 with 50 mg/L kanamycin for selection. These single colonies were used to prepare a overnight liquid culture in YEP medium plus antibiotics (rifampicin 25 mg/L, gentamycin 25 mg/L and kanamycin 50 mg/L).

For *Arabidopsis* transformation, the floral dip method was performed (Clough and Bent, 1998). Six to eight *Arabidopsis* (ecotype Columbia) plants were grown in a pot at 22^oC, and initial shoots were detached to induce an increase number of secondary shoots. These plants were grown until the flowering stage. The fresh overnight culture of *A. tumefaciens* was harvested and resuspended in inoculation medium (IM) (Clough and Bent, 1998). Floral buds were immersed in the solution for 5 min, and then transferred to a high humidity (80%) chamber. Seeds were harvested from the plants and screened for transformation as described below.

Tobacco plants (*Nicotiana tobaccum,* cv Havana and petite SR1) were transformed by a modified leaf-disc transformation method described previously (Horsch *et al,* 1985). Briefly, an overnight culture of *A. tumefaciens* was harvested and resuspended in 10 mM $MgSO_4$, 5% sucrose and 0.005% Silwett L-77 (Clough and Bent, 1998). Leaves were injured by inducing cuts and were immersed in resuspended *A. tumefaciens* suspension for 10 min. These leaves were co-cultivated for 48h on MS medium (described above), and grown on plates until visible bacterial growth is formed around the leaf edge. After 48 h, the leaves were washed with sterile water and transferred to callus inducing medium (0.8 mg/L indole acetic acid (IAA), 2.0 mg/L kinetin in MS media supplemented with 30 mg/L hygromycin and 200 mg/L carbenicillin, Figure 3.3). The leaves were incubated for 3-4 weeks on this media, during which the *A. tumefaciens* transformed cells produced calli and preliminary shoot formation took place (Fig. 3.4). Each individual plantlet was separated and transferred to root inducing media (0.5 mg/L naphthalic acetic acid (NAA) in MS media supplemented with 30 mg/L hygromycin and 200 mg/L carbenicillin). After 1-2 weeks, the roots were induced from the plantlets and they were transferred to soil for subsequent growth.

3.3.4 Plant screening

3.3.4.1 Screening for herbicide-resistance plants

Seeds were sterilized and sown on MS medium containing 30 mg/L hygromycin. Resistant seedlings were transferred to soil 3 weeks post-germination. In the T_1 generation, plants heterozygous for the insert were taken as positive tranformants. At T_2 generation, plants were screened for segregation and plants showing 3:1 segregation were selected as containing a single locus insertion. These plants were analyzed for homozygosity by testing the hygromycin resistance in the progeny. Homozygous T_3 plants were propagated and used for the experiments.

3.3.4.2 Screening for Reporter genes

All herbicide resistant plants were tested for the expression of the reporter genes. For N::LUC lines, a charges couple device (CCD) camera (Gloor Instruments, Basel, Switzerland) was used (Fig. 3.5), while for the N::GUS lines, a histochemical staining method was used (Fig. 3.6). Plants showing uniform and consistent expression through out the T_1, T_2 and T_3 generations were utilized for the experiments.

3.3.5 Reporter gene analysis

3.3.5.1 Luciferase expression analysis using CCD camera

A CCD camera was used for the analysis of the luciferase expression. For preparation of luciferin solution, 1 mg of luciferin powder was dissolved in diethyl sulphoxide (DMSO), and made to a final concentration of 0.5 mM. Plants were sprayed with this luciferin solution and incubated in dark for 30 min. Plants were placed in the CCD camera chamber and the camera was fine focused on the plants. Images were taken using Pixcel software 2.8 (PerkinElmer, Cambridge, UK). One control image with light was taken first with a 1 min exposure time. Two subsequent dark images were taken with 10 min exposure each. These images were processed using analySIS software 3.2 (Soft Imaging Systems, Munster, Germany). In brief, the control image taken in the presence of light was given green color (Fig. 3.7). The two dark images were superimposed to remove all the background fluorescence. A color code was defined to give gradual intervals of the intensity of LUC expression (Fig. 3.8). This was required to analyze any change in the intensity of the LUC expression over time.

3.3.5.2 Luciferase expression analysis using a quantification assay

The luciferase quantification assay was performed as mentioned by Kartikeyan, *et al.* (2002), with modifications. It allows analysis of minute differences in concentration of reporter proteins in the total amount of protein. For the analysis, plant tissue was collected and flash frozen in liquid nitrogen. This tissue was ground to fine powder and homogenized with 500 μL of LUC extraction buffer. The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was taken to a fresh tube. Total protein concentration was measured for these samples using the Nano Orange Protein Quantification Kit (Invitrogen, Burlington, Ontario). Briefly, for each sample, 195 μL of Nano Orange working solution was taken into the well of a microplate. 5 μL of the extract was added to each well. The plate was incubated at 90°C for 10 min and cooled down to room temperature. The amount of fluorescence was measured using a microplate reader (FL_X 800, Bio-Teck Instruments Inc. Winooski, USA). For standards, various known concentrations of bovine serum albumin (BSA) were used.

Flash assay for luciferase quantification assay was carried out using the same microplate reader. The sample extracts were standardized to have the same total protein concentration by diluting them with LUC extraction buffer. In a microplate, 100 μL of LUC assay buffer and 50 μL of the sample extract were added to the same well. The plate was placed in the carrier of microplate reader. Fifty μL of 1 mM of luciferin solution was added to each well to a final concentration of 0.25 mM. Immediately, the reading was taken by microplate reader using the KC4 software 3.02 revsion 4 (Bio-Teck Instruments Inc. Winooski, USA). The readings from the samples were compared for the analysis. All the software settings were done prior to starting with the assay. Reading was carried out using the luminescence mode with the end point mode. The luciferase activity in form of relative luminescence units (RLU) is calculated as luminescence units per mg total protein. The RLU values were different in independent experiments, hence they can not be compared between two different experiments.

3.3.5.3 GUS expression analysis using histochemical staining

The procedures of the histochemical analysis were performed according to Jefferson (1987). The staining buffer consisted of 100 mg 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; Jersey Labs Inc., USA) with 0.05% NaN_3, 1 mL dimethylformamide, and 0.05% Tween 80 in 200 mL of 0.1 M sodium phosphate buffer (pH 7.0). *Arabidopsis* and tobacco plants were placed in tubes (1.5-50 ml), suspended in the staining buffer, and vacuum infiltrated for 15 min. Subsequently, plants were placed at 37°C for 48 h. The plants were bleached for better observation of the GUS expression. For this, staining buffer was replaced with 70% ethanol and plants were incubated at 37°C. Ethanol was changed frequently until the plants were sufficiently bleached.

3.3.6 Expression pattern analysis of tobacco N::GUS lines

Lines SGUS S1-9, SGUS S12-4 and SGUS S2-7 were used in the experiments. For control, two independent lines, 35S::GUS 1-9 and 35S::GUS 1-16, that have the GUS gene controlled by the CaMV 35S constitutive promoter, were used. Plants were grown under uniform conditions. Whole seedlings were harvested at 2, 4, and 6 weeks of age. Histochemical staining was performed according to the aforementioned procedure. After staining, plants were analyzed using a stereomicroscope.

3.3.7 Analysis of the effect of virus on the LUC activity

Lines SLUC P2-1 and SLUC P6-1 were used for the experiment. Plants were grown in growth chambers for 6 to 8 weeks. TMV strain U1 was used for infection. TMV concentration was measured and diluted to a concentration of 5 μg/mL in buffer (10 mM sodium phosphate, pH 7.0). Two types of inoculation and analysis were performed.

Luciferin was applied to single leaf and the leaf was incubated for 30 min in dark. This leaf was then infected with TMV or mock-treated with help of carborundum powder and the residues were washed with sterile water. The CCD camera was then fine focused on the infected region of the leaf for very detailed pictures. Continuous dark frames were taken in the region until 2 h post-infection. All the frames were processed as mentioned before, and analyzed for any minute changes in LUC expression.

Alternatively, carborundum powder and 50 μL of distilled water were rubbed gently into the selected leaves. Residues were washed away using a spray of sterile distilled water. 20 μL of the virus was then gently rubbed into the injured area. For a control treatment, 20 μL of buffer (10 mM sodium phosphate, pH 7.0) was used. At 0, 15 min, 30 min, 1 h for the first hour and then at each half hour until 7 h, tissues infected region were collected from these plants. The tissue was analyzed for specific activity of the luciferase.

3.3.8 Analysis of the effect of virus and high temperature on LUC activity

Plants from the lines SLUC P2-1 and SLUC P6-1 were grown in growth chamber and were used at 6-8 week of age. For this experiment plants were shifted to a chamber at 32°C. Light and humidity conditions were maintained at the same level as for the control. Under this temperature, TMV and mock inoculation were carried out, taking care that the temperature did not drop down in the growth chamber. All the solutions and water were pre-incubated to 32°C. For CCD image analysis, the plants were incubated for 24 h at 32°C temperature. Subsequently, they were shifted to 24°C for various time periods from 0 to 15 min, 30 min, 1 h and then at each half hour until 7 h. The expression of TMV and buffer treated plants was compared with control plants that were not inoculated at all. For the quantification assay, plants were kept in this condition for 8 to 10 days, until they started showing virus symptoms. As soon as symptoms appeared, they were incubated in the 24°C chamber (same light and humidity conditions) for 0 h to 7 h. Reporter expression analysis was carried out at these time points using a quantification assay, and four plants in each treatment at each time point were analyzed.

3.3.9 Analysis of the effect of high temperature on LUC activity

SLUC P2-1 and SLUC P6-1 plants were grown under normal growth conditions in a growth chamber until 6-8 weeks of age. They were divided into two treatments. For the high temperature treatment, plants were shifted to a chamber at 45°C. Control treatment plants were maintained under control conditions in the chamber at 24°C. At 0, 15 min, 30 min, 1 h, 1 h 30 min, 2 h, 3 h, 4 h and 6 h time points, reporter expression analysis was done from four plants from each treatment using the CCD camera. For quantification assay, tissues from the treated plants were collected at different time points mentioned in the previous experiments and flash frozen.

3.3.10 Analysis of the effect of low temperature on the LUC activity

Plants used in the experiment were grown in growth chambers until 6-8 weeks of age. They were divided into two treatments. Low temperature treatment plants were moved to a chamber set at 4°C, and control plants were maintained in the 24°C control growth chamber. The same light, humidity and day length conditions for both of the chambers were maintained. At 0, 15 min, 30 min, 1 h, 1 h 30 min, 2 h, 3 h, 4 h and 6 h time points, four plants were analyzed using the CCD camera. For measuring the specific activity, tissues from four plants from each treatment were collected at the time points as mentioned in the virus experiment and quantification assay was carried out.

3.3.11 Analysis of the effect of salicylic acid on the LUC activity

The SA solution consisted of 5 mM SA in distilled water with 0.1% Tween 80. As a control, distilled water containing 0.1% Tween 80 was used. The N::LUC plants were grown until 6-8 weeks of age. For the SA and control treatments, SA and mock solution was uniformly applied on the leaves by using a fine spray. At different time points as mentioned in the first experiment, starting from time point zero after treatment application, four plants from both the treatments were analyzed using the quantification assays.

3.4 Results

3.4.1 Transformation and screening of plants

The seeds of the *Arabidopsis* plants harvested following the floral dip transformation procedure were subjected to the herbicide screening. More than 120 transformed plants with N::GUS and N::LUC constructs were identified. The expression of the reporter gene from these 120 plants was evaluated by the reporter gene assays. Based on the expression level of the luciferase reporter gene, plants were differentiated into high expression (N::LUC S), moderate expression (N::LUC P) or low expression (N::LUC N) groups. In the next generation, along with reporter gene expression, the segregation ratios to the herbicide treatment of about 103 lines were analyzed (Table 3.1). As a result, 57 individual plant lines having single locus insertion of T-DNA were identified. Segregation analysis performed on T_3 plants revealed 11 homozygous lines. The lines from NLUC construct were NLUCP 6-6, 19-8, 26-11 and 28-1. While from NGUS construct the lines were NGUSP 1-3, 5-8, 7-4, 9-1, 12-5, 14-2 and 16-1. In future experiments, these single T-DNA insertion plants will be used for detecting factors involved in the regulation of the N gene promoter.

From tobacco, successfully transformed plants from the SR1 background were given a SLUC prefix, and Havana plants were given a HLUC prefix. More emphasis was given to the SR1 plants as they were to be used for subsequent experiments and were differentiated to groups based on their reporter gene expression, as previously mentioned. Briefly, out of 95 positive transformants from the T_1 generation, 43 had a single locus insertion in the T_2 generation. By testing the T_3 generation, 8 lines from the SLUC plants were identified to be homozygous. Their expression was examined, and the plant lines with moderate and uniform expression were selected. Two lines, SLUC P2-1 and SLUC P6-1, had a similar expression level, and were selected for use in further experiments (Table 3.2). Similarly, three lines, SGUS S1-9, SGUS S12-4 and SGUS S2-7, were selected from the SGUS plants for the expression pattern analysis.

3.4.2 Expression pattern analysis of N::GUS plants

Comparison of the expression patterns of the GUS reporter gene in N::GUS and control 35S::GUS plants was carried out. There was a difference in the expression pattern between the lines.

In two-week-old tobacco seedlings, the expression of GUS in N::GUS lines was limited to the midveins of the cotyledons, with no expression in the lamella. In contrast, the control 35S::GUS tobacco plants displayed uniform expression throughout the cotyledon. Furthermore, there was no expression in the roots of N::GUS seedlings, whereas the 35S::GUS seedlings showed uniform expression in the roots (Fig. 3.9). At four-weeks of age, the GUS activity began to spread into the lamella of tobacco true leaf, and at about six weeks of age there was uniform expression throughout the leaf (Fig. 3.10).

Primary roots from the N::GUS line do not show any GUS expression in the early seedling stages. This absence of expression was found even in four-week-old seedlings and beyond. In contrast, the expression of GUS in roots of control plants was uniform from germination stage throughout maturity (Fig. 3.11).

3.4.3 Effect of TMV on LUC activity

The effect of TMV on LUC activity was analyzed by infecting the N::LUC plants with TMV and checking the expression level of the reporter gene, as previously mentioned.

When a single leaf was infected, very minute changes in LUC expression in that same leaf analyzed over time with a CCD camera, were observed. In the virus treated leaf, there was a very minor increase in the intensity of LUC expression at the 1 h time point. In contrast, the buffer treatment revealed a small decrease in the expression (Fig. 3.12). The experiment was repeated on five leaves.

The effect of TMV on the N gene promoter was also analyzed using a quantitative LUC assay. Leaves treated with either TMV or control buffer were harvested from 0 h to 7 h post-infection, and the specific activity was measured (Appendix 6.1). An average of the specific activity at each time point was calculated from the four different plants used (Table 3.3). when we compared the values of individual treatment over time, the buffer treatment contained statistically identical averages (One-way analysis of variance (ANOVA), $F_{15,63}$=0.889, P=0.581), as did the TMV treatment (One-way ANOVA, $F_{16,63}$=1.197, P=0.306). While when we compared the values of buffer and virus at particular time point, between treatments only the 1h time point showed a significantly higher LUC activity of luciferase, 53.93 RLU, when compared to control treatment, 36.94 RLU (Students t-test assuming unequal variances, T=2.255, df=4, P=0.043, Table 3.3). Hence, the results indicated a pattern similar to that observed in the CCD experiment (Fig 3.13).

3.4.4 Effect of virus and high temperature on LUC activity

In this experiment, tobacco plants were incubated at 32°C and infected with TMV or a control buffer. When the infected plants started showing symptoms, they were incubated at 24°C for different time durations and the reporter gene activity was analyzed.

CCD image analysis revealed that there was a clear induction of N::LUC expression when plants were incubated for 2 h at 24°C after being transferred from 32°C. This induction was not observed in the subsequent time points. Furthermore, the area of high intensity corresponded to the likely area of TMV spread after a 24 h incubation period (Fig. 3.14). The experiment was repeated twice.

For the quantitative LUC assay, the specific activity of plants that were incubated at 22°C for 0 h to 7 h after the shift from 32°C to 22°C was assayed (Appendix 6.2). The averages of four plants from each time point were analyzed. The overall pattern for the specific activities indicated a trend as seen in CCD analysis (Fig. 3.15). in the comparison of values of treatments over time it was found that the control plants had statistically identical means (One-way ANOVA, $F_{15,63}=0.542$, $P=0.903$), as did the TMV treatments (One-way ANOVA, $F_{15,63}=0.779$, $P=0.694$). While the statistical analysis did not show any significant differences among the treatments for any given time points when the values between the buffer and virus treatments were compared at perticular time point. However, at the 1h 30min time point, the specific activity in TMV treatment plants was 1.2-fold higher than control treatment plants.

Similarly, the TMV treatment plants had 1.46-fold higher specific activity than control plants at the 2h time point, which suggest an increase in the reporter gene activity (Table 3.4).

3.4.5 The effect of high temperature on the LUC activity

This experiment revealed the effect of high temperature on the activity of LUC. With CCD image analysis, it was observed that there was a rapid increase in the intensity of expression within 15 min of incubation at 45°C. This increase eventually subsided with prolonged incubation. After 3 h, the expression pattern matched the intensity observed in the control plants. After 3 h time point, the intensity started decreasing, and after 6 h of incubation at this temperature there was no visible luciferase expression in the plant (Fig. 3.16).

In an alternative assay, plants were incubated at 45°C and 24°C for 0 h to 7 h time durations, and luciferase activity was quantified (Appendix 6.3). The mean activity was found to be identical among the control plants compared to high temperature treatment plants at all time points (One-way ANOVA, $F_{15,63}$=0.652, P=0.816, Table 3.5). The comparison of values in control and high temperature treatment revealed that the means were significantly different in the samples prepared from heat-treated plants for all time (One-way ANOVA, $F_{15,63}$=2.181, P=0.0210). Specifically, it was found that the 30 min treatment differed from the 0 h treatment (Students t-test assuming equal variances, T=2.73, df=6, P=0.017). A further examination of the trend found that the values at the 15 min to 3 h 30 min were significantly different than the later time points from the 5 h mark and beyond (All Pairs Students T-test,

$P<0.05$). When the comparison was done between 45°C temperature treatment with the control treatments, it revealed a trend relating to that of the CCD camera images (Fig. 3.17).

3.4.6 The effect of low temperature on the LUC activity

CCD image analysis revealed that there was high increase in the intensity of luciferase activity at 15 min after incubation at 4°C. This increase was reduced at later time points. The expression at 3h post-treatment was similar between low and control temperatures. At the subsequent time points, the expression of reporter gene reduced further (Fig 3.18).

Alternatively, the LUC activity of luciferase was measured in a quantification assay (Appendix 6.4). The data analysis was done by calculating and comparing the average of four plants at each time point from both the treatments (Table 3.6). The averages observed within the 24°C control were found to be statistically identical (One-way ANOVA, $F_{15,63}=0.359$, $P=0.983$), while the averages within the cold treatment revealed statistically significant differences (One-way ANOVA, $F_{15,63}=2.243$, $P=0.0175$). Further analysis found that the 0 h time point was identical to all of the other time-points, except the initial times of 15 min to 1h 30min and the later times of 5 h 30min and beyond (All pairs student t-test, $P<0.05$). Also, the control and low temperature treatments were significantly different at 6h 30min time point (Paired Student's T-test, $p<0.01$, Table 3.6). The trend showed certain similarities to the low temperature experiment using CCD camera (Fig. 3.19) and the heat treatment. The differences observed between the control groups and the cold treatments, were similar to those observed for the heat treatment. In both cases, the increase of LUC activity was observed in plants exposed to either 4°C or 45°C from 15 min to 1 h 30 min after the exposure. Similarly, in both cases, the activity of LUC decreases at 5 to 6 h from the beginning of the exposure. These experiments suggest that N gene promoter is temperature sensitive. Also there were no reports on temperature sensitivity of the luciferase enzyme, it would be important, in the future, to test the activity of LUC after the exposure of 35S:LUC plants to either temperature extreme.

3.4.7 The effect of Salicylic acid on the LUC activity

The N::LUC plants were either sprayed with SA or buffer and the quantitative LUC assay was performed at different time points from 0 h to 7 h (Appendix 6.5). The values from four plants were averaged and analyzed. The averages within the buffer treatment (One-way ANOVA, $F_{15,63}=0.370$, $P=0.981$) and the SA treatment (One-way ANOVA, $F_{15,63}=0.331$, $P=0.989$) were found to be identical. The averages were quite uniform and there were no significant differences among the plants of the various treatments at the various time points (Student's T-test, $p<0.05$, Table 3.7). The specific activity remained between 9.8 to 12.4 RLU and gave a very uniform trend between various time points (Fig. 3.20).

3.5 Discussion

In order to understand the function and regulation of the N gene, it is very important to study the activity of the N gene promoter. Characterization of this promoter will also help in understanding the other *R* genes, as the N gene promoter may become a model for other R gene promoters, as the structure and function of plant *R* genes are conserved.

Analysis of expression of the GUS gene controlled by the N gene promoter in leaves and roots gave a better insight about the tissue specific expression directed by this promoter. The fact that the N gene promoter is not as active in the early developmental stages suggests that tobacco plants could be particularly sensitive to TMV at the young age. Similarly, it could be predicted that the roots of the plants of different age would be also sensitive to TMV infection. This, however, poses not threat to the plant, since roots are not a typical place for viral infection.

It is very important to understand the role of the N gene promoter region in the interaction with TMV as it can regulate the level of transcription (Alberts *et al.*, 2002). When N promoter::reporter plants were inoculated with TMV and analyzed for the reporter gene expression, it shows an induction corresponding to 1 h to 1 h 30 min after inoculation. The CCD images show localized areas of induction, which is likely due to the method of inoculation infecting a limited number of cells on the leaf. Further variation in this quantitative method could be due to some of the following reasons. Whole leaves were harvested for the infected tissue, resulting in the analysis of infected as well as uninfected cells; there is a high variability in the expression level in control plants, indicating high plant-to-plant variation. This may explain why the quantification assay failed to give more significant results. However, the induction as revealed by CCD analysis suggests that the transcription level of the N gene increases in the presence of TMV. As this increase early in the N gene mediated response to TMV, it can be suggested that this increase is important in the onset of resistance.

The N gene does not protect the tobacco plant against TMV at temperatures above 28°C. When the plants were inoculated with TMV at 32°C, and then transferred to 24°C after the occurrence of virus symptoms, they show an induction in expression of he LUC reporter gene at the 2 h time point. The CCD images show a clear difference in the luciferase activity at this time. The results from the quantitative assay, although not significant, indicate a 1.46 fold increase in the reporter gene activity in TMV treated plants at the 2 h time point when compared to control plants. With the apparent variability in the reporter gene expression in plants and due the fact that changes in LUC expression can be visualized using CCD analysis, it is very likely that the induction is higher than we observe in the experiment. This early induction, as seen in the prior experiment, may be vital for N gene regulated resistance. In such a situation, if this induction is necessary for resistance, in its absence, the N protein may not be as efficient or completely non-functional. This may be the reason why N gene mediated resistance is not functional at high temperature.

The increase in the reporter gene activity may be due to increase in the transcription, given that there is no post transcription regulation for either LUC or GUS has been reported in the literature. It might be possible that accumulation of a high amount of N protein may be necessary for an efficient response again TMV. These predictions contradict the previous reports that there is no change in the transcription level of N gene following TMV infection (Dinesh-Kumar and Baker, 2000). However, this recent report suggests that the N gene regulated by the 35S promoter and terminator also imparts resistance. The transcription level of such a construct would be higher than the native promoter, as 35S is a constitutive promoter. This indicates that N protein is functional when present in high amount. More experiments are obviously needed to analyze the changes in accumulation of N transcripts or protein.

When the N-LUC plants were subjected to the high temperature treatment alone, the CCD images show that the LUC activity increased 30 min -1 h 30 min after treatment. However, prolonged incubation in these temperatures results in very little or no activity. The quantification assay also suggested a similar trend. In plants incubated at 45°C, the LUC activity was 1.34 fold higher than the control at 1 h of exposure, and decreases to 1.36 fold lower than the control at 5 h 30 min time point. If at high temperature there was less N protein activity, it was logical to assume that this would result in less efficient response to TMV. Hence, the results predict that if the plants are incubated at 32°C for more than 6h, they will not be able to resist pathogen infection and systemic spread. Furthermore, the results also predict that if the plant would be infected in the initial 1 h of incubation at 32°C, it would able to form necrotic lesions and suppress systemic infection as this time point likely had sufficient transcriptional activity to actively mount the resistance response.

The CCD images revealed that at low temperature the increase and decrease in LUC activity is not as drastic as that seen in high temperature. The quantification assay indicates that in plants subjected to low temperatures, the reporter gene activity increases during the initial time points (1.17-1.24 fold) when compared to control plants. At 6 h 30 min of incubation, the activity of low temperature treatment plants was 1.46 fold lower than control plants. These results suggest that in the plants that are incubated at low temperatures for more than 5 h there may be a decrease in the transcription of N gene. Hence, lower temperatures should be hindering the normal N gene derived resistance response, as the high temperatures do. Expression of reporter genes driven by 35S promoter do not change after three days of 4°C treatment, while, reporter genes driven by cold inducible promoters show increase in there accumulation (Gu *et al.*, 2006; Shimamura *et al.*, 2006). Hence, it becomes clear that the change in the LUC expression in N::LUC plant is due to N promoter function and not due to change in plant metabolism due to cold stress. It should be analyzed whether plants grown at 4°C retain the ability to resist TMV infection.

As revealed by the quantification assay, external application of SA does not interfere with the LUC activity. It can be suggested that there is no influence of SA on the N gene promoter region. Report from Levy *et al.* (2004) indicates an increase in N gene transcription after 72 hours, in inoculated as well as non-inoculated leaves. This increase in transcription may be due to influence of SA accumulation. However, the result suggests that there is no positive feedback mechanism for regulating *R* genes activity by SA accumulation.

3.6 Conclusion

The function of the N gene promoter region is developmentally regulated. The results suggest that the N protein might not be present in leaf lamella during early growth phases. Also, the roots may lack the N protein through out the growth phase. In such a scenario N protein mediated resistance response might be absent from the lamella of young plants and the roots of plants of different ages. Experiments with inoculation of TMV in these tissues might reveal validity of this postulation. Infection with TMV leads to the immediate increase in the activity of LUC. This induction may be important for development of resistance. The role of this induction and mechanism responsible for it still need to be revealed. High as well as low temperatures can influence the function of N gene promoter, whereby these temperature extremes can hinder its induction. The results suggest that there is no influence of SA on N gene promoter region. Hence, it can be concluded that the efficiency of the R genes might be subjective to various biotic and abiotic factors. The recognition of these influences may aid in understanding host pathogen interactions and development of pathogen resistant plants.

3.7 References

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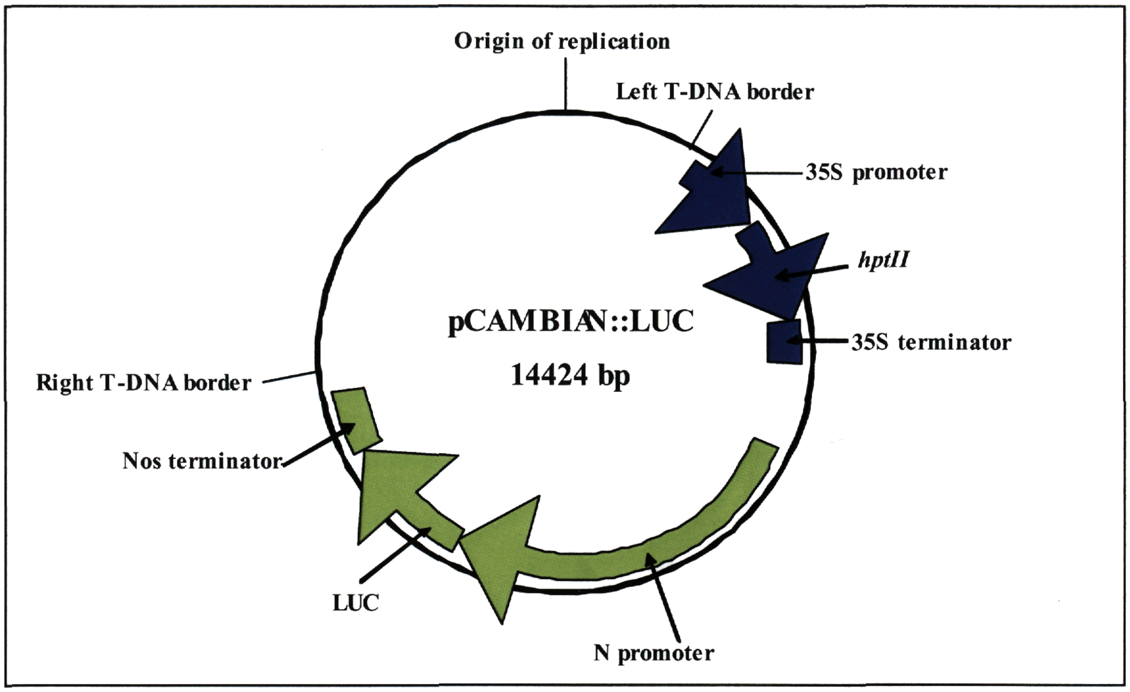


Fig 3.1 Drawing of pCAMBIA N::LUC vector

Similar to the N::GUS vector, the pCAMBIA N::LUC vector consisted of a hygromycin resistance gene as a selection marker near the left T-DNA border. The LUC cDNA was followed by a Nos terminator sequence and the *hptII* gene was followed by 35S terminator sequence.

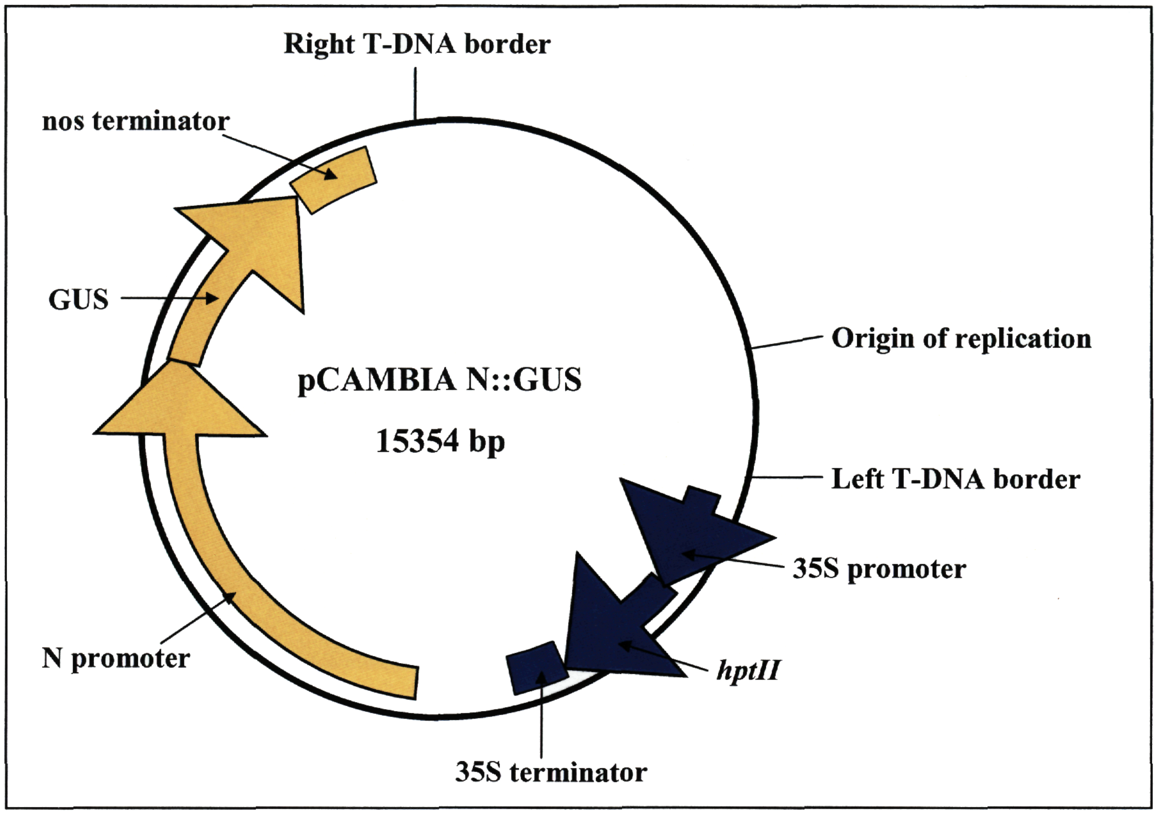


Fig 3.2 Drawing of pCAMBIA N::GUS vector

The vector consisted of the hygromycin resistance gene (*hptII*) as a selection marker. It was near the left border of T-DNA. The 35S promoter was present at the 5' end and the 35S terminator sequence was present at the 3' end of *hptII*. The GUS cDNA was under the control of N promoter at 5' end and the Nos terminator was located at its 3' end.

Fig 3.3 Regeneration of transformed tissues from explants

The leaf of tobacco placed on callus inducing medium after incubation with *Agrobacterium tumefaciens* for 48 h. The medium consisted of 0.8 mg/L IAA, 2.0 mg/L kinetin in MS medium with 30 mg/L hygromycin and 200 mg/L carbenicillin. This regeneration will result in the formation of a callus from the transformed tissue.

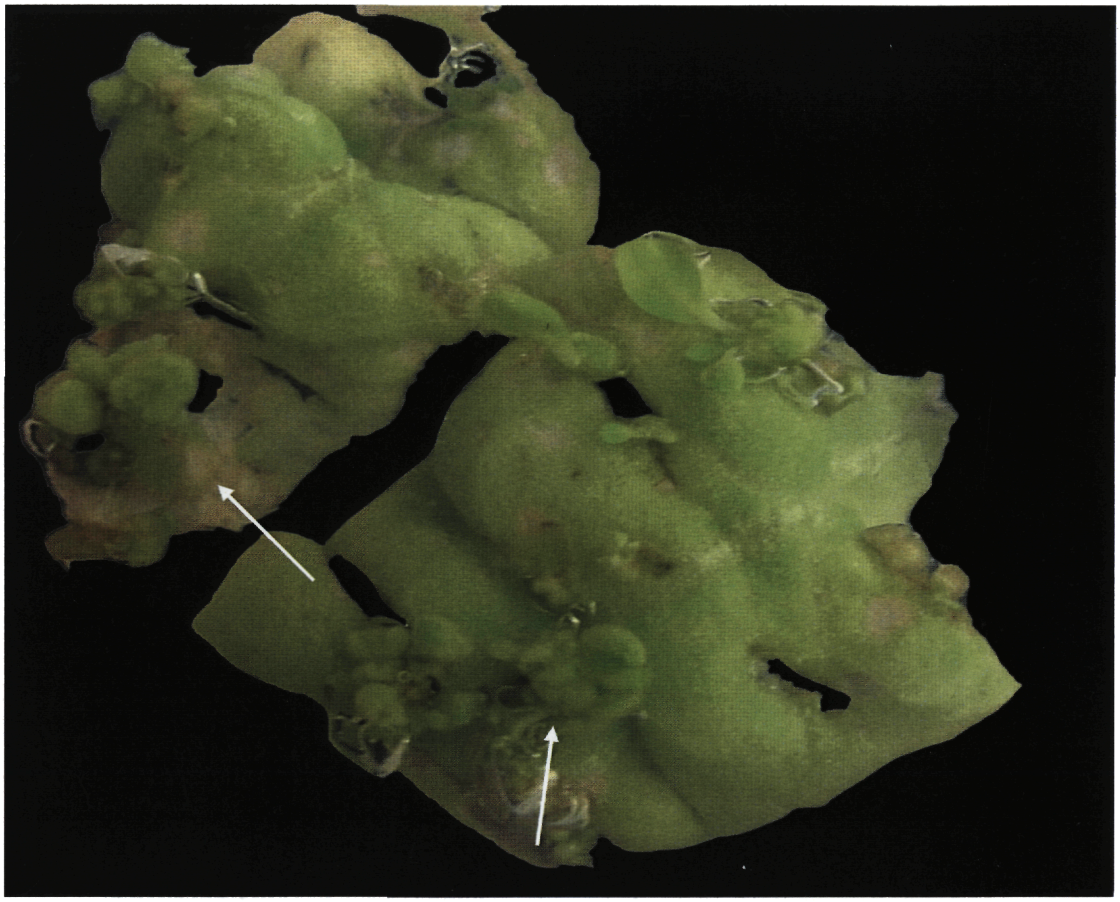


Fig 3.4 Induction of shoot from callus

This figure shows shoot induction from the leaf incubated on callus regeneration medium. These shoots originated from the transformed cells as they were the only ones to be resistant to the herbicide.

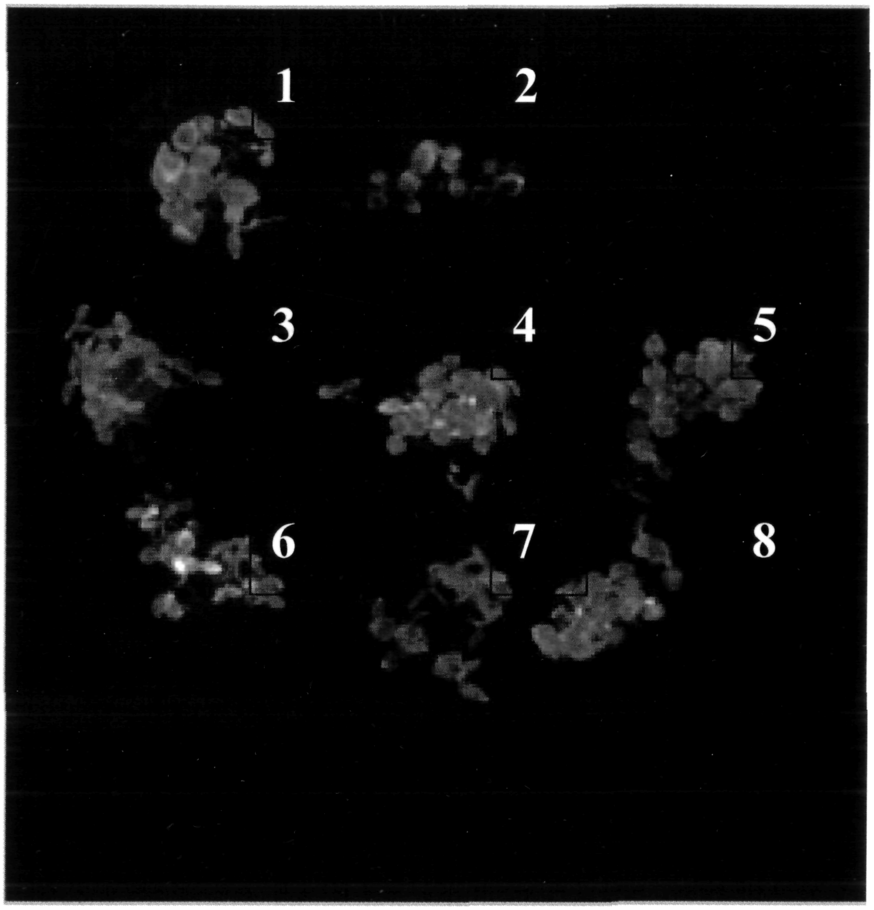


Fig 3.5 Screening of transformed plants on the basis of LUC expression

This CCD image shows different level of luciferase expression from different tobacco plant lines. 1) SLUC P5-4 2) SLUC P9-5 3) SLUC P6-3 4) SLUC Pn3-6 5) SLUC Pn6-5 6) SLUC P2-5 7) SLUC P3-4 8) SLUC Pn1-5

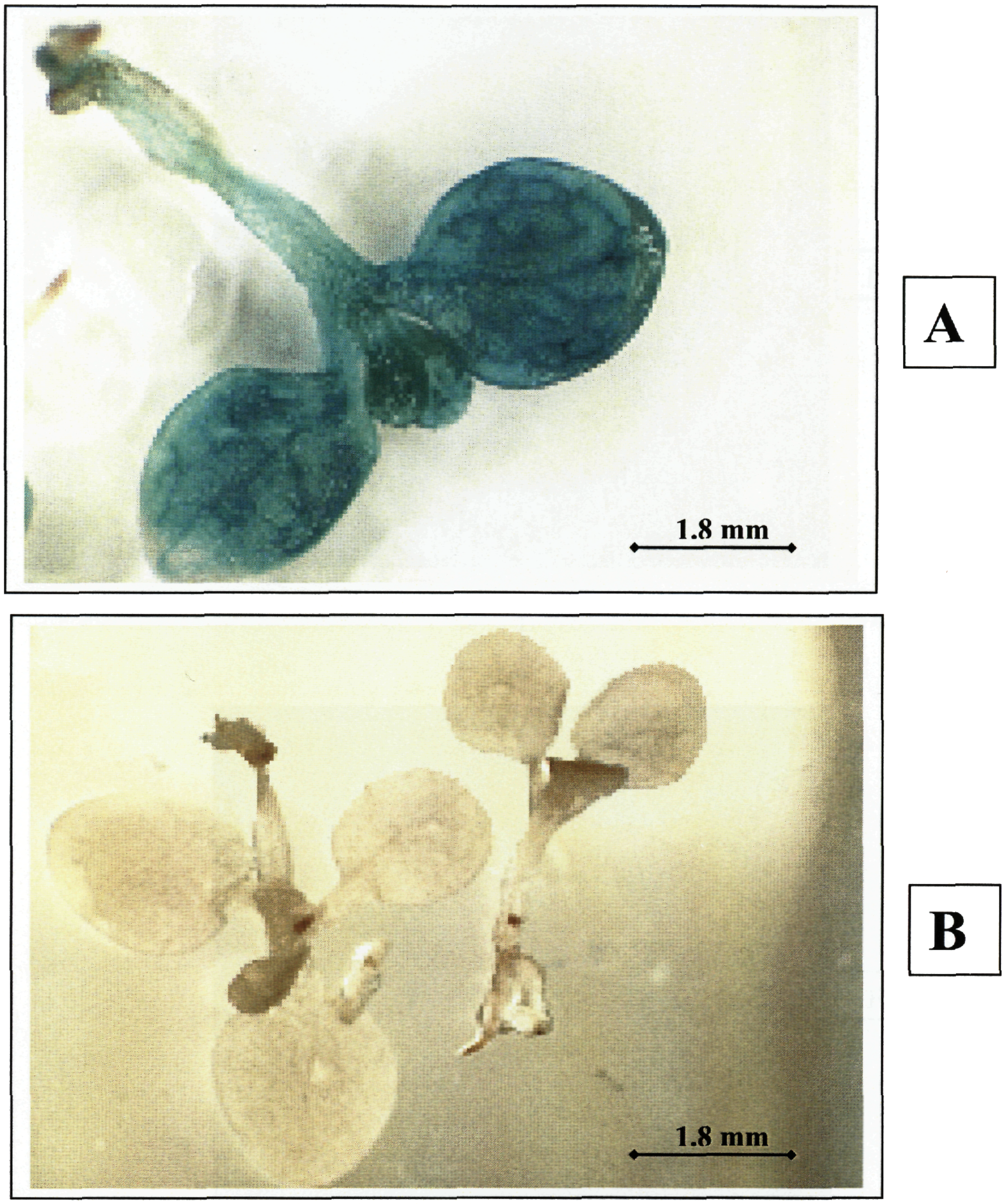


Fig 3.6 Screening of transformed plants by GUS expression

The *Arabidopsis* seedlings represent the differences observed in the expression of the GUS reporter gene. These plants were transformed with the N::GUS construct and have a stable integration of the construct within their genomes. A) Line N::GUS 3 expressing the reporter gene. B) No expression of reporter gene was detected in line N::GUS 24.

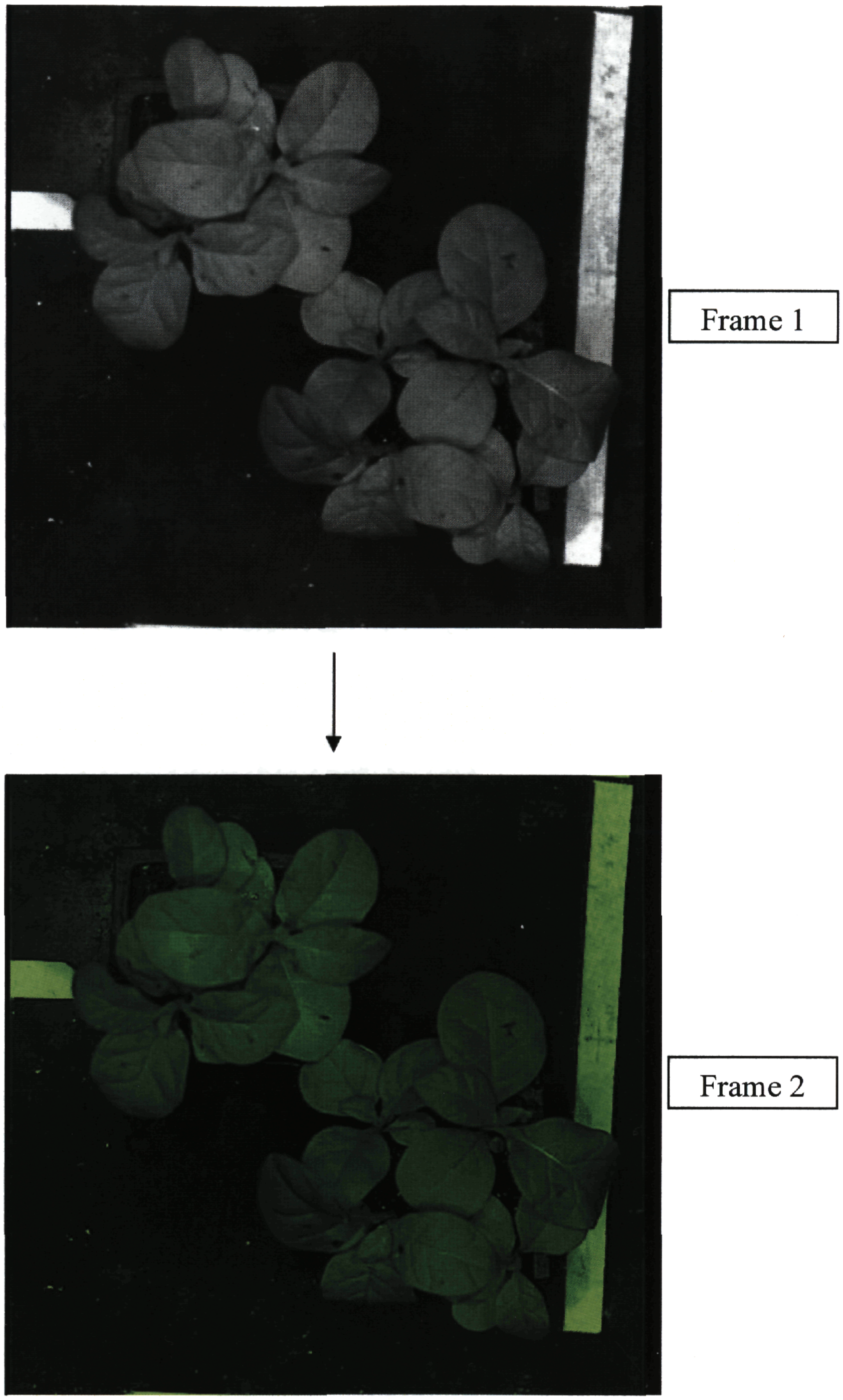


Fig 3.7 Processing of the control image obtained from the CCD camera

A single CCD image of the tobacco plant is taken in the presence of light (frame 1). This frame is given a green color (frame 2). This image is used for localizing the position of the plants.

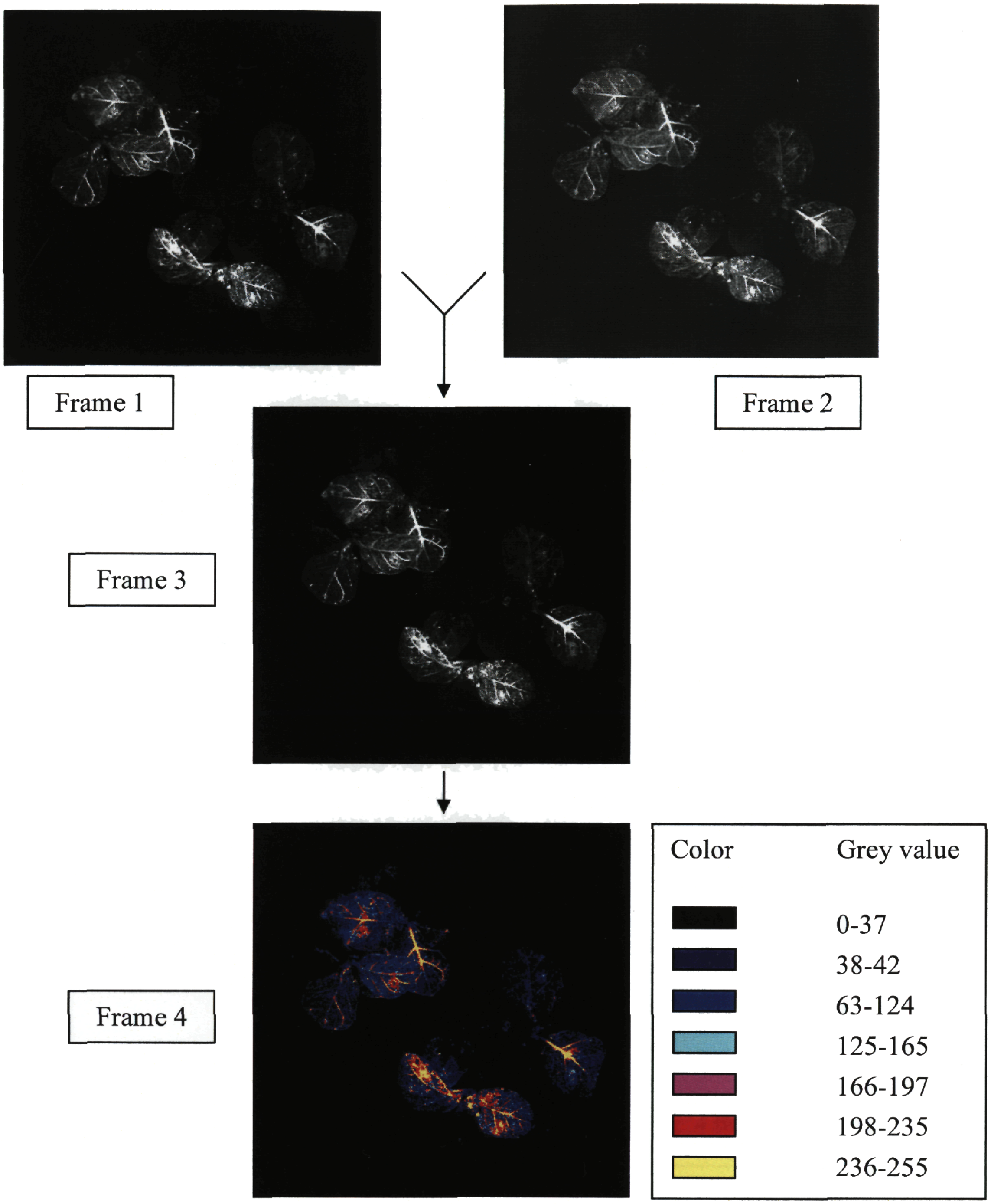


Fig 3.8 Processing of the dark image obtained from the CCD camera

Two frames of tobacco plants are taken in dark (frame 1 and 2). These frames are combined to give common expression area (frame 3). Various colors are allotted to different ranges of intensity of expression (frame 4) as mentioned in the table.

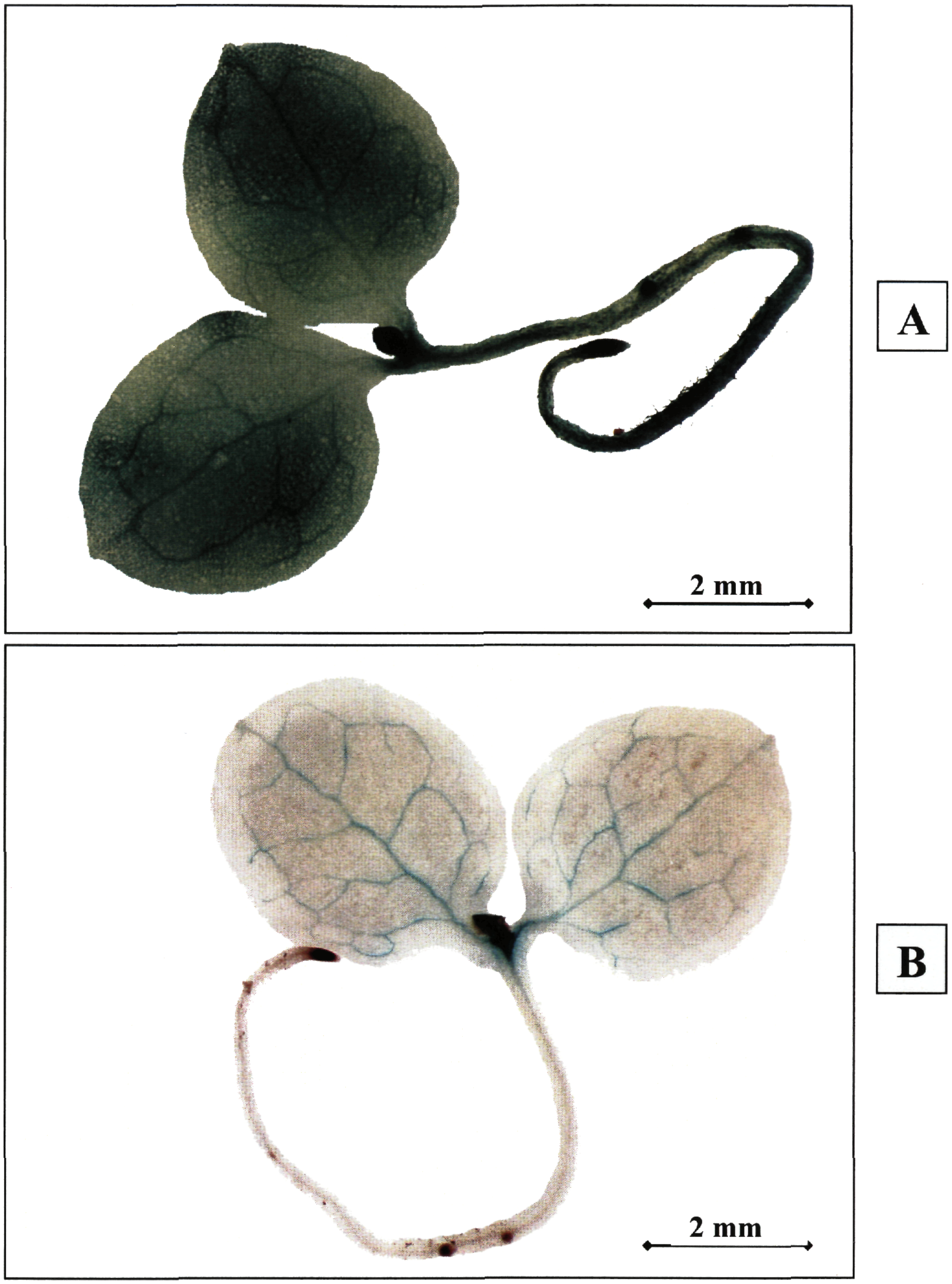


Fig 3.9 Expression of the GUS reporter gene in different tobacco plant organs at 2 weeks after germination

A) 35S::GUS (35SGUS 1-16) tobacco seedling with expression in all tissues, and B) N::GUS (SGUSS12-4) seedling with expression limited to vascular tissue of cotyledons.

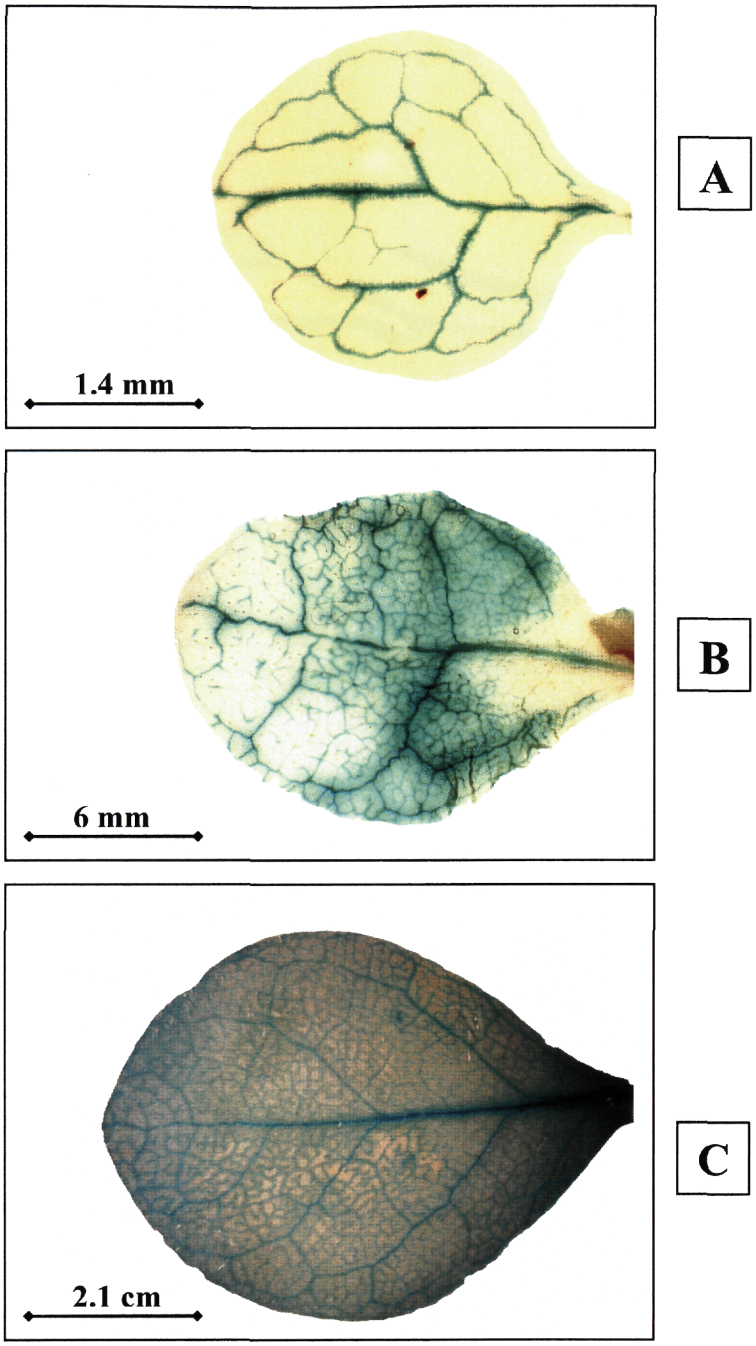


Fig. 3.10 The pattern of N::GUS expression at various developmental stages in the tobacco leaf

A) N::GUS (SGUSS 12-4) expression in 2 week old tobacco cotyledons is limited to the subsidiary and mid veins. B) At 4 weeks of age expression begins to spread in the leaf lamella. C) By 6 weeks, the N::GUS expression is present uniformly throughout the leaf.

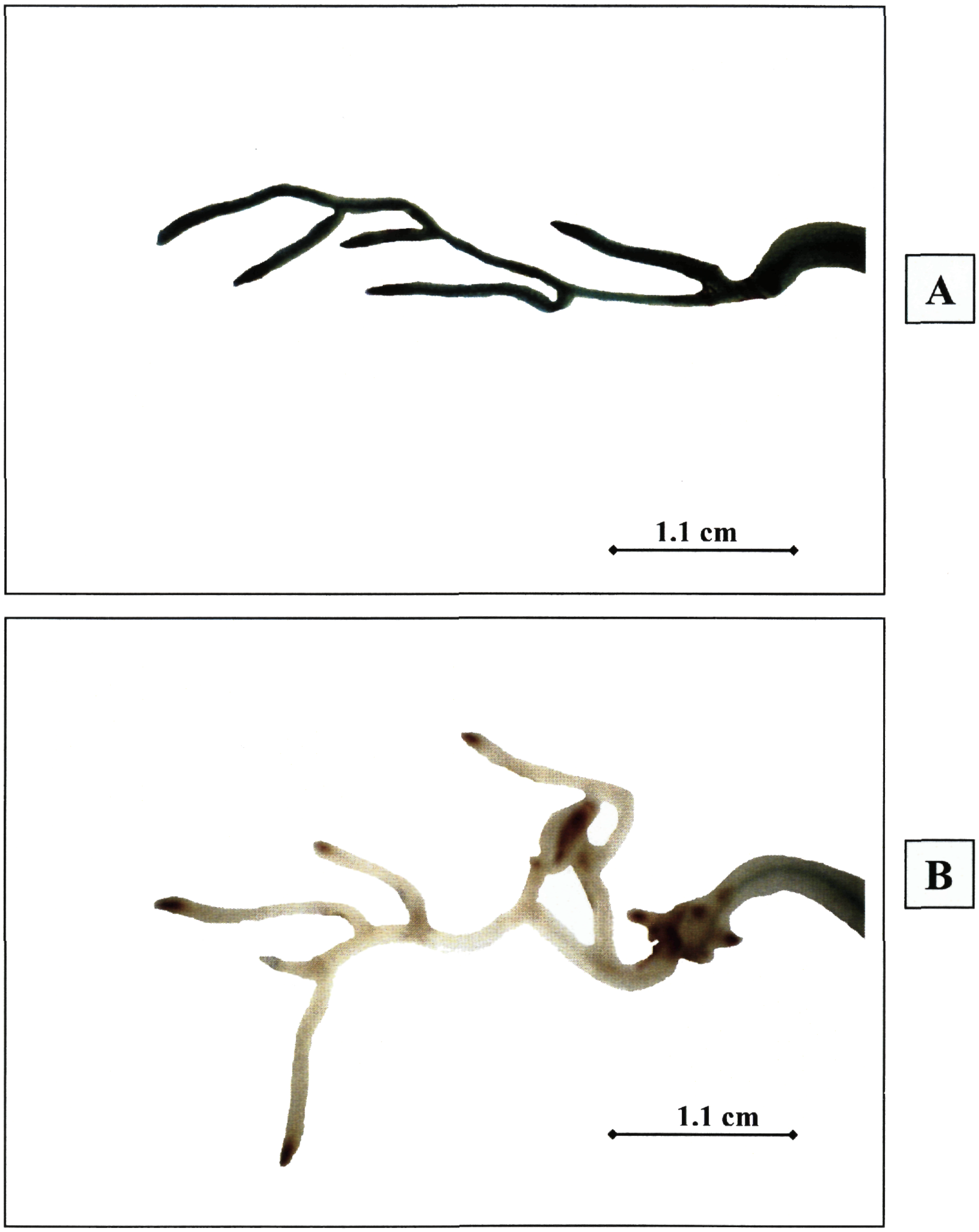


Fig 3.11 Expression of GUS in roots of 4 week old tobacco N::GUS and 35S::GUS plants

A) Roots of 35S::GUS (35SGUS 1-16) plant showing expression throughout the roots. B) No GUS expression was detected in roots of the 4 week old tobacco N::GUS (SGUSS 12-4) plant.

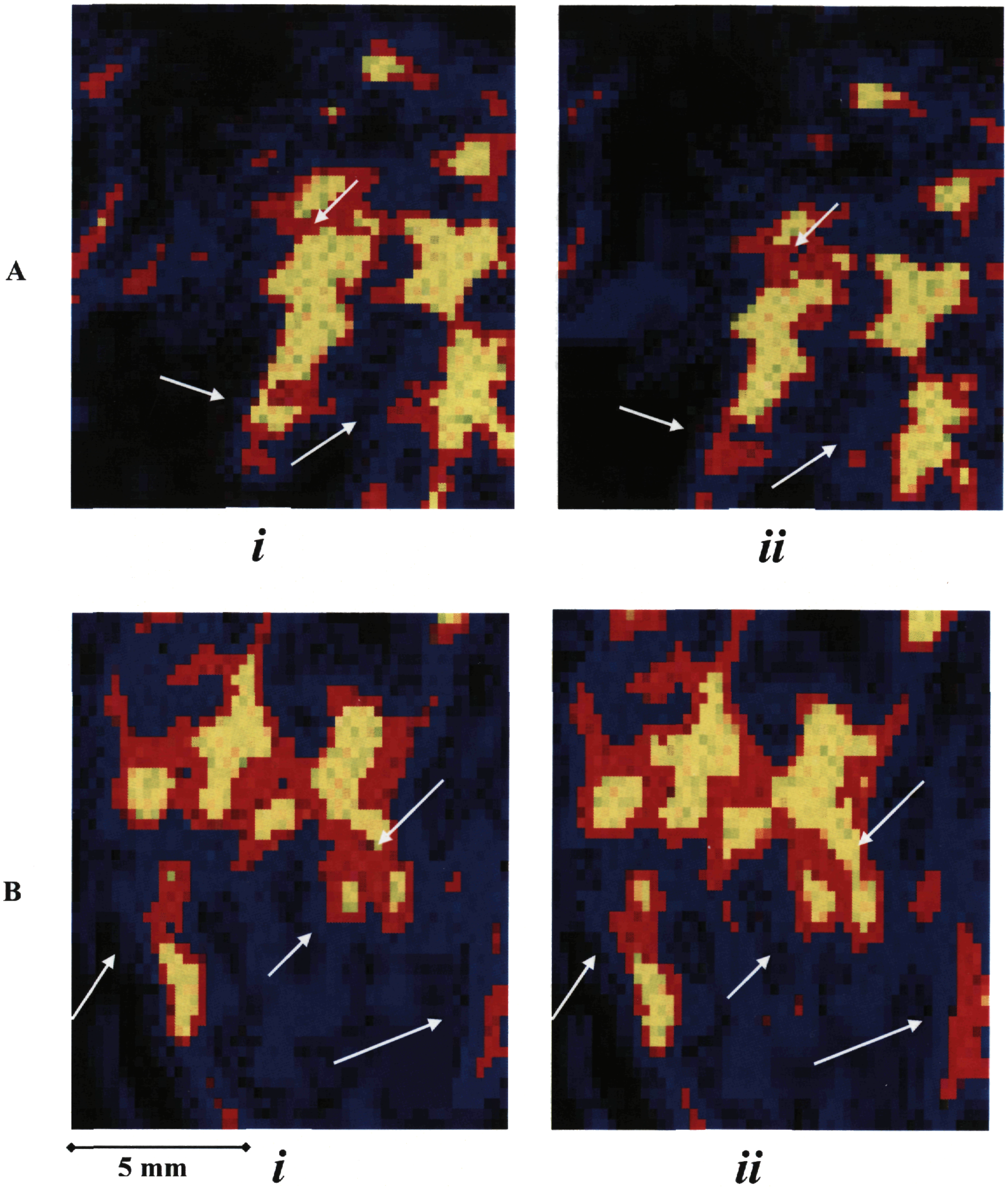


Fig 3.12 CCD images of LUC expression upon virus infection in tobacco N::LUC plants

Decrease in the expression on LUC in buffer treatment at 0 h (A*i*) and at 1 h (A*ii*) time points in tobacco SLUCP 6-1 plants (arrows). Increase in expression at 1 h (B*i*) as compared to 0 h (B*ii*) after infection with TMV (arrows).

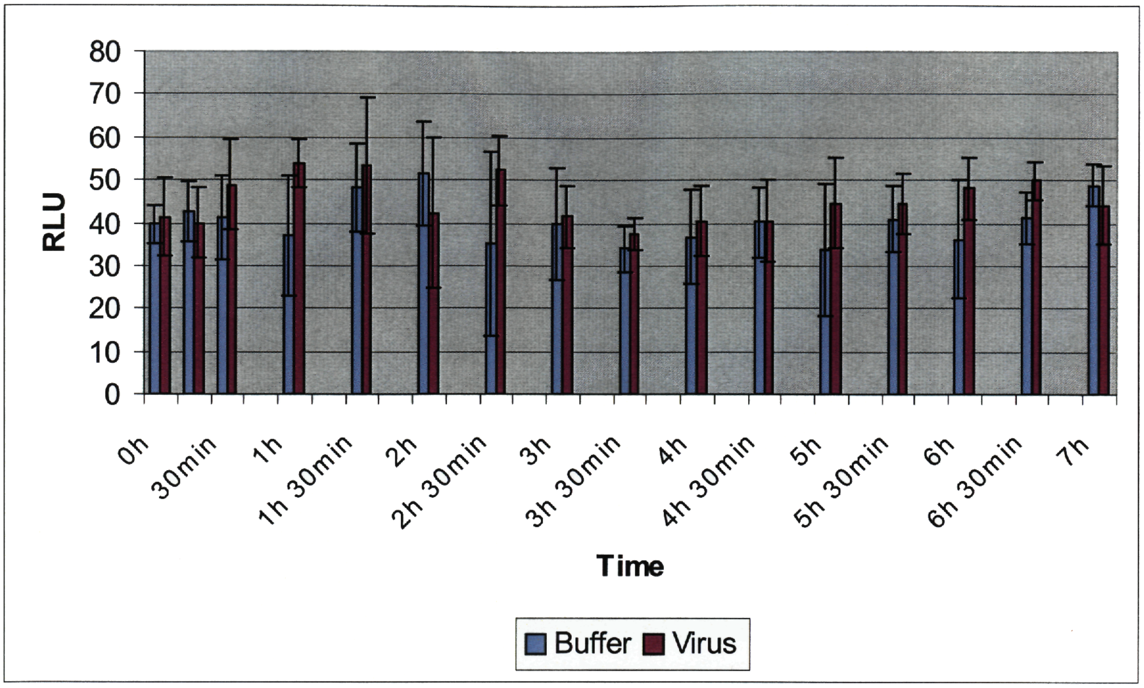


Fig 3.13 The effect of TMV on the activity of LUC in tobacco SLUCP 6-1 plants

Tobacco N::LUC plants were infected with either TMV or mock treated. The graph indicated the specific activity of luciferase in these plants at various time points post infection (X axis) as measured by quantitative assay (RLU in Y axis).

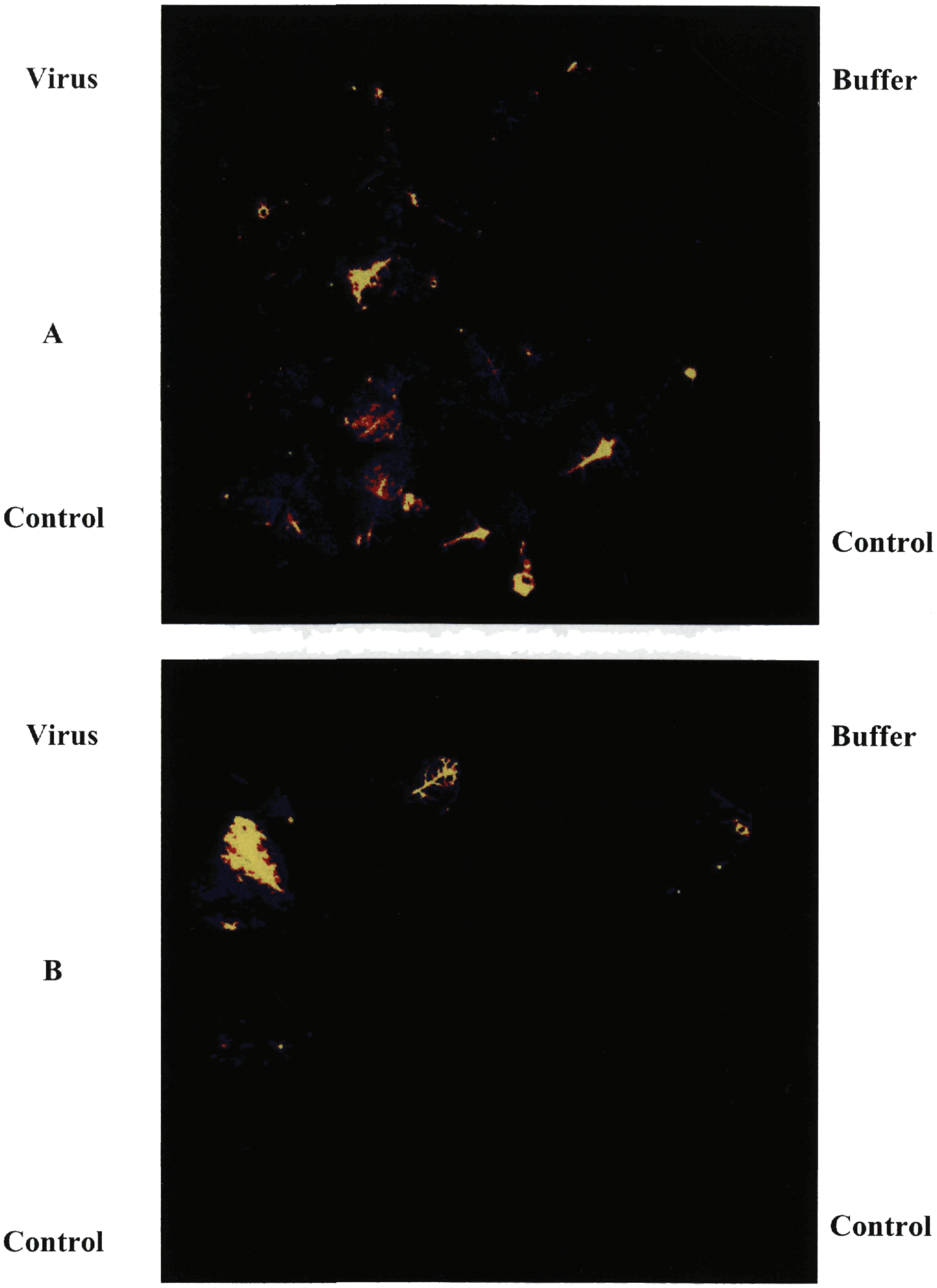


Fig 3.14 CCD image analysis of N::LUC tobacco plants from systemic virus experiment

One tobacco SLUCP 6-1 plant from virus and buffer treatment and two plants from control treatment were analyzed simultaneously. A) Virus (upper left), buffer (upper right) and control plants (bottom) incubated for 1 h at 24°C. B) Plant having systemic virus and incubated for 2 h show high LUC activity compared to other treatment plants.

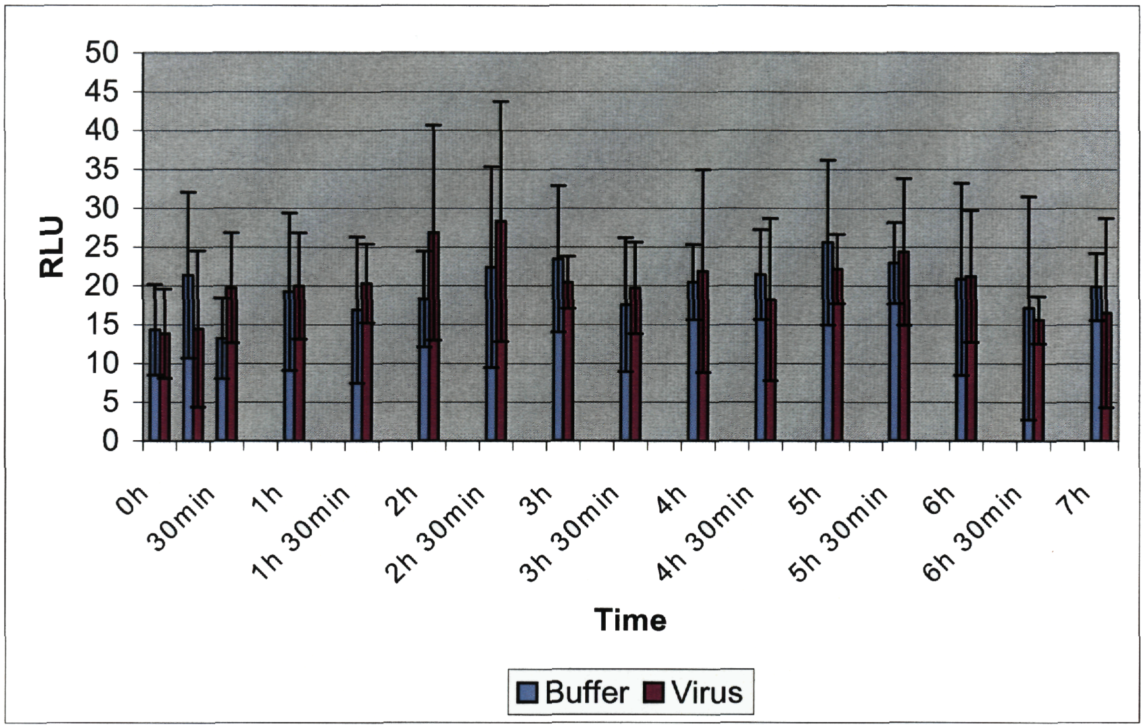


Fig 3.15 Effect of TMV and temperature on LUC activity in tobacco SLUCP 6-1 plants

N::LUC tobacco plants were incubated at 32°C. They were treated with TMV or buffer. These plants were allowed to produce virus symptoms and then shifted back to 24°C temperature. Following this shift, tissues were collected and specific activity was measure from four plants at each time point. The graph shows the average of the values from four plants at each time point post infection (X axis) as measured by quantitative assay (RLU in Y axis).

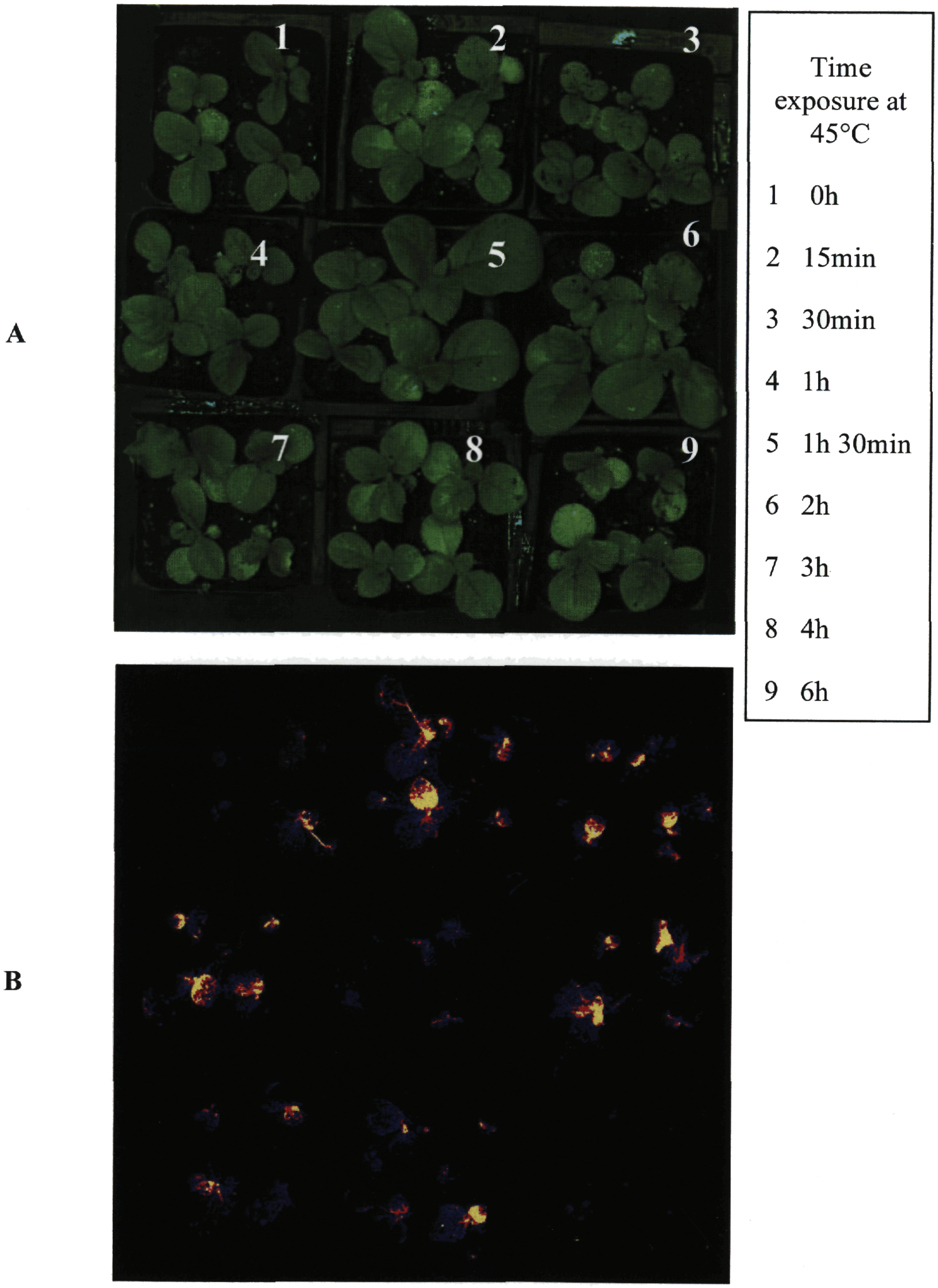


Fig 3.16 CCD images of N::LUC tobacco plants from the high temperature experiment

Four tobacco SLUCP 6-1 plants at each time point were analyzed after incubation at 45°C. A) Control image showing the placement of treated plants. B) Dark image showing the difference in expression level.

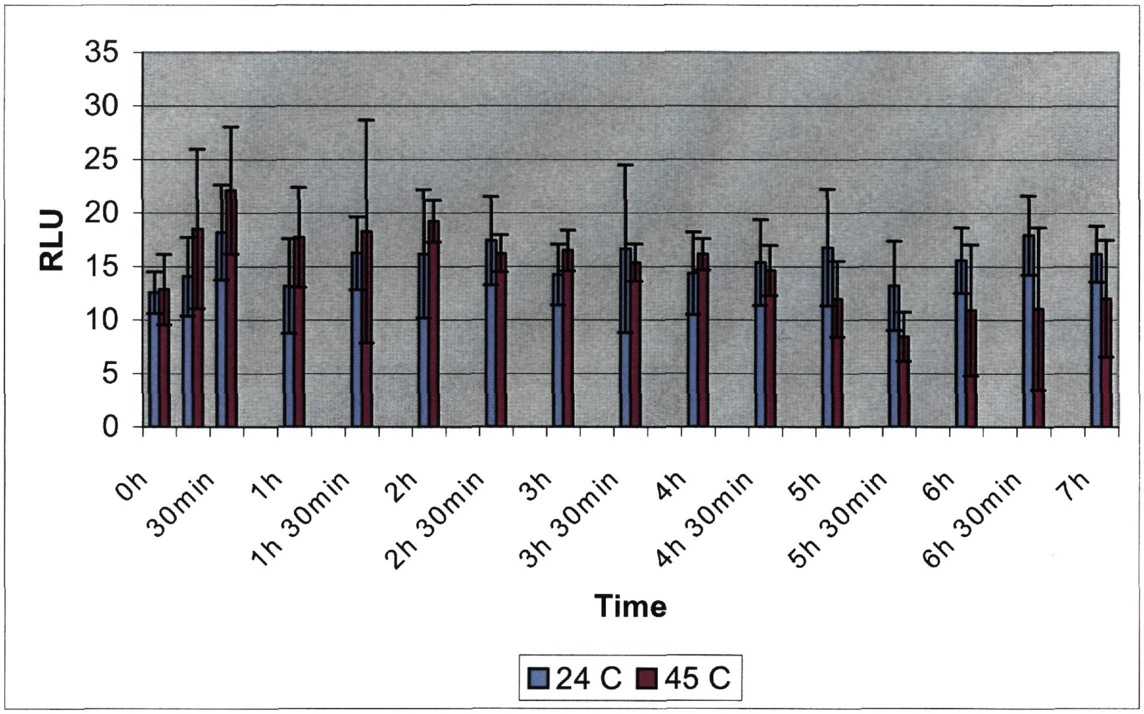
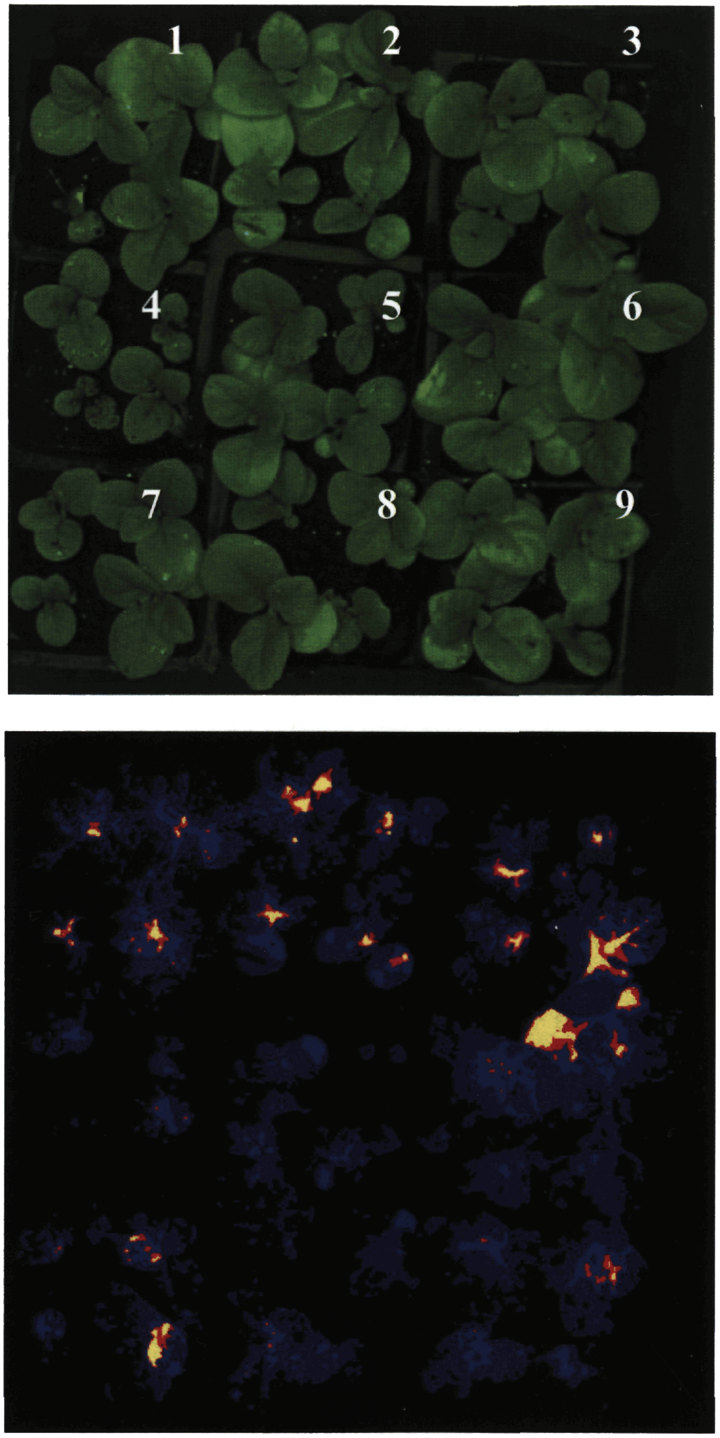


Fig 3.17 The effect of high temperature on the LUC activity in tobacco SLUCP 6-1 plants

N::LUC tobacco plants were either incubated at 24°C or 45°C. At different time points tissue was collected from the plants to study the reporter gene activity. The graph shows the average of the values from four plants at each time point post treatment (X axis) as measured by quantification assay (RLU in Y axis).



Time exposure at 4°C	
1	0h
2	15min
3	30min
4	1h
5	1h 30min
6	2h
7	3h
8	4h
9	6h

Fig 3.18 CCD images of N::LUC tobacco plants from the low temperature experiment

Four SLUCP 6-1 plants were analyzed for each time point. A) Control image shows the placement of treated plants. B) Differences in expression level in the same plants in the dark image.

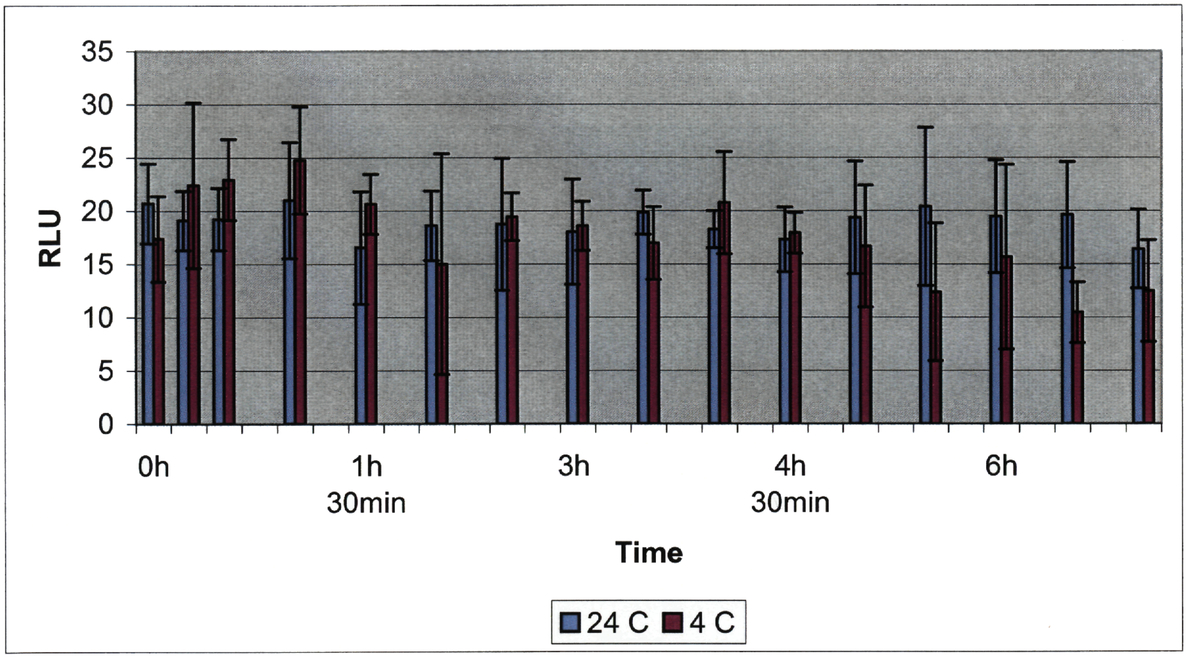


Fig 3.19 The effect of low temperature on the LUC activity in tobacco SLUCP 6-1 plants

Four tobacco N::LUC plants were incubated at either 24°C or 4°C. Luciferase activity was measured from the tissues of these plants. The graph shows the average of the values from four plants at each time point post treatment (X axis) as measured by quantification assay (RLU in Y axis).

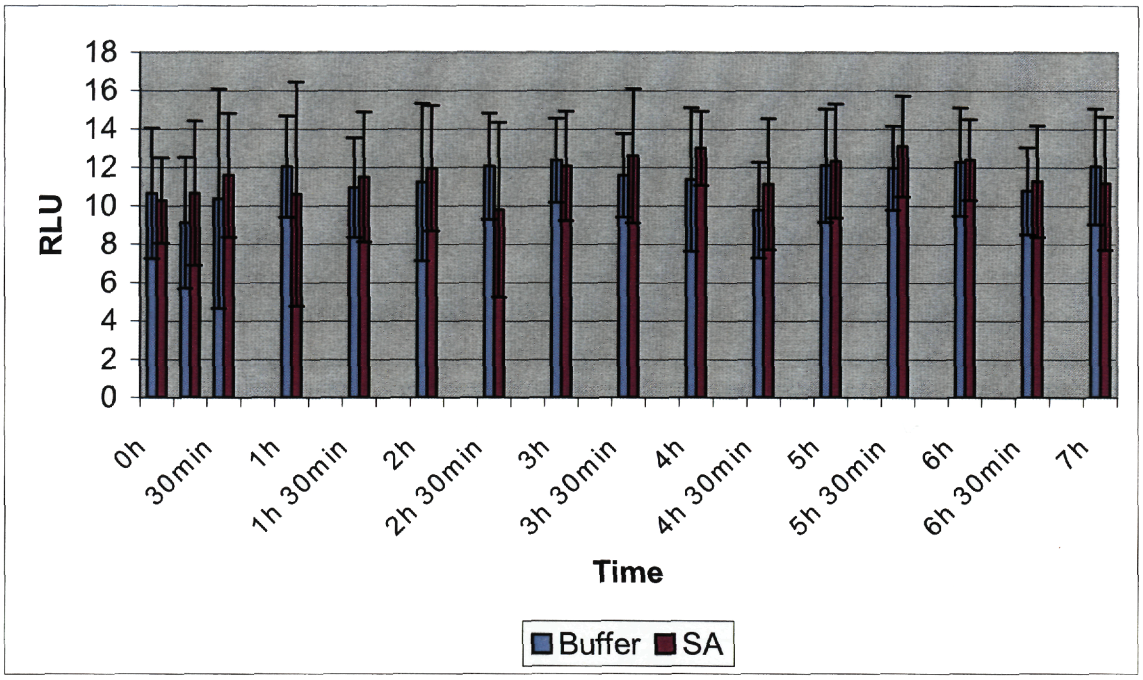


Fig 3.20 The effect of salicylic acid on the LUC activity in tobacco SLUCP 6-1 plants

Either SA or buffer was exogenously applied on the N::LUC tobacco plants. At each time point tissues were collected from four plants. The graph shows the average of the values from four plants at each time point post treatment (X axis) as measured by quantification assay (RLU in Y axis).

Table 3.1 The screening of single T-DNA insertion in *A. thaliana* based on segregation ratio

In T_2 generation, Arabidopsis plants were analyzed for the number of loci of T-DNA insertion. The table shows the Chi^2 result ($p<0.05$, df 1) for segregation ratio of resistant (R) and sensitive (S) plants grown in the presence of 30 mg/L hygromycin in MS media. It also indicated the lines that were selected to have single locus T-DNA insertion.

Line		**Observed R**	**Observed S**	**Ratio (R/S)**	**Expected R**	**Expected S**	**Chi^2**	**Single locus selection**
N-GUS	**N01**	105	19	5.53	93	31	6.19	**No**
N-GUS	**N02**	83	33	2.52	87	29	0.74	**Yes**
N-GUS	**N04**	115	42	2.74	118	39	0.26	**Yes**
N-GUS	**N09**	31	9	3.44	30	10	0.13	**Yes**
N-GUS	**N14**	138	22	6.27	120	40	10.80	**No**
N-GUS	**N18**	63	18	3.50	61	20	0.33	**Yes**
N-GUS	**N22**	87	33	2.64	90	30	0.40	**Yes**
N-GUS	**N23**	59	32	1.84	68	23	5.01	**No**
N-GUS	**N29**	110	25	4.40	101	34	3.02	**No**
N-GUS	**N32**	83	57	1.46	105	35	18.44	**No**
N-GUS	**N34**	141	49	2.88	143	48	0.06	**Yes**
N-GUS	**N40**	127	41	3.10	126	42	0.03	**Yes**
N-GUS	**N41**	116	73	1.59	142	47	18.71	**No**
N-GUS	**N43**	125	55	2.27	135	45	2.96	**Yes**
N-GUS	**N45**	135	55	2.45	143	48	1.58	**Yes**
N-GUS	**N48**	80	10	8.00	68	23	9.26	**No**
N-GUS	**N50**	148	76	1.95	168	56	9.52	**No**
N-GUS	**N51**	110	6	18.33	87	29	24.32	**No**
N-GUS	**N52**	53	17	3.12	53	18	0.02	**Yes**
N-GUS	**N53**	98	37	2.65	101	34	0.42	**Yes**
N-GUS	**N54**	143	41	3.49	138	46	0.72	**Yes**
N-GUS	**N55**	117	10	11.70	95	32	19.87	**No**
N-GUS	**N56**	124	39	3.18	122	41	0.10	**Yes**
N-GUS	**N57**	110	49	2.24	119	40	2.87	**Yes**
N-GUS	**N59**	79	39	2.03	89	30	4.08	**No**
N-GUS	**N60**	137	13	10.54	113	38	21.34	**No**
N-GUS	**N61**	127	50	2.54	133	44	1.00	**Yes**
N-GUS	**N73**	192	47	4.09	179	60	3.63	**No**
N-GUS	**N79**	143	53	2.70	147	49	0.44	**Yes**

Line		Observed R	Observed S	Ratio (R/S)	Expected R	Expected S	Chi^2	Single locus selection
N-GUS	**N80**	114	31	3.68	109	36	1.01	**Yes**
N-GUS	**N81**	120	36	3.33	117	39	0.31	**Yes**
N-GUS	**N82**	140	47	2.98	140	47	0.00	**Yes**
N-GUS	**N83**	95	39	2.44	101	34	1.20	**Yes**
N-GUS	**N84**	154	11	14.00	124	41	29.58	**No**
N-GUS	**N86**	144	31	4.65	131	44	4.95	**No**
N-GUS	**N91**	222	80	2.78	227	76	0.36	**Yes**
N-GUS	**N92**	199	20	9.95	164	55	29.41	**No**
N-GUS	**N94**	103	50	2.06	115	38	4.81	**No**
N-GUS	**P01**	165	60	2.75	169	56	0.33	**Yes**
N-GUS	**P02**	165	23	7.17	141	47	16.34	**No**
N-GUS	**P03**	180	26	6.92	155	52	16.83	**No**
N-GUS	**P04**	133	1	133.0	101	34	42.04	**No**
N-GUS	**P05**	171	47	3.64	164	55	1.38	**Yes**
N-GUS	**P06**	176	53	3.32	172	57	0.42	**Yes**
N-GUS	**P07**	101	25	4.04	95	32	1.79	**Yes**
N-GUS	**P08**	43	24	1.79	50	17	4.18	**No**
N-GUS	**P09**	165	43	3.84	156	52	2.08	**Yes**
N-GUS	**P10**	105	19	5.53	93	31	6.19	**No**
N-GUS	**P11**	108	22	4.91	98	33	4.52	**No**
N-GUS	**P12**	78	22	3.55	75	25	0.48	**Yes**
N-GUS	**P14**	81	25	3.24	80	27	0.11	**Yes**
N-GUS	**P16**	108	39	2.77	110	37	0.18	**Yes**
N-GUS	**S01**	136	30	4.53	125	42	4.25	**No**
N-GUS	**S02**	92	23	4.00	86	29	1.53	**Yes**
N-GUS	**S03**	144	69	2.09	160	53	6.21	**No**
N-GUS	**S06**	386	72	5.36	344	115	21.03	**No**
N-GUS	**S08**	146	56	2.61	152	51	0.80	**Yes**
N-GUS	**S11**	118	37	3.19	116	39	0.11	**Yes**
N-GUS	**S12**	150	78	1.92	171	57	10.32	**No**
N-GUS	**S15**	66	23	2.87	67	22	0.03	**Yes**
N-GUS	**S16**	124	43	2.88	125	42	0.05	**Yes**
N-GUS	**S17**	99	25	3.96	93	31	1.55	**Yes**
N-GUS	**S18**	170	74	2.30	183	61	3.69	**Yes**
N-GUS	**S19**	66	31	2.13	73	24	2.51	**No**
N-GUS	**S20**	38	5	7.60	32	11	4.10	**No**
N-GUS	**S21**	319	111	2.87	323	108	0.15	**Yes**
N-GUS	**S23**	304	136	2.24	330	110	8.19	**No**
N-GUS	**S24**	762	180	4.23	707	236	17.44	**No**

Line		**Observed R**	**Observed S**	**Ratio (R/S)**	**Expected R**	**Expected S**	**Chi^2**	**Single locus selection**
N-GUS	**S26**	84	32	2.63	87	29	0.41	**Yes**
N-GUS	**S27**	128	27	4.74	116	39	4.75	**No**
N-GUS	**S28**	49	19	2.58	51	17	0.31	**Yes**
N-GUS	**S29**	62	27	2.30	67	22	1.35	**Yes**
N-GUS	**S30**	238	86	2.77	243	81	0.41	**Yes**
N-GUS	**S31**	243	120	2.03	272	91	12.57	**No**
N-GUS	**S32**	176	52	3.38	171	57	0.58	**Yes**
N-Luc	**N01**	131	31	4.23	122	41	2.97	**No**
N-Luc	**N04**	151	32	4.72	137	46	5.51	**No**
N-Luc	**N05**	126	20	6.30	110	37	9.95	**No**
N-Luc	**N06**	86	34	2.53	90	30	0.71	**Yes**
N-Luc	**N08**	123	37	3.32	120	40	0.30	**Yes**
N-LUC	**N09**	97	24	4.04	91	30	1.72	**Yes**
N-Luc	**N10**	128	52	2.46	135	45	1.45	**Yes**
N-Luc	**P02**	33	16	2.06	37	12	1.53	**Yes**
N-Luc	**P06**	106	42	2.52	111	37	0.90	**Yes**
N-Luc	**P07**	19	36	0.53	41	14	48.01	**No**
N-Luc	**P10**	84	9	9.33	70	23	11.65	**No**
N-Luc	**P13**	12	62	0.19	56	19	136.38	**No**
N-Luc	**P17**	29	11	2.64	30	10	0.13	**Yes**
N-Luc	**P18**	149	8	18.63	118	39	33.17	**No**
N-Luc	**P19**	79	34	2.32	85	28	1.56	**Yes**
N-Luc	**P20**	69	24	2.88	70	23	0.03	**Yes**
N-Luc	**P22**	85	44	1.93	97	32	5.71	**No**
N-Luc	**P26**	80	35	2.29	86	29	1.81	**Yes**
N-Luc	**P28**	158	57	2.77	161	54	0.26	**Yes**
N-Luc	**S07**	57	30	1.90	65	22	4.17	**No**
N-Luc	**S14**	51	17	3.00	51	17	0.00	**Yes**
N-Luc	**S15**	115	34	3.38	112	37	0.38	**Yes**
N-Luc	**S17**	94	39	2.41	100	33	1.33	**Yes**
N-Luc	**S18**	119	51	2.33	128	43	2.27	**Yes**
N-Luc	**S19**	90	33	2.73	92	31	0.22	**Yes**
N-Luc	**S20**	137	73	1.88	158	53	10.67	**No**
N-Luc	**S22**	74	66	1.12	105	35	36.61	**No**

Table 3.2 The screening of tobacco N::LUC lines for homozygosity and expression

Tobacco N::LUC plants were tested for homozygosity in T_3 generation by single plants progeny testing. If they show 100% resistance, it indicated that the plant was homozygous. The table indicated the homozygous plants identified. It also identifies which plants were selected for experiments based on homozygosity and expression.

Line		**Homozygous progenies**	**Expression**	**Final selection**
SLUC	**Pn1**	Pn1-1	Low	No
SLUC	**Pn1**	Pn1-3	Low	No
SLUC	**Pn1**	Pn1-4	Low	No
SLUC	**Pn1**	Pn1-5	Low	No
SLUC	**Pn3**	Pn3-2	High	No
SLUC	**Pn3**	Pn3-6	High	No
SLUC	**Pn3**	Pn3-7	Low	No
SLUC	**Pn6**	Pn6-1	Moderate, inconsistent	No
SLUC	**Pn6**	Pn6-2	Low	No
SLUC	**Pn6**	Pn6-3	High	No
SLUC	**Pn6**	Pn6-4	Low	No
SLUC	**Pn6**	Pn6-5	Low	No
SLUC	**P2**	P2-1	Moderate, consistent	**Yes**
SLUC	**P2**	P2-2	Moderate, inconsistent	No
SLUC	**P2**	P2-3	High	No
SLUC	**P2**	P2-5	Moderate, inconsistent	No
SLUC	**P3**	P3-1	Moderate, inconsistent	No
SLUC	**P3**	P3-4	Low	No
SLUC	**P5**	P5-1	Moderate, consistent	No
SLUC	**P5**	P5-2	Low	No
SLUC	**P5**	P5-3	Moderate, consistent	No
SLUC	**P5**	P5-4	Moderate, consistent	No
SLUC	**P6**	P6-1	Moderate, consistent	**Yes**
SLUC	**P6**	P6-3	Moderate, consistent	No
SLUC	**P9**	P9-5	Low	No

Table 3.3 The effect of TMV on the LUC activity in tobacco SLUCP 6-1 plants

N::LUC tobacco plants were analyzed for the effect of virus and control on the specific activity of luciferase. The table indicated the averages from 4 treated plants at various time points and the difference in LUC activity of virus and control plants.

Time	Buffer (RLU)	Virus (RLU)	Change as compared to buffer		
	Average	Average		% Change	
0h	39.58	41.36	Increase	4.49	
15min	42.50	39.84	Decrease	6.26	
30min	41.25	48.88	Increase	18.48	
1h	36.94	53.93	Increase	45.98	$p<0.06$
1h 30min	48.15	53.41	Increase	10.93	
2h	51.66	42.19	Decrease	18.34	
2h 30min	35.13	52.20	Increase	48.61	
3h	39.73	41.44	Increase	4.30	
3h 30min	34.05	37.56	Increase	10.32	
4h	36.72	40.40	Increase	10.04	
4h 30min	40.08	40.42	Increase	0.84	
5h	33.73	44.58	Increase	32.16	
5h 30min	40.88	44.24	Increase	8.21	
6h	36.20	48.06	Increase	32.77	
6h 30min	41.26	49.93	Increase	21.02	
7h	48.74	44.17	Decrease	9.38	

Table 3.4 The effect of TMV and high temperature on the LUC activity in tobacco SLUCP 6-1 plants

The tobacco N::LUC plants were treated with TMV or buffer, at 32°C. These plants were incubated at 24°C and the activity of the luciferase was analyzed. The table shows the averages of the expression of the plants in each treatment at various time points and the difference in comparison to control.

Time	Buffer (RLU)	Virus (RLU)	Change as compared to buffer		
	Average	Average		% Change	
0h	14.33	13.84	Decrease	3.40	
15min	21.38	14.45	Decrease	32.40	
30min	13.19	19.72	Increase	49.52	
1h	19.22	19.96	Increase	3.88	
1h 30min	16.89	20.32	Increase	20.31	
2h	18.28	26.83	Increase	46.78	
2h 30min	22.39	28.28	Increase	26.32	
3h	23.42	20.42	Decrease	12.81	
3h 30min	17.53	19.69	Increase	12.35	
4h	20.48	21.89	Increase	6.90	
4h 30min	21.43	18.20	Decrease	15.07	
5h	25.59	22.18	Decrease	13.36	
5h 30min	22.93	24.36	Increase	6.26	
6h	20.84	21.20	Increase	1.74	
6h 30min	17.13	15.60	Decrease	8.95	
7h	19.87	16.50	Decrease	16.93	

Table 3.5 The effect of high temperature on LUC activity in tobacco SLUCP 6-1 plants

The tobacco N::LUC plants were incubated at either 24°C or 45°C. The table shows the difference in the averages of expression calculated from 4 plants per time point per treatment.

Time	24°C (RLU)	45°C (RLU)	Change as compared to buffer		
	Average	Average		% Change	
0h	12.55	12.85	Increase	2.35	
15min	14.05	18.50	Increase	31.64	
30min	18.18	22.11	Increase	21.64	
1h	13.16	17.73	Increase	34.79	
1h 30min	16.25	18.28	Increase	12.46	
2h	16.18	19.24	Increase	18.92	
2h 30min	17.39	16.21	Decrease	6.81	
3h	14.23	16.48	Increase	15.85	
3h 30min	16.65	15.36	Decrease	7.78	
4h	14.35	16.12	Increase	12.31	
4h 30min	15.38	14.63	Decrease	4.88	
5h	16.78	11.95	Decrease	28.76	
5h 30min	13.19	8.42	Decrease	36.19	
6h	15.56	10.90	Decrease	29.93	
6h 30min	17.93	11.05	Decrease	38.36	
7h	16.16	12.00	Decrease	25.75	

Table 3.6 The effect of low temperature on the LUC activity in tobacco SLUCP 6-1 plants

Tissue was collected from the tobacco N::LUC plants treated at 24°C and 4°C. The averages of RLU were calculated from 4 plants per time point per treatment.

Time	**24°C (RLU)**	**4°C (RLU)**	**Change as compared to buffer**		
	Average	Average		% Change	
0h	20.68	17.38	Decrease	15.97	
15min	19.08	22.40	Increase	17.37	
30min	19.23	22.93	Increase	19.21	
1h	20.99	24.75	Increase	17.94	
1h 30min	16.53	20.63	Increase	24.81	
2h	18.60	14.99	Decrease	19.42	
2h 30min	18.73	19.44	Increase	3.79	
3h	18.01	18.58	Increase	3.16	
3h 30min	19.83	16.95	Decrease	14.52	
4h	18.25	20.75	Increase	13.69	
4h 30min	17.29	17.92	Increase	3.63	
5h	19.35	16.66	Decrease	13.89	
5h 30min	20.38	12.36	Decrease	39.36	
6h	19.46	15.66	Decrease	19.51	
6h 30min	19.60	10.45	Decrease	46.71	$p<0.01$
7h	16.40	12.46	Decrease	24.04	

Table 3.7 The effect of salicylic acid on the LUC activity in tobacco SLUCP 6-1 plants

Tobacco N::LUC plants were treated with either salicylic acid or buffer. From four plants from each time point, tissues were sampled and the luciferase activity was measured. The table shows the activity at various time points after the treatment and the differences in the control and SA treatment.

Time	**Buffer (RLU)**	**SA (RLU)**	**Change as compared to buffer**		
	Average	Average		% Change	
0h	10.65	10.29	Decrease	3.40	
15min	9.11	10.66	Increase	17.02	
30min	10.37	11.60	Increase	11.91	
1h	12.05	10.61	Decrease	11.91	
1h 30min	10.95	11.51	Increase	5.07	
2h	11.24	11.96	Increase	6.48	
2h 30min	12.07	9.80	Decrease	18.79	
3h	12.37	12.08	Decrease	2.34	
3h 30min	11.59	12.60	Increase	8.76	
4h	11.38	13.00	Increase	14.28	
4h 30min	9.78	11.13	Increase	13.83	
5h	12.10	12.35	Increase	2.02	
5h 30min	11.98	13.12	Increase	9.52	
6h	12.29	12.41	Increase	0.96	
6h 30min	10.78	11.28	Increase	4.66	
7h	12.05	11.18	Decrease	7.22	

4.0 Changes in virus tolerance, genome stability and DNA methylation in the progeny of plants infected with compatible pathogen

4.1 Abstract

Infection with compatible viral pathogen results in generation of systemic recombination signal (SRS) that is capable of spreading faster than the virus and promoting changes in the frequency of somatic and meiotic recombination. This signal can have a role in the induction of various plant mechanisms against pathogen infection. To understand the transgenerational effect of the SRS more clearly, the progeny of pathogen-infected plants were analyzed. These plants showed a significant delay in symptom development and a delay in the progression of the virus in the plants. Methylation analysis revealed that the genomes of these plants were hypermethylated, with a sequence specific pattern of hypomethylation at *R* gene-like loci. The control loci, actin, RENT, and 5.8S ribosomal RNA, were either significantly hypermethylated or not changed. Changes in methylation were coupled with an increased frequency of rearrangements in *R* gene-like loci when compared to control loci. It is hypothesized that the global genome hypermethylation is part of a plant mechanism to protect the genome, while the increased rate of rearrangements at specific loci due to the selective hypomethylation serves a role in increasing sequence variability. Such variation could result in production of novel *R* gene specificities that can recognize pathogens and lead to resistance response. Hence, the SRS can play a key role in the evolution of *R* genes against virulent pathogens and the increased tolerance to pathogens in the next generation.

4.2 Introduction

Day to day, all organisms are faced with a variety of external influences from the environment, and some of these influences are detrimental. These influences are known as stresses (Arnholdt-Schmitt, 2004; Madlung and Comai, 2004). Plants, being sessile organisms, have developed special mechanisms to counteract these stresses (Shinozaki *et al.,* 2003; Sung *et al.,* 2003). Infection with pathogens is one of the stresses that induce many different pathways in plants, including resistance (*R*) gene-mediated resistance (Whitham *et al.,* 1994). Plants are also able to produce mobile signals in response to pathogen invasion, such as in SAR (Dong, 2001) and systemic post-transcriptional RNA silencing (Waterhouse *et al.,* 2001; Mlotswa *et al.,* 2002). It has previously been shown that pathogens alter the genome stability in plants by changing the frequency of homologous recombination (Lutch *et al.,* 2002). They also reported on HR frequency increase upon the infection of a whole plant with fungal pathogen. Previous report by Kovalchuk et al. (2003) also showed the influence of pathogens on HR frequency; local (single leaf) infection of tobacco plants with TVM or oil-rape mosaic virus resulted in systemic increase of somatic and meiotic recombination frequency (Kovalchuk *et al.,* 2003). It was proposed that recombination increase was promoted by generation of a systemic recombination signal (SRS) that could travel faster than the virus to systemic tissue (Kovalchuk *et al.*, 2003). The nature and specificity of this signal still remains enigmatic.

Here, the trasnsgenerational changes in plants that exhibiteded the SRS response were analyzed. For the experiments, 20 individual tobacco (*Nicotiana tabaccum, cv.* SR1) plants were infected with TVM or mock-inoculated. The progeny of the plants that were exposed to the SRS through TMV infection were termed the progeny of infected (PofI), whereas the progeny of mock-infected plants were termed the progeny of control (PofC).

These experiments were aimed at the study of the effect of the SRS on the plant genome and the plant ability to tolerate the compatible pathogen. The fact that the spread of the SRS is systemic raises the question of whether the SRS is able to trigger some sort of plant mechanism that can result in a higher tolerance to the incoming virus. To address this question, the study of the virus titer and the viral symptoms in TMV infected PofI and PofC plants was carried out. The concentration of the virus present in these plants at 6, 9 and 12 days after infection was evaluated.

Reduced genome stability can be a function of the alteration in cytosine methylation status, whereby a decrease in methylation status can lead to an increase in the rate of somatic recombination events (Lowe *et al.,* 1992). If the frequency of rearrangements is increased, it is logical to assume the generation of increased variability in these regions. Hence, it can be hypothesized that the creation of higher variability in a gene that is involved in pathogen resistance can result in novel phenotype in plants which might better resist pathogens. Upon interaction with pathogens, such a plant will have better chances to survive. Plant resistance (*R*) proteins mediate active resistance to pathogen infection. They possess specific recognition receptors for *Avr* factors from each particular pathogen. Creation of higher variation by recombination in these genes may lead to the formation of new recognition specificity to a virulent pathogen (Ritcher *et al.,* 1995; Tornero *et al.,* 2002).

It can be hypothesized that the SRS might be able to trigger rearrangements in pathogen-related genes, like *R* genes, which can result in the formation of novel *R* genes, which can recognize and resist virulent pathogens.

To analyze this hypothesis, an initial examination of the global genome methylation status of PofI and PofC plants was done. Secondly, investigation of sequence specific changes in methylation status was carried out. For these experiments the methylation status of *R* gene-like loci, actin, repetitive elements in *Nicotiana tabacum* (RENT), and 5.8S ribosomal RNA loci were compared.

4.3 Materials and Methods

4.3.1 Generation of transgenic tobacco plants carrying a homologous recombination substrate

The grafting procedure on the plants carrying recombination substrate is mentioned previously (Kovalchuk *et al.,* 2003). Seeds from the newly emerged tissue of SR1 plants (line LU#1) grafted with leaves derived from the pathogen (TMV) infected plants and mock-treated plants were used for the experiments (Fig. 4.1A). 21 lines of Progeny of infected were named PofI and 20 lines of progeny of control were named PofC.

4.3.2 Visualization of luciferase activity

Luciferin was applied to the leaves and plants were incubated in the dark for 1-2 hours. The recombination events in form of spots of luciferase activity were analyzed using the CCD camera (Fig. 4.1B).

4.3.3 Plant inoculation and virus titer analysis

Firstly, single leaves of 7-10 two-month old plants of PofI and PofC lines were infected with 300 ng of TMV RNA. Symptom appearance was monitored and the averages were calculated.

In the second experiment, five-week-old PofI plants (lines 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20; twenty plants for each line, 360 plants total) and PofC plants (lines 1,2,3,4 and 5; five plants from eight different lines, 40 plants in total) were used. Plants were infected with 200 ng of TMV RNA and tissue samples (1.5x1.5 cm, ~200mg) from lower non-inoculated leaves were collected at 6, 9 and 12 days post infection (dpi). Parallel to this, plants were closely monitored for the appearance of virus symptoms. From the collected tissue viral RNA was extracted and virus concentration was measured. In brief, tissue was crushed to powder and homogenized with 400 µL of 0.5M phosphate extraction buffer pH 7.0 (4.1 g Na_2HPO_4, 2.5g NaH_2PO_4, 100µL ß-mercaptoethanol per 100 mL). Samples were purified using equal volumes (400 µL) of chloroform and 1-butanol. Virions were precipitated using separate volumes of 50 µL of 40% polyethyleneglycol 6000 and 10% NaCl solution. The pellet was resuspended in a 1:50 dilution of phosphate buffer (10 mM sodium phosphate buffer, pH 7.0) and viral concentrations were quantified. The concentration of virus was calculated in mg/ml of virus particles (Register and beachy, 1988)

4.3.4 DNA extraction

DNA extraction was carried out from whole plants using the Nucleon PhytoPure total DNA isolation kit (Amersham Life Science) following the manufacturer's protocol.

4.3.5 RFLP of N gene-like resistance gene, actin, RENT and 5.8S loci

Restriction fragment length polymorphism (RFLP) analysis for N gene-like loci was performed on 149 PofI plants and 147 PofC plants, stemming from 21 PofI and 20 PofC lines, respectively. Five µg of genomic DNA was digested with either *Hin*dIII or *Eco*RI. Hybridization was carried out with a probe derived from the 4th exon of the N gene. The frequency of rearrangements in the *R* gene loci was calculated according to the formula, $f_r = n_r/p \times 30$. Where f_r is the frequency of rearrangements; n_r is the number of rearranged loci; p is the number of plant screened (149 and 147); and 30 is the number of loci that carry homology to N-gene.

The RFLP analysis of the actin loci was performed on 491 PofI plants and 479 PofC plants by digestion of genomic DNA with the *Hin*dIII and probing restricted DNA with the actin Tob71 probe (298bp; 5' TTGTGTTGGACTCTGGTGATGGTG and 3' AATGGTGATCACCTGCCCATCTGG primers). This region carries significant (>90%) homology to the same region in the other tobacco actin genes such as Tac9, Tob93, Tob103, Tob54, etc.

The RFLP analysis of the RENT loci was performed on 142 PofI plants and 137 PofC plants by digestion with *Hin*dIII or *Eco*RI. The DNA was probed with a 514 bp probe obtained with RENT1 specific primers (forward 5' TCTGATTTATCCGACTCAGATC 3', reverse 5' TTTAGAAGGGACTTAAACAGAG 3'). This region has over 90% homology to the other known

RENT sequences. The RFLP analysis of the 5.8S ribosomal RNA loci was also performed on 142 PofI plants and 137 PofC plants by digestion with *Hin*dIII. Digested DNA was probed with a 370 bp fragment (forward 5' TAAATTGAAAGCCTGCCTCTC 3', reverse 5' ACCACTTGTCGTGACGTCCG 3'). The blots were scanned using HP Scanjet 4850 photo scanner. Fragment intensity was quantified using "Image J" (NIH, www.rsb.info.nih.gov/ij).

4.3.6 Analysis of global genome methylation

Genomic DNA was extracted as mentioned before. Subsequently, the DNA was digested overnight with a 10-fold (U: µg DNA) excess of *Hpa*II according to the manufacturers protocol (New England Biolabs, Beverly, MA). An additional DNA aliquot was incubated without a restriction enzyme and served as a background control. Single nucleotide extension reaction was performed on 2 µg of DNA, 1X Polymerase chain reaction (PCR) buffer II, 1.0 mM $MgCl_2$, 0.25 units of AmpliTaq DNA Polymerase (Perkin Elmer, Foster City, CA), $[^3H]$dCTP (57.4 Ci/mmol) (NEN, Boston, MA) as previously described (Pogribny et al., 1999). The incorporation of $[^3H]$dCTP was shown in dpm/µg of DNA. For the control of *HpaII* activity, similar procedure was followed with *MspI* enzyme. The values are correlated with completeness of the DNA digestion with methylation sensitive enzymes. A higher level of methylation would result in a decrease in digestion with the methylation sensitive *Hpa*II and, thus, a lower incorporation of radioactively labelled cytosine ($[^3H]$-dCTP) and lower values. These values were compared with MspI by taking the ratio of *HpaII* to *MspI* values.

4.3.7 Analysis of methylation of N gene-like and actin loci

Genomic DNA (10 µg) was digested with the methylation sensitive enzyme *Hpa*II and probed with either the 4th exon of the N gene or with the actin Tob71 probe mentioned previously. The intensity of the fragment is inversely correlated with the methylation status at that particular locus. Tissues with hypermethylated loci have less genomic DNA cut in the loci and, thus, will have loci (fragments) with a lower intensity. Hypomethylated loci are restricted more frequently and, thus, will have fragments with a higher intensity. Plants were also analyzed with methylation insensitive enzyme *Msp*I (identical recognition sequence as *Hpa*II). Up to fifteen plants per each group were analyzed. All the data for the N and actin gene was analyzed using the Microcal Origin 6.0. The blots were scanned using HP Scanjet 4850 photo scanner. Fragments intensity was quantified using "Image J" (NIH, www.rsb.info.nih.gov/ij).

4.3.8 Bisulfite treatment

As a pre-requisite for the combined bisulfite restriction analysis (COBRA) assay, the bisulphate treatment of DNA was carried out (Frommer *et al.,* 1992). In brief, 1 µg of DNA was denatured in 50 µL of 0.2 mM NaOH at 37°C for 20 min. In the next step, 30 µl of freshly prepared 10 mM hydroquinone (Sigma) and 520 µL of 3 M sodium bisulfite (Sigma), pH 5 were added. Samples were incubated under a layer of mineral oil at 55°C for 16h. The DNA was purified using the DNA cleanup system (Promega Corporation, Madison, WI, USA) and eluted with 100 µL of water. The DNA was subsequently desulfonated with 0.3 M NaOH at 37°C, followed by an ethanol precipitation. The DNA pellet was resuspended in 1 mM Tris-HCl, pH 8, and further used for PCR.

4.3.9 Combined bisulfite restriction analysis of DNA

The COBRA assay was carried out as mentioned in a previously published protocol (Xiong *et al.*, 1997). Briefly, sodium bisulfite-treated DNA as mentioned previously, was PCR-amplified with the following sets of primers: actin, forward 5'TTGTGTTGGACTCTGGTGATGGTG, reverse 5' AATGGTGATCACCTGCCCATCTGG 3'; RENT, forward 5' TCTGATTTATCCGACTCAGATC 3', reverse 5' TTTAGAAGGGACTTAAACAGAG 3'; 5.8S, forward 5' TAAATTGAAAGCCTGCCTCTC 3', reverse 5' ACCACTTGTCGTGACGTCCG 3'. The expected size of the product was 298 bp, 51 bp, 370 bp, for actin, RENT and 5.8S, respectively. The PCR products were digested with either *Hpy*CH4IV or *Taq*I and separated on a 3% agarose gel. Fragment intensity was quantified using "Image J" (NIH, www.rsb.info.nih.gov/ij).

4.3.10 Statistical treatment of the data

The statistical analysis of the experiments was carried out by performing either a Students t-test (two-tailed paired or non-paired) or a Single factor ANOVA. Statistical analyses were performed using the MS Excel software version 11.8012.6568 and Microcal Origin 6.0.

4.4 Results

4.4.1 Response of PofI and PofC plants to TMV

Several independent experiments were carried out by infecting plants at various ages. In the first experiment, plants were infected at 10-week age. The results showed that at least 4 out of 20 PofI lines had delayed symptom appearance after being infected with TMV (data not shown).

The second experiment was carried out on 5-week old plants. Twenty plants from eighteen PofI lines (360 plants in total) and 5 plants from eight PofC lines (40 plants in total) were used for the experiment. Plants were infected with 200 ng of TMV RNA. Symptom appearance was monitored daily. Virus concentration in the lower non-inoculated leaves was analyzed at 6, 9 and 12 dpi. PofC plants developed symptoms much faster than PofI plants (Table 4.1). More than 50% (23 /40) of PofC plants had symptoms on day 5 and all of them had symptoms on day 7. In contrast, less than 30% (on average) of PofI plants had developed symptoms on day 5 and approximately 70% on day 7 (Table 4.1). In lines 8, 9, 10, 13, and 20, some plants showed no symptoms up to day 15. The analysis of virus concentration confirmed, however, that these plants were infected.

Analysis of the concentration of virus revealed that there are differences in various PofI and PofC lines (Fig. 4.2). At 6 dpi, 13 out of 18 PofI lines had a significantly lower concentration of virus when compared to PofC plants (Fig. 4.3; Appendix 6.6). A similar trend was observed at 9 dpi (Fig. 4.4; Appendix 6.7). Importantly, 10 out of 18 lines (lines 1, 2, 3, 7, 9, 14, 15, 16, 18 and 20) had lower concentrations of the virus at both 6 and 9 dpi. Lines 4, 8, 10, 12, 13 and 19 had lower virus concentration either at 6 or 9 dpi. Two of the lines, line 5 and line 11, had no difference in virus concentration when compared to PofC. In most of the PofI lines, the virus concentrations were higher at 12 dpi when compared to PofC. Virus concentrations in PofC plants peaked at 9 dpi, and were the lowest at 12 dpi (Fig 4.5; Appendix 6.8). In contrast, most of the PofI lines had the lowest virus concentrations at 6 dpi. The concentrations were maximum at 9 dpi and remained nearly the same at 12 dpi (in some lines it continued to rise, whereas in the other lines it started declining, Figure 4.2).

4.4.2 Epigenetic changes in PofI and PofC

The investigation of global genome methylation in both PofI and PofC revealed that there is a difference in their methylation status. It was found the PofI plants had hypermethylated genomes when compared to the PofC plants (fold:ct, 1.2-2.1 fold difference, Table 4.2). The variation methylation status among various samples may be due to the difference in variation in the response of individual plant to the stress.

In the next experiment, the methylation patterns of the resistance gene and actin loci were studied. The RFLP analysis of the *R* gene-like or actin-like loci, using methylation sensitive enzyme *Hpa*II, revealed most of the fragments were of the same intensity and, therefore, had a similar methylation status (20 and 6 loci for *R* genes and actin genes, respectively). The progeny of infected plants contained 3 *R* gene loci with fragments of 20-50% (statistically insignificant) lower intensity, hence, were hypermethylated (Table 4.3). Importantly, there were 5 different *R* gene loci in the progeny of infected plants with fragments of 400-800% higher intensity, representing largely hypomethylated loci (Figure 4.7 A, B; Table 4.3). In contrast, among the 9 actin loci, there were 3 heavily hypermethylated loci and no hypomethylated loci (Figure 4.7 C, D; Appendix 6.9).

These results indicate that the genomes of PofI plants undergo hypermethylation, while the *R* gene-like loci show hypomethylation, in contrast to the complete hypermethylation of the control loci.

4.4.3 Confirmation of methylation data –COBRA

To support our data on the epigenetic changes that were obtained using the *Hpa*II enzyme and RFLP analysis, a COBRA analysis on PofI plants (lines 3, 8, 9, 10 and 19) and PofC plants was performed.

For the amplification of the actin fragment, same primers as that in RFLP analysis were used. The expected fragment of 298 nt was obtained. Several possibilities for the digestion pattern in this fragment exist.

Digestion with *Hpy*CH4IV would yield two fragments of 180 and 118 bp if the DNA was fully methylated (the enzyme recognition sequence ACGT is preserved from conversion by bisulfite due to the methylation of cytosine) and one fragment of 298 bp if the DNA was not methylated (ACGT is converted to ATGT by bisulfite; Figure 4.8 A). Partial methylation would result in the presence of all three fragments with differing intensity (Figure 4.8 B). Additional fragments of ~240 and ~60 bp may form if an alternative *Hpy*CH4IV cleavage site was located in other homologous loci.

Analysis of the structure of seven different actin genes indeed showed the absence of the original *Hpy*CH4IV site in three different genes (data not shown). A lower intensity of the main, undigested fragment of 298 bp (nearly 2-fold difference; 49.7±14.8 versus 95±12.2, $P<0.01$, $n=5$) and higher intensity of the smaller 60 bp fragment (~40% difference; 50.5±5.5 versus 36.7±3.4, $P<0.01$, $n=5$) is an indication of a higher degree of methylation of some of the actin loci in the PofI as compared to PofC (Figure 4.8B, Table 4.4).

Digestion of the actin PCR product with *Taq*I will result in 4 fragments of 132, 72, 66 and 26 bp if the DNA was methylated (Figure 4.8A) and one fragment of 298 nt if the DNA was not methylated (Figure 4.8A). The fragment of ~230 nt is the result of methylation at TCGA site and not at the CCGA sites (Figure 4.8A). Similar to the results with *Hpy*CH4IV, the lower intensity of the main, undigested fragment (~25% difference; 45.2±5.5 versus 57.1±6.2, $P<0.01$, $n=5$) and higher intensity of smaller 66.72 bp fragment (~40% difference; 56.3±6.8 versus 40.8±9.3, $P<0.05$, $n=5$) was observed in PofI as compared to PofC (Figure 4.8B).

This experiment confirmed the hypermethylation status of the PofI loci obtained with the RFLP analysis of *Hpa*II cut DNA. The difference in methylation observed in the COBRA was not as high as that observed with the RFLP, due to the fact that COBRA determines the average methylation data for all the actin loci, while the RFLP assay allows analysis of each locus individually.

COBRA performed for the RENT and 5.8S loci did not reveal any significant difference in methylation between PofI and PofC plants (Figure 4.8 C, D; quantification data not shown). However, the 5.8S loci in general were severely hypermethylated when compared to the RENT or actin loci (indicated by complete absence of undigested fragment, Figure 4.8 C, D). This was also confirmed upon RFLP analysis of the 5.8S loci after digestion of genomic DNA with *Hpa*II enzyme, as very little DNA was digested in both, PofI and PofC plants.

4.4.4 Stability of *R* gene loci

To analyze whether a change in recombination frequency would occur in *R* gene-containing loci, the rearrangements of N gene-like loci were quantified. It was previously reported that the tobacco genome contains a number of loci carrying a high degree of homology to the N gene (Whitham *et al.,* 1994). Genomic DNA was digested either with *Hin*dIII or *Eco*RI restriction enzymes (Figure 4.6A). Hybridization of the SR1 tobacco genome with a probe based on the sequence of the 4th exon of the N gene showed that the SR1 tobacco cultivar contained 30 loci with various degrees of homology to the N gene (Figure 4.6 A, B). Analysis of the RFLP of these 30 N gene-like loci was carried out in the initially created PofI and PofC plants. Individual plants which were are either PofC or PofI were analyzed, in which, the PofI plants were found to have 8.2-fold increase in instability of N gene-like *R* gene loci when digested with *Hin*dIII (Figure 4.6 B). Whereas the RFLP of 149 PofI revealed the difference in 33 loci, the RFLP of 147 PofC plants revealed the difference only in 4 fragments. This rearrangement frequency in *R* gene loci was calculated to be 7.4×10^{-3} per plant for PofI and 9×10^{-4} per plant for PofC. The PofC and PofI plants were created in one individual experiment and the progenies of the plants were analyzed for various experiments.

Hybridization of genomic DNA digested with the *Eco*RI enzyme revealed a similar pattern with a 6.5 fold difference in *R* gene loci instability (14 fragments out of 98 PofI and 2 fragments out of 91 PofC, Figure 4.6 C). Hence, the data from both restriction enzymes *Hind*III and *Eco*RI reveal that following the SRS there is increase in the frequency of rearrangements in *R* gene like loci.

4.4.5 Stability of actin and actin-like loci

To analyze the stability of the actin and actin-like loci, one of the regions with a high homology to actin genes was used for the RFLP analysis. Hybridization of the genomic DNA of nearly 700 plants from PofI and PofC with the actin probe revealed no significant difference among all 13 actin-like loci (Figure 4.6 D).

4.4.6 Stability of RENT and 5.8S loci

In the RFLP analysis, membranes used for hybridization with N-gene 4th exon were stripped and on these membranes, the hybridizations with the RENT or 5.8S loci probes were done.

RFLP of the RENT loci revealed 45 fragments of different size and intensity (Figure 4.6 E). No difference in RFLP was found, and there were 3 fragments of different size from PofI, and 2 fragments differing from PofC plants. This resulted into a statistically similar rearrangement frequency of 4.47×10^{-4} and 3.02×10^{-4} for PofI and PofC, respectively.

RFLP analysis of the 5.8S loci revealed 52 fragments of different size and intensity. There were two loci of very high intensity and, thus, multiple film exposures had to be done (Figure 4.6 F). Screening of 149 PofI and 147 PofC plants revealed a similar frequency of rearrangements. There were 7 different fragments in PofI and 5 different fragments in PofC (Figure 4.6 H). This resulted into a rearrangement frequency of 9×10^{-4} and 6.5×10^{-4} for PofI and PofC, respectively.

These results indicate that there is no difference in the frequency of rearrangements in at least three different loci of PofI and PofC lines.

4.5 Discussion

During an incompatible plant-pathogen interaction, the plant is able to resist pathogen infection. However, little is known about what happens when a plant faces a virulent pathogen. Recent evidence suggests that an oxidative burst upon the infection of a pathogen occurs regardless of whether the pathogen is virulent or avirulent (Grant et al., 2000). Supporting this evidence, it was observed that the plant is able to produce a mobile signal, which increases the recombination frequency before the spread of the virus. This signal was termed as SRS (Kovalchuk et al., 2003).

The SRS is capable of spreading faster than TMV, this implements that the signal might have a role in warning the distant tissues of an incoming pathogen. Furthermore, this warning signal may be involved in signaling mechanisms, which enable these tissues to better deal with the incoming stress. To characterize the effects of the SRS various experiments on the PofI and PofC lines were carried out.

When the time of symptom appearance in PofI and PofC lines was investigated, it was observed that PofI lines were able to delay symptom appearance in comparison to PofC lines. In addition, PofI lines showed a significantly lower virus concentration in tissues at 6 and 9 days after infection. In comparison, the virus titers in PofC plants reach the maximum at 9 dpi and show the decrease at 12 dpi. A similar picture was observed in tomato plants infected with TMV; virus concentration peaked at 7 dpi and declined steadily at later days (Balogun *et al.,* 2002) The analysis of virus progression in PofI and PofC lines indicates that PofI plants are able to delay the infection from the virus and, hence, delay the process of disease onset . This delay of disease onset can provide the plants to reach the reproductive phase and to pass the genetic information to next generation. How these plants are able to delay the virus propagation is still not understood. Thus, further research needs to be carried out in this direction.

Previously, it was observed that there is an increase in the frequency of HR in response to the pathogen (Kovalchuk *et al.*, 2003). As HR is a major threat to genome stability, it is undesirable for the plant to increase the HR frequency as it might lead to alteration of important genetic information. This increase in HR is also observed in plants that are subjected to environmental changes like temperature, radiation, and salt stress (Boyko *et al.,* 2005). Hence, this increase in HR is likely a plant-derived mechanism against stress, and not pathogen derived.

The rate of recombination in any area of the genome is influenced by the cytosine methylation status of the area. An increase in methylation results in a decrease in the occurrence of recombination in the area (Lowe *et al.,* 1992). Hence, methylation increases genome stability, and *vice versa.*

If the HR in a certain region can be upregulated via an alteration in methylation status, it could result in new combination of genetic information. This would create new variation for evolutionary selection to act upon. One of the obvious candidates where such an induction of variation can be beneficial to plants is in the plant resistance (*R*) genes. When one considers that it has been suggested that gene duplication with subsequent diversification events is the major cause of *R* gene evolution (Whitham *et al.,* 1994; Lawrence *et al.,* 1995), evidence of which is that most *R* genes are members of multigene families and very often are found in large clusters at a single locus (Song *et al.,* 1995), this hypothesis seems more plausible.

Most of the *R* genes contain NBS and LRR domains. The LRR region is responsible for the recognition of the specific pathogen elicitors (Dangl and Jones,, 2001). If the SRS can increase the frequency of rearrangements, and thereby the genetic variability in the LRR domains, plants could generate alternate recognition sites by which they could resist the same or another pathogen. Hence, the SRS can increase the probability of converting a virulent pathogen to avirulent pathogen.

To check the aforementioned hypothesis, analysis of the methylation status of the PofI and PofC plant genomes was carried out. These results indicated that the PofI plant genomes are 1.2 to 2.1 fold more methylated when compared to PofC plant genomes. In the next step, the sequence specific methylation statuses of various loci were investigated. Methylation-sensitive RFLP analysis revealed that the *R* gene loci are significantly hypomethylated. In contrast, the actin loci were either hypermethylated or there was no change in the methylation status. The results from COBRA assay again indicated that the actin loci are either hypermethylated or there was no change. RENT and 5.8S loci were also analyzed using COBRA and the results showed either hypermethylation or no differences in their methylation in either PofI or PofC plant genomes. As the *N*-gene-like *R* loci are found in clusters and contain repetitive elements, it was important to use control loci that contained these factors. The RENT loci were also used as a control for the repetitive elements, while the 5.8S loci were used for the cluster control. High methylation status of the rRNA coding loci is not surprising as only a subset of the total rRNA gene pool is active at a given time (Lawrence and Pikaard, 2004). All the above results suggest that in PofI plants, the *R* gene-like loci have significant hypomethylation as compared to PofC plants, while the actin, RENT and 35S loci have either hypermethylation or no change in methylation in PofI plants as compared to PofC plants. If changes in methylation patterns differed between these loci, it would lend more evidence to a locus-specific methylation mechanism as opposed to a structure-specific mechanism. As this is what has been observed, there appears to be a sequence- or locus-specific mechanism involved.

As a change in methylation status can influence the rate of recombination in particular region, the frequency of rearrangements occurring in the hypomethylated *R* gene-like and unchanged or hypermethylated other loci using RFLP were analyzed.

In PofI plants, RFLP of R gene-like loci showed 6.5 to 8.2-fold decrease in the stability. There was no change in the stability of actin loci, which is logical as actin is a housekeeping gene, and the decreased stability would result in deleterious effects. RFLP analysis of RENT and 5.8S loci did not show any difference between PofI and PofC plants. Hence, it can be concluded that following the SRS, the genome stability of the *R* gene-like loci is significantly reduced. These results support our initial hypothesis.

4.6 Conclusion

Our data suggest the existence of a specific epigenetically controlled mechanism that promotes rearrangements in *R* gene loci upon contact with a compatible pathogen. It also suggests SRS can result in a higher tolerance to the future pathogen infection. Future studies are clearly needed to understand the signal specificity and the mechanism underlying the methylation changes. Recognition and future use of such mechanisms might enhance our capacities to create more tolerant plants.

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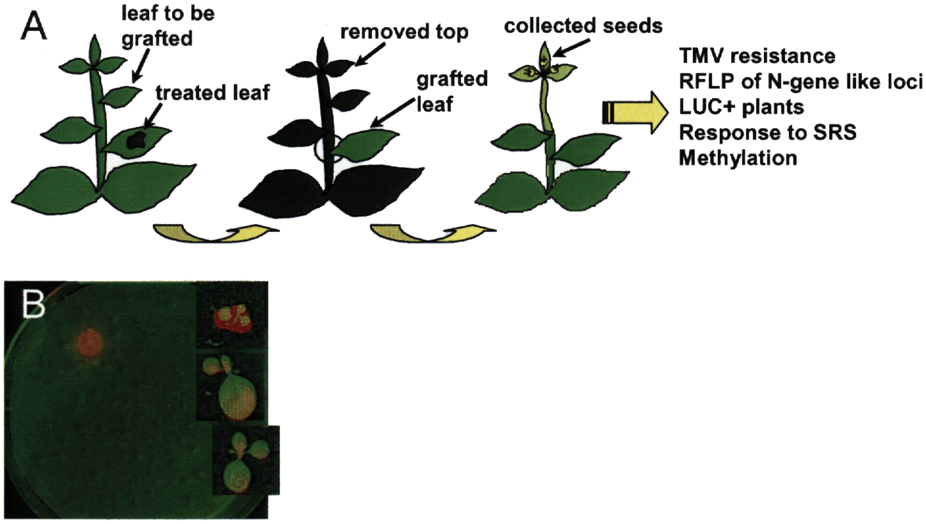

Fig. 4.1 Analysis of the progeny of infected and progeny of control plants

A. Schematic representation of the experimental set up. Briefly, in a previous experiment, single leaves of 10-week old SR1 tobacco plants were inoculated with 300ng of TMV RNA (21 plants) or mock treated (20 plants). 24 hours after inoculation, the upper, non-treated leaves (virus-free, checked as previously published, Kovalchuk et al., 2003) from these plants were grafted onto 10 week-old healthy plants (21 plants with leaves from virus-treated and 20 plants with leaves from mock-treated plants), from which the tops were previously removed. The seeds derived from the newly emerged tissue were collected and named progeny of infected (PofI) or progeny of control (PofC). These seeds were used to analyze pathogen resistance, response to SRS, appearance of LUC+ plants, RFLP of various loci, global and loci-specific methylation.

B. Screening for LUC+ plants (plants with fully recombined transgene) using CCD camera. Plants were sprayed with luciferin and the expression of the luciferase was analyzed. A spot of luciferase expression reveals fully recombined transgene.

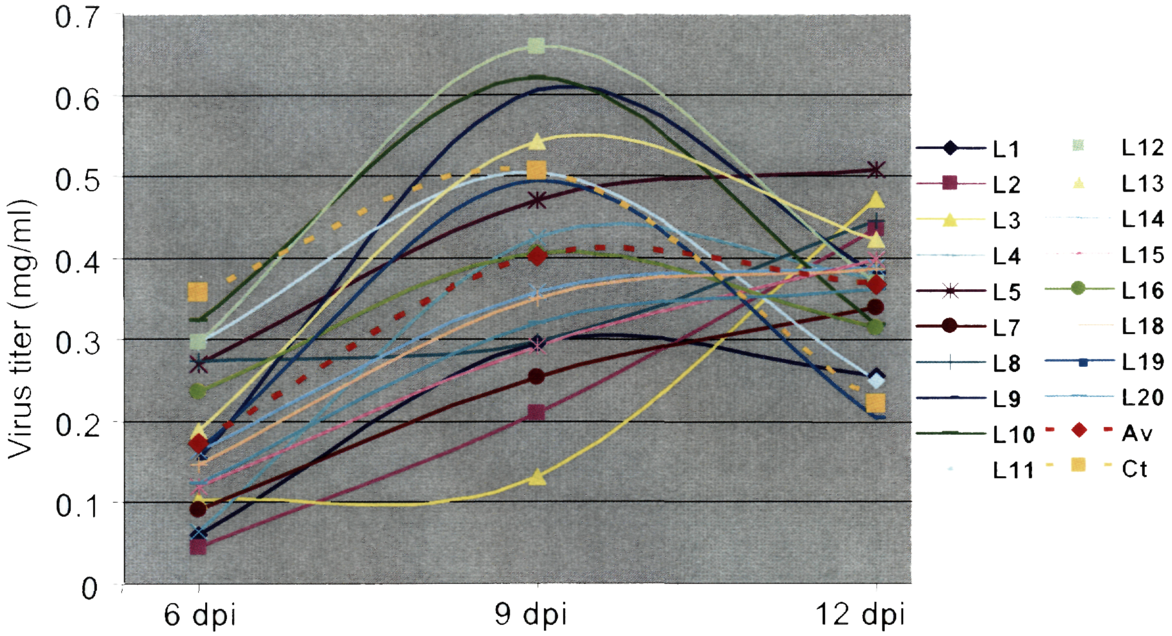

Fig. 4.2 Virus concentrations in PofI and PofC plants infected with TMV

Virus concentrations were measured in infected plants at 6, 9 and 12 dpi. Lines show the data for virus concentrations as an average from 20 to 40 plants. Red dashed line shows the average of PofI lines, whereas orange dashed line shows the average of PofC lines.

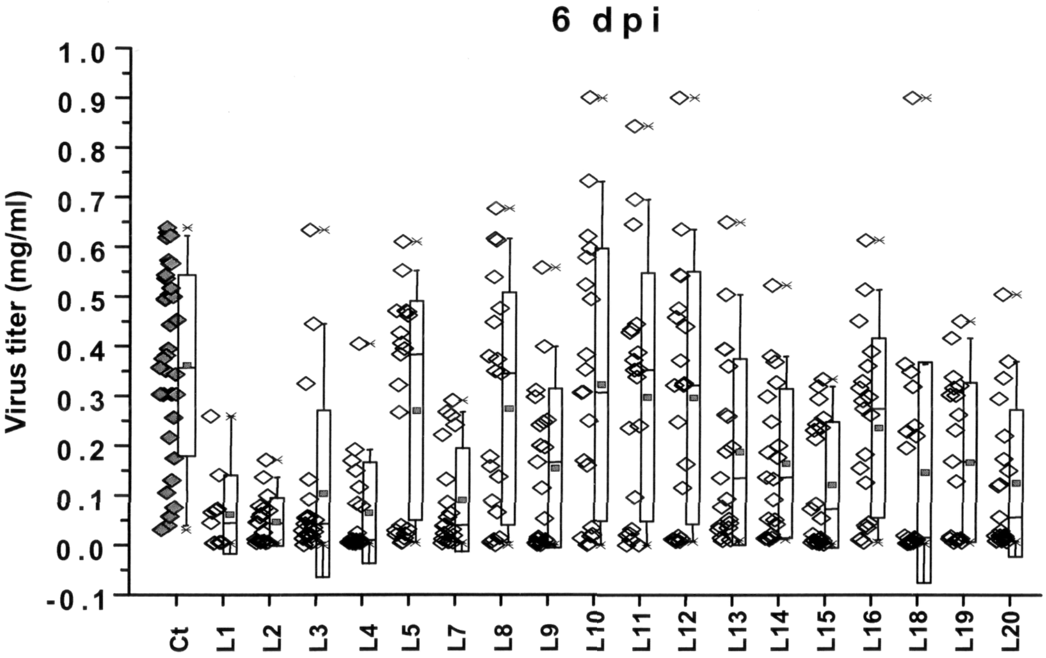

Fig. 4.3 Virus concentration in different lines at 6 dpi

Each line indicates the concentration (diamond), maximum, minimum, 1-99% confidence interval (bars) and SD values (rectangles). In the control, none of the 40 plants shows zero concentration, while few plants in almost all other lines have plants showing zero concentration.

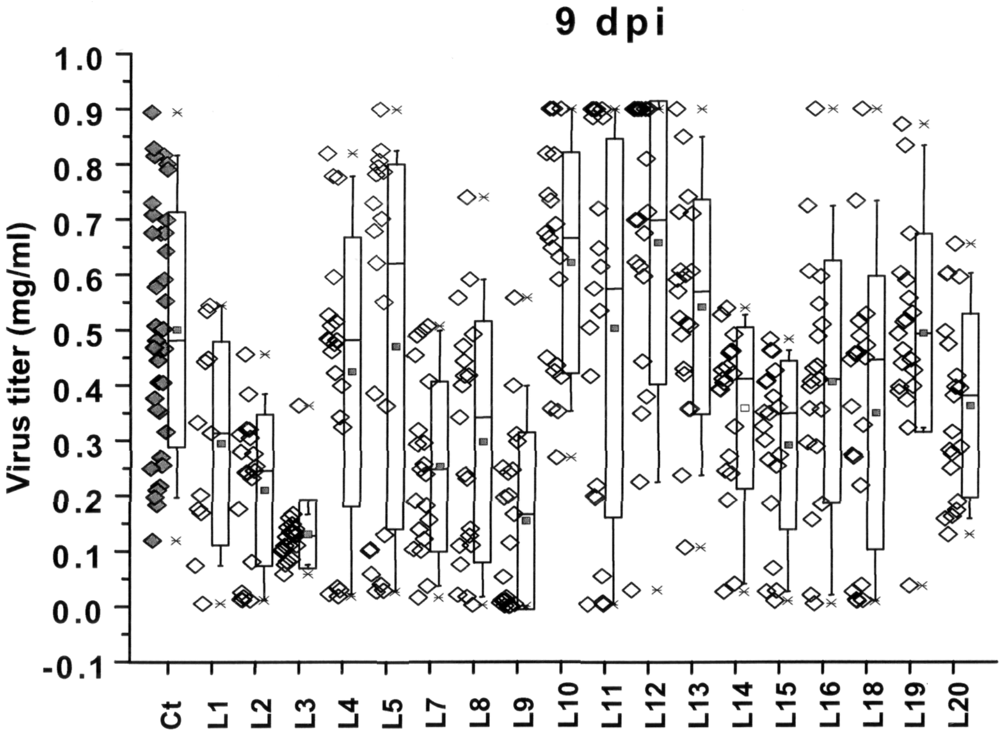

Fig. 4.4 Virus concentrations in different lines at 9 dpi

At 9 dpi, few plants from line 2, 9, and 18 were found with zero virus concentration. Line 11 and 12 as well as the control, had plants that the virus concentration reached maximum level. Each line indicates the concentration (diamond), maximum, minimum, 1-99% confidence interval (bars) and SD values (rectangles).

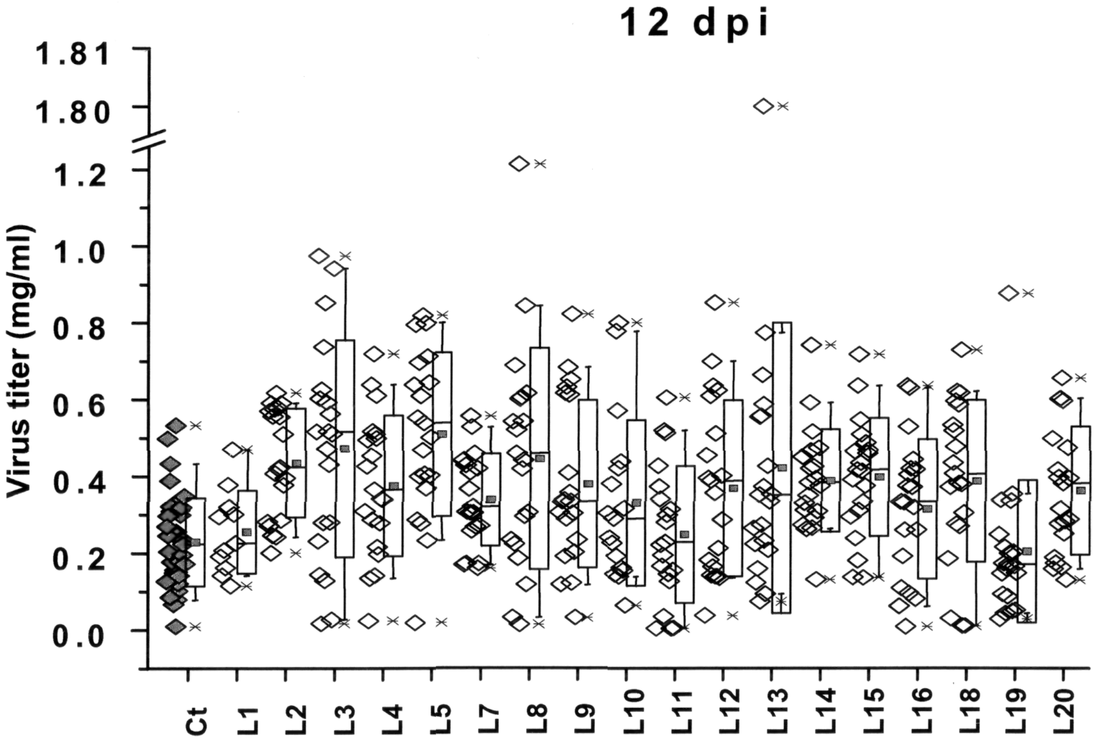

Fig. 4.5 Virus concentration in different lines at 12 dpi

As compared to other lines, the virus concentration in PofI was significantly lower. In some lines, few plants reached the maximum level at 12 dpi, which indicates that the process of virus replication and disease onset was significantly delayed. Each line indicates the concentration (diamond), maximum, minimum, 1-99% confidence interval (bars) and SD values (rectangles).

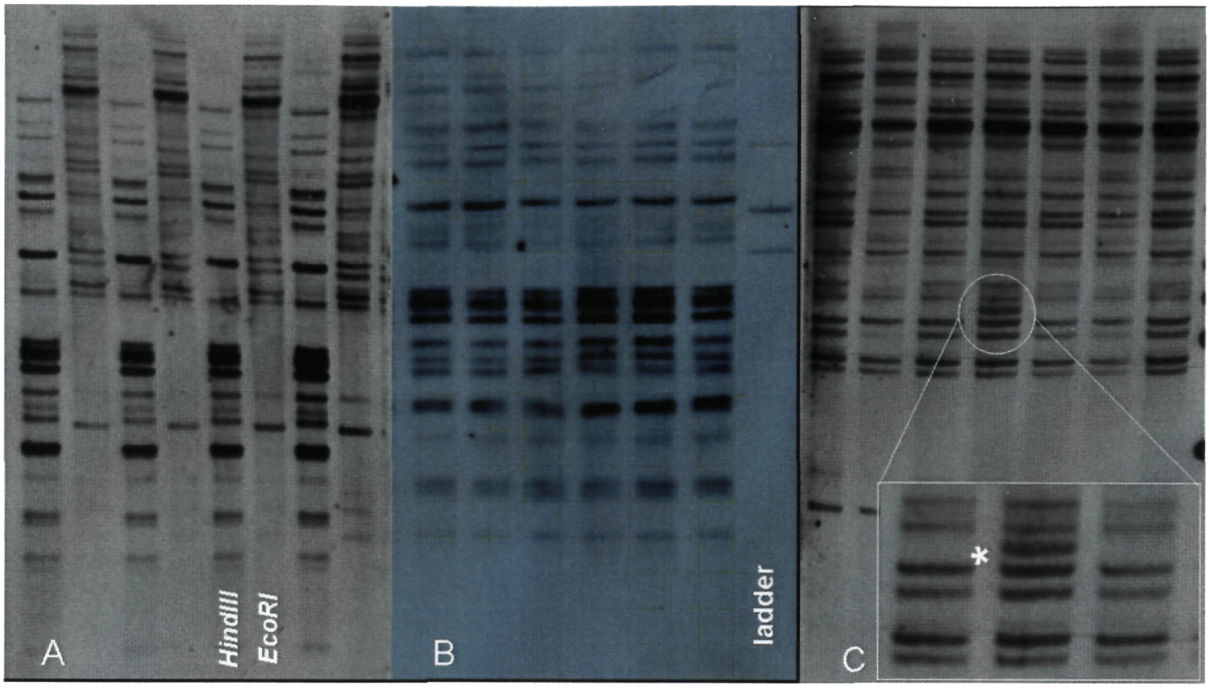

Fig. 4.6a RFLP analysis of the N gene-like resistance gene loci

(A) RFLP analysis of N gene-like loci revealed 30 different loci with homology to the 4th exon of the N gene. Most of the analyzed progeny of infected (149 plants) and progeny of control (147 plants) had similar fragment patterns after being digested with either *Eco*RI (A) or *Hin*dIII (A,B). Occasional differences were mostly observed in the progeny of infected plants (C).

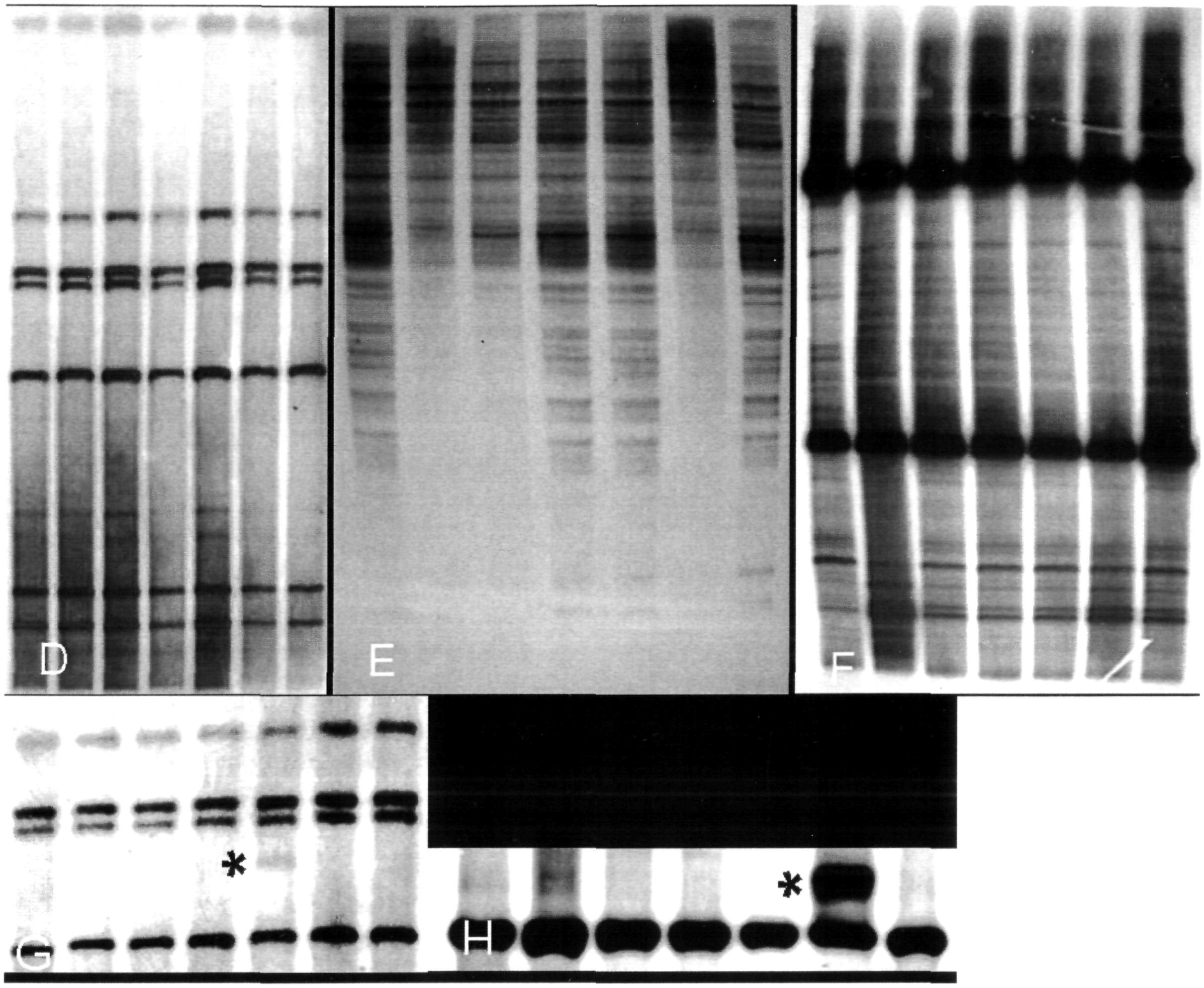

Fig. 4.6b RFLP analysis of the actin, RENT and 5.8S loci

RFLP analysis of the actin loci showed 13 different fragments (D). The actin fragments were observed to be stable and only a single polymorphic fragment was found in PofC (G). Digestion of genomic DNA with *Hin*dIII and probing with either RENT (E) or 5.8S-specific (F) probe revealed 45 and 52 fragments, respectively. Occasional differences in both (H) were found (additional fragment in one of the PofI samples).

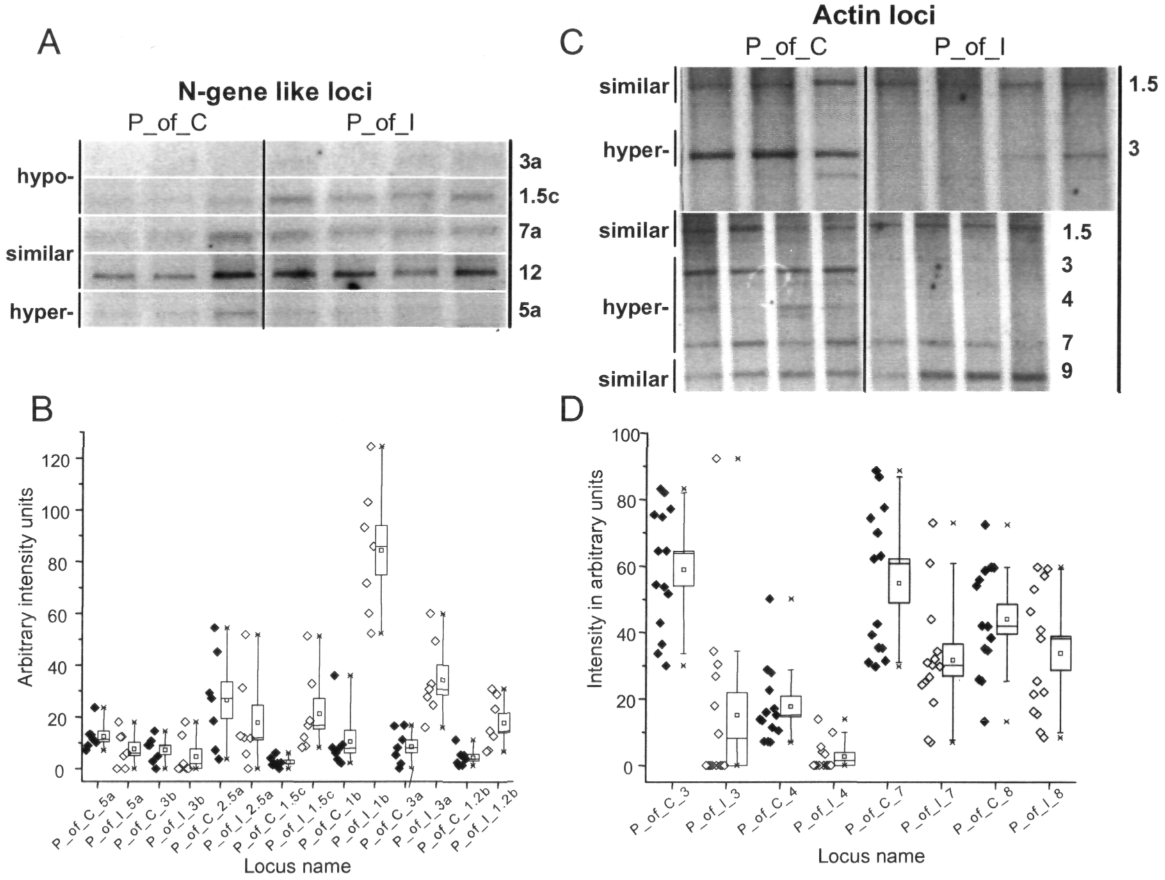

Fig. 4.7 Analysis of methylation in *R* gene and actin loci

Each "stripe" represents an individual locus with homology to the N-gene (A) or Tob71 actin gene (C). "Similar" methylation shows the loci with methylation that was similar (on average) between PofI and PofC for both N and actin genes (A, C). "Hyper-" and "hypo-" shows hyper- and hypomethylation of the PofI loci. Each graphic (B,D) shows all the data points, the mean, the maximum and the minimum, the 1-99% confidence interval, as well as SE calculated from 7-15 individual plant samples from each of PofC or PofI groups. Individual numbers (PofI3 or PofC1.5b) on the graphic represent a particular locus and correspond to the loci labeled on the blot.

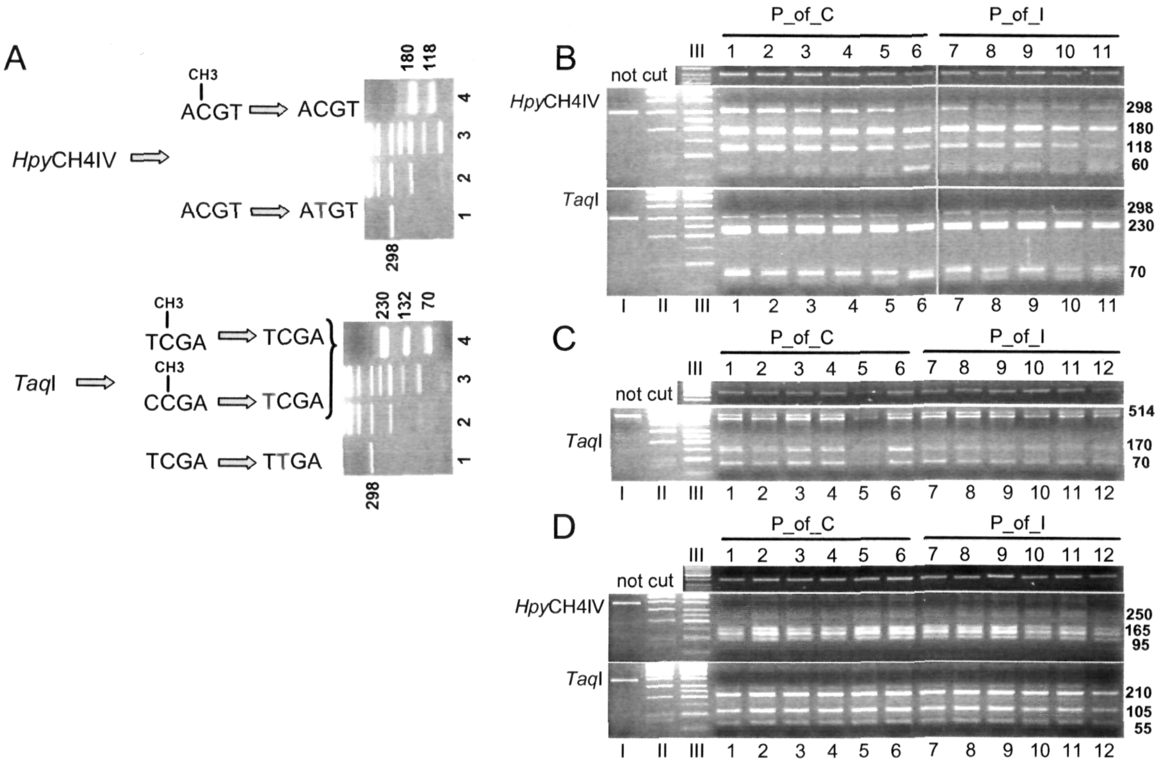

Fig. 4.8 Methylation status of actin, RENT and 5.8S loci in PofI and PofC performed using COBRA

Bisulfite treated genomic DNA from 6 PofI and 6 PofC lines were used for amplification of the actin, RENT and 5.8S loci.

A. Schematic presentation of digestion of the actin PCR product (298 bp, line 1) with *Hpy*CH4IV or *Taq*I enzymes. Bisulfite-converted nucleotide is labelled in red. Methylated ACGT recognition site for *Hpy*CH4IV was retained after bisulfite treatment to allow the enzyme to cut DNA yielding two fragments of 180 and 118 nt (line 4). Recognition sequence for *Taq*I TCGA can either be preserved from conversion by bisulfite while methylated or can be created from conversion of CCGA site if the only methylated cytosin is at a CpG site. Lines 2 and 3 show 100 bp and 50 bp ladders.

B. Comparison of digestion patterns in actin loci of PofI and PofC plants after 16h incubation with *Hpy*CH4IV or *Taq*I. The upper panel shows the undigested PCR product serving as a loading control. Line "I" is the undigested PCR product, lines "II" and "III" show 100 and 50 nt ladders, respectively. Lines 1-6 represent individual PofC plants; lines 7-11 represents individual plants from PofI (lines 3, 8, 9, 10 and 19). Bands used for quantification by "Image J" are labelled on the right side. Note: band "70" in *Taq*I digested DNA marks two fragments of 72 and 66 bp that were used for quantification.

C. Digestion pattern in RENT loci. Coding is similar to that used for actin, except that there were 6 PofI samples, lines 7-12.

D. Digestion pattern in 5.8S loci. Coding is similar to that used for RENT.

Table 4.1 Symptom appearance in the progeny of infected (PofI, 1-20) and control (PofC, ct) plants

	ct	**#1**	**#2**	**#3**	**#4**	**#5**	**#7**	**#8**	**#9**	**#10**
5 dpi	23/40	4/20	5/20	3/20	6/20	10/20	5/20	6/20	2/20	7/20
%	57.5	20	25	15	30	50	25	30	10	35
6 dpi	32/40	11/20	9/20	8/20	12/20	15/20	8/20	10/20	5/20	11/20
%	80	55	45	40	60	75	40	50	25	55
7 dpi	40/40	14/20	15/20	13/20	17/20	18/20	14/20	13/20	12/20	13/20
%	100	70	75	65	85	90	70	65	60	65
9 dpi		19/20	20/20	18/20	20/20	20/20	20/20	15/20	16/20	18/20
%		95	100	90	100	100	100	75	80	90
12 dpi		20/20		20/20				16/20	17/20	18/20
%		100		100				80	85	90
15 dpi								18/20	18/20	20/20
%								90	90	100
	#11	**#12**	**#13**	**#14**	**#15**	**#16**	**#18**	**#19**	**#20**	
5 dpi	11/20	4/20	6/20	4/20	5/20	3/20	4/20	9/20	7/20	
%	55	20	30	20	25	15	20	45	35	
6 dpi	16/20	7/20	9/20	9/20	8/20	6/20	7/20	14/20	10/20	
%	80	35	45	45	40	30	35	60	50	
7 dpi	19/20	15/20	13/20	16/20	13/20	12/20	11/20	17/20	16/20	
%	95	75	65	80	65	60	55	85	80	
9 dpi	20/20	19/20	17/20	19/20	18/20	19/20	17/20	20/20	18/20	
%	100	95	85	95	90	95	85	100	90	
12 dpi		20/20	18/20	20/20	20/20	20/20	20/20		19/20	
%		100	90	100	100	100	100		95	
15 dpi			20/20						20/20	
%			100						100	

Symptom appearance was monitored on 5, 6, 7, 9, 12 and 15 dpi. Numbers in the upper rows show the number of plants with symptoms and the number of infected plants. Numbers in the lower rows show the percentage of infected plants at certain dpi.

Table 4.2 Global genome methylation analysis of PofC and PofI plants using cytosine extension assay

*Hpa*II	*Msp*I	*Msp*I: *Hpa*II	*Hpa*II	*Msp*I	*Msp*I: *Hpa*II	Fold: ct (t-test)	*Hpa*II	*Msp*I	*Msp*I: *Hpa*II	Fold: ct (t-test)
PofC			**PofI 8**				**PofI 19**			
2663.7	6383.4	2.4	1609.2	5287.2	3.3		387.0	1317.6	3.4	
5262.7	8475.7	1.6	236.1	1186.6	5.0		135.9	619.1	4.6	
3549.6	10353.3	2.9	886.0	2188.7	2.5		47.9	328.9	6.9	
2154.5	3952.2	1.8	**910.4**	**2887.5**	**3.2**	1.6 (0.25)	84.2	430.1	5.1	
2778.7	5996.4	2.2	*686.9*	*2137.7*			83.1	508.2	6.1	
1509.5	5096.5	3.4					163.2	593.0	3.6	
5510.7	7475.7	1.4	**PofI 9**				**150.2**	**632.8**	**4.2**	2.1 (0.003)
1243.8	2795.8	2.2	1762.1	3552.5	2.0		*123.1*	*352.0*		
555.2	4282.1	7.7	939.7	2290.4	2.4		**PofI 20**			
1378.6	1825.8	1.3	822.6	2431.4	3.0		1695.0	6760.0	4.0	
1577.4	2885.0	1.8	1110.8	2622.6	2.4		1490.2	2547.8	1.7	
5129.7	8908.2	1.7	700.1	2066.2	3.0		1151.9	2516.6	2.2	
1227.9	2881.7	2.3	321.3	714.4	2.2		2405.5	2612.1	1.1	
727.2	1545.2	2.1	1052.1	2547.9	2.4		2933.3	1867.4	0.6	
3684.9	5420.5	1.5	264.8	551.7	2.1		1788.5	2361.6	1.3	
2596.9	**5218.5**	**2.0**	**871.7**	**2097.1**	**2.4**	1.2 (0.99)	1459.8	1291.1	0.9	
1677.8	*2698.7*		*476.6*	*1003.4*			1429.2	1624.6	1.1	
							2086.6	2718.8	1.3	
							2529.9	6388.6	2.5	
							1897.0	**3068.9**	**1.6**	0.8 (0.19)
							572.7	*1907.4*		

Numbers show the incorporation (dpm/µg of DNA) of radioactively labeled cytosine ([^{3}H]-dCTP) into the genomic DNA of PofC and PofI (8, 9, 19, 20) cut with methylation sensitive (*Hpa*II) and insensitive (*Msp*I) enzymes. Incorporation is directly dependent on the completeness of the DNA digestion with methylation sensitive enzymes - the higher the methylation, the lower the digestion rate, and the lower the methylation, the higher the digestion rate. "*Hpa*II" and "*Msp*I" columns show the data for *Hpa*II and *Msp*I cut DNA. "*Msp*I:*Hpa*II" shows the ratio of radioactive incorporation in "*Msp*I" to "*Hpa*II" cut genomic DNA. The higher the ratio, the higher the methylation status. The averages are shown in bold followed by standard deviation in italics. Difference between PofI and PofC is shown as "Fold: ct". Statistical significance is presented by p- value in the two-tailed non-paired t-test (in parenthesis).

Table 4.3 Methylation analysis of N gene like resistance gene loci

	PofC	PofI	Fold	F	P-value	F crit
5a	12.5±2.0	7.6±2.6	-1.6	2.2	0.17	4.7
3b	7.3±1.9	4.7±2.9	-1.5	0.6	0.47	4.7
2.5a	26.5±7.1	17.8±6.7	-1.5	0.8	0.39	4.7
3a	8.4±2.5	34.3±5.7	4.1	17.1	1.0E-03	4.7
1.5c	2.5±0.7	21.2±5.9	8.4	9.8	9.0E-03	4.7
1.2b	4.4±1.3	17.7±3.8	4.1	11.1	6.0E-03	4.7
1b	10.4±4.4	84.3±9.5	8.1	49.7	1.3E-05	4.7

Genomic DNA was digested with the methylation sensitive enzyme *Hpa*II and probed with the 4th exon of the N-gene. The fragment intensity (see Figure 4.7A) inversely correlates with the methylation status at a particular locus. PofC and PofI stand for progeny of control and progeny of infected plants, respectively. Rows (5a, 3b …) represent loci with changed intensity of the fragments. Data (with SE) shows the average intensity of the fragments (calculated from 9 to 15 different plants). "Fold" shows the difference in fragment intensity between PofI and PofC. Negative values (-1.6; -1.5) correspond to lower average fragment intensity in PofI as compared to PofC and, thus, to hypermethylation (insignificant, see F and P-value) of loci 5a, 3b and 2.5a. Similarly, positive values (4.1; 8.4; 4.1; 8.1) correspond to higher average fragment intensity in PofI as compared to PofC and, thus, to hypomethylation (significant, see F and P-value) of loci 3a, 1.5c, 1.2b and 1b. Statistical analysis was performed by Single factor ANOVA (Excel, Microsoft).

Table 4.4 Analysis of methylation status of actin loci in PofI and PofC using COBRA

Enzyme	***Hpy*CH4IV**			***Taq*I**			
Fragment size	**298nt***	**132nt**	**60nt***	**298nt***	**230nt**	**72nt**	**66nt***
PofI	40.9	35.5	56.3	36.49	52.61	79.88	57.16
PofI	38.7	36.8	48.6	49.03	50.75	74.25	56.73
PofI	46.5	45.0	43.0	43.37	49.05	85.23	45.06
PofI	47.1	51.4	49.1	50.08	51.64	83.78	63.49
PofI	75.4	47.5	55.5	47.14	51.67	91.11	59.18
AV±SD	**49.7±14.8**	**43.2±6.9**	**50.5±5.5**	**45.2±5.5**	**51.1±1.3**	**82.9±6.3**	**56.3±6.8**
PofC	84.2	46.5	39.4	47.44	52.39	87.14	55.85
PofC	100.5	47.6	41.0	64.38	50.57	88.32	42.75
PofC	80.4	51.7	33.1	56.58	47.46	82.39	37.10
PofC	107.4	60.4	35.6	57.56	47.71	81.82	36.70
PofC	104.0	56.6	34.3	59.35	46.08	91.91	31.43
AV±SD	**95.3±12.2**	**52.6±5.9**	**36.7±3.4**	**57.1±6.2**	**48.8±2.6**	**86.1±4.2**	**40.8±9.3**

This table shows the comparison of fragment intensity after COBRA. Fragment intensity was quantified using "Image J" software. The data were obtained by measuring the intensity of the individual fragments, relating it to the background intensity and standardizing it to the undigested 298 bp PCR product. "AV±SD" stands for average plus/minus standard deviation. Fragment size corresponds to the fragment size in Figure 5. Asterisks represent a statistically significant difference between the average intensity of PofI and PofC fragments.

5.0 General Conclusion

The activity of the N gene promoter was developmentally regulated. The expression of the marker gene regulated by the N gene promoter was limited to veins in 2-week-old seedlings and spread to leaf lamella of the true leaves as the plants matured. It was also shown to be different in two different plant organs, high expression in leaves and low expression in roots. The activity of the N gene promoter was also influenced by various biotic as well as abiotic factors. The trend of the increase in promoter activity upon TMV infection and prolonged exposure to high or low temperatures was observed. No influence was found upon the application of exogenous SA on the activity of the N gene promoter.

SRS triggered number of changes in the progeny of infected plants. The genomes of the progenies of infected plants were found to be hypermethylated. On the contrary, several *R* gene-like loci carrying the homology to the N gene were hypomethylated when compared to control loci. This resulted in a significant increase in the frequency of rearrangements in these *R* gene-like loci. The SRS also resulted in an increase in tolerance to virus infection in the next generation, which were observed as a delayed TMV replication in infected progeny. It is possible that this increase in tolerance was a result of the epigenetic changes elicited by the SRS.

Appendix 6.1: Effect of TMV on LUC activity in tobacco SLUCP 6-1 plants

Columns show the raw data for 4 different plants per each experimental group (time points, column 1).

Time point	Treatment (RLU)	
	Buffer	Virus
0h 1	40.3	50.9
0h 2	38.6	47.3
0h 3	34.1	35.6
0h 4	45.3	31.6
15m 1	52.0	47.6
15m 2	36.4	36.8
15m 3	43.2	29.8
15m 4	38.4	45.2
30m 1	50.3	60.3
30m 2	48.3	34.8
30m 3	30.0	51.4
30m 4	36.4	49.0
1h 1	20.0	48.6
1h 2	34.98	49.7
1h 3	54.0	57.2
1h 4	38.8	60.2
1h 30m 1	56.1	68.7
1h 30m 2	37.9	31.4
1h 30m 3	40.6	54.3
1h 30m 4	58.0	59.2
2h 1	61.3	48.3
2h 2	57.0	47.9
2h 3	54.3	56.1
2h 4	34.0	16.4
2h 30m 1	43.0	55.4
2h 30m 2	06.0	61.3
2h 30m 3	56.7	42.5
2h 30m 4	34.8	49.6
3h 1	54.6	51.3
3h 2	23.0	37.0
3h 3	43.2	34.9

Time point	**Treatment (RLU)**	
	Buffer	**Virus**
3h 4	38.1	42.6
3h 30m 1	34.6	36.1
3h 30m 2	41.0	34.8
3h 30m 3	32.6	43.2
3h 30m 4	28.0	36.2
4h 1	21.6	46.3
4h 2	46.2	48.7
4h 3	43.1	32.6
4h 4	35.9	34.0
4h 30m 1	37.0	51.3
4h 30m 2	51.3	31.8
4h 30m 3	32.0	45.9
4h 30m 4	40.0	32.6
5h 1	28.0	56.0
5h 2	50.9	48.3
5h 3	41.0	31.2
5h 4	15.0	42.8
5h 30m 1	46.4	46.3
5h 30m 2	31.2	53.2
5h 30m 3	47.8	37.8
5h 30m 4	38.1	39.6
6h 1	16.0	47.8
6h 2	39.1	38.5
6h 3	43.6	56.1
6h 4	46.1	49.8
6h 30m 1	37.4	46.2
6h 30m 2	34.8	54.1
6h 30m 3	45.3	46.2
6h 30m 4	47.5	53.2
7h 1	41.6	46.2
7h 2	49.8	31.6
7h 3	51.3	52.8
7h 4	52.3	46.1

Appendix 6.2: Effect of TMV and high temperature on LUC activity in tobacco SLUCP 6-1 plants

Columns show the raw data for 4 different plants per each experimental group (time points, column 1).

Time point	**Treatment (RLU)**	
	Buffer	**Virus**
0h 1	11.3	18.4
0h 2	16.8	09.3
0h 3	21.2	08.5
0h 4	08.0	19.2
15m 1	21.4	10.2
15m 2	12.5	06.2
15m 3	15.2	29.1
15m 4	36.4	12.3
30m 1	20.4	25.3
30m 2	08.2	10.3
30m 3	11.2	18.2
30m 4	13.0	25.0
1h 1	22.9	21.0
1h 2	31.4	16.5
1h 3	08.0	29.1
1h 4	14.6	13.2
1h 30m 1	16.4	21.3
1h 30m 2	18.2	26.5
1h 30m 3	30.0	14.2
1h 30m 4	05.0	19.2
2h 1	10.8	28.1
2h 2	17.3	12.4
2h 3	25.9	45.2
2h 4	19.2	21.5
2h 30m 1	12.1	35.2
2h 30m 2	39.1	46.2
2h 30m 3	26.1	20.3
2h 30m 4	12.3	11.4
3h 1	21.9	21.8
3h 2	18.3	16.3
3h 3	16.3	19.32

Time point	**Treatment (RLU)**	
	Buffer	**Virus**
3h 4	37.2	24.2
3h 30m 1	29.8	20.1
3h 30m 2	16.4	12.4
3h 30m 3	14.3	26.8
3h 30m 4	09.6	19.4
4h 1	21.1	41.2
4h 2	27.1	12.6
4h 3	16.5	15.4
4h 4	17.3	18.3
4h 30m 1	16.4	06.3
4h 30m 2	22.5	17.9
4h 30m 3	29.2	31.8
4h 30m 4	17.6	16.8
5h 1	20.5	18.3
5h 2	19.5	19.5
5h 3	21.0	22.6
5h 4	41.5	28.3
5h 30m 1	21.5	19.3
5h 30m 2	29.4	38.2
5h 30m 3	16.9	17.6
5h 30m 4	24.0	22.3
6h 1	12.3	20.0
6h 2	17.3	21.3
6h 3	14.6	11.4
6h 4	39.2	32.1
6h 30m 1	09.8	15.9
6h 30m 2	14.1	11.4
6h 30m 3	06.5	16.5
6h 30m 4	38.9	18.6
7h 1	19.3	14.0
7h 2	16.5	06.0
7h 3	17.6	34.1
7h 4	26.1	11.9

Appendix 6.3: Effect of high temperature on LUC activity in tobacco SLUCP 6-1 plants

Columns show the raw data for 4 different plants per each experimental group (time points, column 1).

Time point	**Treatment (RLU)**	
	24°C	**45°C**
0.00h 1	13.2	14.1
0.00h 2	14.7	08.0
0.00h 3	12.4	15.3
0.00h 4	10.0	14.0
15m 1	16.3	13.1
15m 2	13.7	23.6
15m 3	09.0	26.1
15m 4	17.2	11.2
30m 1	20.1	24.7
30m 2	15.8	14.2
30m 3	13.4	28.1
30m 4	23.4	21.4
1h 1	16.3	20.1
1h 2	17.3	23.1
1h 3	08.0	14.3
1h 4	11.0	13.4
1h 30m 1	19.7	24.3
1h 30m 2	12.3	25.8
1h 30m 3	18.4	19.9
1h 30m 4	14.6	03.1
2h 1	18.3	21.9
2h 2	09.0	19.5
2h 3	14.3	17.3
2h 4	23.1	18.3
2h 30m 1	12.3	16.5
2h 30m 2	16.3	18.3
2h 30m 3	18.9	14.1
2h 30m 4	22.1	15.9
3h 1	10.5	17.2
3h 2	17.3	16.4
3h 3	14.1	18.4

Time point	**Treatment (RLU)**	
	24°C	**45°C**
3h 4	15.0	13.9
3h 30m 1	13.2	16.2
3h 30m 2	26.2	14.0
3h 30m 3	19.2	13.8
3h 30m 4	08.0	17.4
4h 1	16.5	17.6
4h 2	13.7	16.0
4h 3	18.0	14.2
4h 4	09.2	16.7
4h 30m 1	15.4	12.3
4h 30m 2	21.0	14.3
4h 30m 3	12.0	17.9
4h 30m 4	13.1	14.0
5h 1	15.3	15.8
5h 2	16.2	13.9
5h 3	24.3	10.1
5h 4	11.3	08.0
5h 30m 1	14.3	09.0
5h 30m 2	7.23	11.4
5h 30m 3	14.3	07.0
5h 30m 4	16.9	06.3
6h 1	14.7	04.8
6h 2	19.3	10.4
6h 3	12.0	19.4
6h 4	16.2	09.0
6h 30m 1	19.4	21.1
6h 30m 2	18.0	7.21
6h 30m 3	12.8	03.5
6h 30m 4	21.5	12.4
7h 1	18.3	12.4
7h 2	12.3	09.0
7h 3	17.2	19.5
7h 4	16.8	07.1

Appendix 6.4: Effect of low temperature on LUC activity in tobacco SLUCP 6-1 plants

Columns show the raw data for 4 different plants per each experimental group (time points, column 1).

Time point	**Treatment (RLU)**	
	24°C	**4°C**
0h 1	19.2	21.5
0h 2	25.3	19.5
0h 3	21.7	12.3
0h 4	16.5	16.2
15m 1	19.3	25.1
15m 2	22.7	29.1
15m 3	18.2	24.2
15m 4	16.1	11.2
30m 1	23.2	22.1
30m 2	16.4	18.2
30m 3	17.9	24.1
30m 4	19.5	27.3
1h 1	14.3	29.4
1h 2	21.6	23.5
1h 3	20.5	27.9
1h 4	27.6	18.2
1h 30m 1	15.7	23.5
1h 30m 2	10.0	19.4
1h 30m 3	22.8	17.3
1h 30m 4	17.6	22.3
2h 1	20.1	20.5
2h 2	19.5	23.1
2h 3	13.8	16.4
2h 4	21.0	0.00
2h 30m 1	12.3	21.9
2h 30m 2	27.1	20.1
2h 30m 3	16.9	19.3
2h 30m 4	18.6	16.5
3h 1	24.1	21.2
3h 2	19.3	19.8
3h 3	16.2	16.3

Time point	**Treatment (RLU)**	
	24°C	**4°C**
3h 4	12.4	17.0
3h 30m 1	21.1	21.3
3h 30m 2	18.2	17.2
3h 30m 3	18.0	16.3
3h 30m 4	22.1	13.0
4h 1	19.2	23.1
4h 2	15.7	15.4
4h 3	19.4	26.2
4h 4	18.7	18.3
4h 30m 1	16.3	16.5
4h 30m 2	19.4	17.9
4h 30m 3	20.0	20.6
4h 30m 4	13.4	16.6
5h 1	16.5	19.7
5h 2	27.2	13.4
5h 3	16.0	23.0
5h 4	17.6	10.5
5h 30m 1	28.1	10.5
5h 30m 2	16.1	11.4
5h 30m 3	12.3	06.1
5h 30m 4	25.1	21.4
6h 1	18.3	14.2
6h 2	19.7	12.3
6h 3	26.3	28.1
6h 4	13.5	8.04
6h 30m 1	18.5	11.3
6h 30m 2	14.0	7.98
6h 30m 3	26.1	08.4
6h 30m 4	19.7	14.1
7h 1	16.5	08.0
7h 2	21.3	10.4
7h 3	12.4	19.1
7h 4	15.4	12.4

Appendix 6.5: Effect of salicylic acid on LUC activity in tobacco SLUCP 6-1 plants

Columns show the raw data for 4 different plants per each experimental group (time points, column 1).

Time point	Treatment (RLU)	
	Buffer	SA
0h 1	10.3	10.2
0h 2	09.5	08.2
0h 3	07.4	13.4
0h 4	15.4	09.3
15m 1	10.3	14.1
15m 2	13.2	05.3
15m 3	05.3	12.1
15m 4	07.6	11.1
30m 1	13.4	11.3
30m 2	17.0	14.3
30m 3	06.2	07.2
30m 4	05.0	13.6
1h 1	14.3	09.1
1h 2	08.3	13.8
1h 3	12.1	16.5
1h 4	13.5	03.1
1h 30m 1	14.8	08.2
1h 30m 2	09.6	15.3
1h 30m 3	09.2	13.4
1h 30m 4	10.2	09.1
2h 1	08.2	15.2
2h 2	14.3	08.1
2h 3	15.2	14.1
2h 4	07.1	10.5
2h 30m 1	1345	03.5
2h 30m 2	14.4	14.2
2h 30m 3	12.37	10.1
2h 30m 4	08.1	11.5
3h 1	09.4	15.4
3h 2	12.1	09.1

Time point	**Treatment (RLU)**	
	Buffer	**SA**
3h 3	14.4	13.4
3h 4	13.7	10.4
3h 30m 1	14.6	16.5
3h 30m 2	11.4	12.4
3h 30m 3	09.5	8.07
3h 30m 4	10.9	13.4
4h 1	08.5	13.5
4h 2	12.4	10.8
4h 3	16.2	15.4
4h 4	08.4	12.4
4h 30m 1	08.0	13.4
4h 30m 2	12.4	06.2
4h 30m 3	11.4	11.4
4h 30m 4	07.3	13.5
5h 1	14.6	16.4
5h 2	08.2	12.3
5h 3	14.1	09.3
5h 4	11.6	11.3
5h 30m 1	14.2	13.5
5h 30m 2	09.4	16.4
5h 30m 3	13.2	10.1
5h 30m 4	11.1	12.5
6h 1	09.3	14.3
6h 2	10.5	10.2
6h 3	14.8	11.0
6h 4	14.7	14.1
6h 30m 1	13.7	07.3
6h 30m 2	10.2	11.2
6h 30m 3	08.2	14.2
6h 30m 4	11.0	12.3
7h 1	14.3	16.2
7h 2	11.1	10.2
7h 3	14.6	08.2
7h 4	08.2	10.1

Appendix 6.6: Single factor ANOVA for the virus concentrations at 6 dpi in PofC and PofI plants infected with TMV

Statistical significance of the difference in the virus concentration between the PofC and PofI plants was analyzed with single factor ANOVA. In bold are lines with statistically significant difference.

Groups	*Count*	*Sum*	*Average*	*Variance*
Ct	40	14.32	0.358	0.033
#1	11	0.68	0.061	0.006
#2	20	0.93	0.046	0.002
#3	20	2.06	0.103	0.028
#4	20	1.29	0.065	0.010
#5	20	5.40	0.270	0.049
#7	20	1.81	0.090	0.011
#8	20	5.48	0.274	0.055
#9	20	3.09	0.154	0.026
#10	20	6.46	0.323	0.075
#11	20	5.95	0.297	0.062
#12	20	5.93	0.296	0.065
#13	20	3.75	0.187	0.035
#14	20	3.29	0.164	0.023
#15	20	2.42	0.121	0.016
#16	20	4.70	0.235	0.033
#18	20	2.92	0.146	0.049
#19	20	3.33	0.166	0.026
#20	20	2.49	0.124	0.022

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Ct:#1	Between Groups	0.76	1	0.759	27.26	**3.61E-06**	4.04
	Within Groups	1.36	49	0.028			
Ct:#2	Between Groups	1.30	1	1.296	55.86	**4.68E-10**	4.0
	Within Groups	1.35	58	0.023			
Ct:#3	Between Groups	0.87	1	0.866	27.33	**2.44E-06**	4.0
	Within Groups	1.84	58	0.032			
Ct:#4	Between Groups	1.15	1	1.148	44.47	**1.05E-08**	4.0
	Within Groups	1.50	58	0.026			
Ct:#5	Between Groups	0.10	1	0.104	2.70	0.11	4.0
	Within Groups	2.22	58	0.0384			
Ct:#7	Between Groups	0.95	1	0.955	36.77	**1.07E-07**	4.0
	Within Groups	1.51	58	0.026			
Ct:#8	Between Groups	0.09	1	0.094	2.33	0.13	4.0

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Within Groups	2.34	58	0.040			
Ct:#9	Between Groups	0.55	1	0.553	17.97	**8.17E-05**	4.0
	Within Groups	1.79	58	0.031			
Ct:#10	Between Groups	0.02	1	0.016	0.35	0.56	4.0
	Within Groups	2.73	58	0.047			
Ct:#11	Between Groups	0.05	1	0.049	1.14	0.29	4.0
	Within Groups	2.49	58	0.043			
Ct:#12	Between Groups	0.05	1	0.051	1.16	0.29	4.0
	Within Groups	2.53	58	0.044			
Ct:#13	Between Groups	0.39	1	0.388	11.43	**1.30E-03**	4.0
	Within Groups	1.97	58	0.034			
Ct:#14	Between Groups	0.50	1	0.499	16.74	**1.34E-04**	4.0
	Within Groups	1.73	58	0.030			
Ct:#15	Between Groups	0.75	1	0.751	27.12	**2.63E-06**	4.0
	Within Groups	1.60	58	0.028			
Ct:#16	Between Groups	0.20	1	0.201	6.07	**0.017**	4.0
	Within Groups	1.92	58	0.033			
Ct:#18	Between Groups	0.60	1	0.600	15.54	**2.20E-04**	4.0
	Within Groups	2.24	58	0.039			
Ct:#19	Between Groups	0.49	1	0.490	15.88	**1.91E-04**	4.0
	Within Groups	1.79	58	0.031			
Ct:#20	Between Groups	0.73	1	0.727	24.58	**6.55E-06**	4.0
	Within Groups	1.72	58	0.030			

Appendix 6.7: Single factor ANOVA for the virus concentrations at 9 dpi in PofC and PofI plants infected with TMV

Statistical significance of the difference in the virus concentration between the PofC and PofI plants was analyzed with single factor ANOVA. In bold are lines with statistically significant difference.

Groups	*Count*	*Sum*	*Average*	*Variance*
Ct	40	20.22	0.506	0.046
#1	11	3.24	0.295	0.034
#2	20	4.20	0.210	0.019
#3	20	2.61	0.131	0.004
#4	20	8.49	0.424	0.059
#5	20	9.42	0.471	0.109
#7	20	5.06	0.253	0.024
#8	20	5.94	0.297	0.048
#9	20	3.09	0.154	0.026
#10	20	12.42	0.621	0.040
#11	20	10.07	0.503	0.117
#12	20	13.16	0.658	0.066
#13	20	10.84	0.542	0.038
#14	20	7.17	0.358	0.021
#15	20	5.83	0.292	0.023
#16	20	8.12	0.406	0.048
#18	20	7.00	0.350	0.061
#19	20	9.88	0.494	0.032
#20	20	7.25	0.362	0.028

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Ct:#1	Between Groups	0.38	1	0.383	8.87	**0.004**	4.04
	Within Groups	2.11	49	0.043			
Ct:#2	Between Groups	1.16	1	1.164	31.66	**5.58E-07**	4.0
	Within Groups	2.13	58	0.037			
Ct:#3	Between Groups	1.88	1	1.876	58.88	**2.17E-10**	4.0
	Within Groups	1.85	58	0.032			
Ct:#4	Between Groups	0.09	1	0.088	1.75	0.191	4.0
	Within Groups	2.90	58	0.050			
Ct:#5	Between Groups	0.02	1	0.016	0.24	0.623	4.0
	Within Groups	3.85	58	0.066			
Ct:#7	Between Groups	0.85	1	0.852	22.21	**1.57E-05**	4.0
	Within Groups	2.23	58	0.038			
Ct:#8	Between Groups	0.58	1	0.579	12.53	**7.95E-04**	4.0
	Within Groups	2.68	58	0.046			

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Ct:#9	Between Groups	1.65	1	1.646	42.22	**2.03E-08**	4.0
	Within Groups	2.26	58	0.039			
Ct:#10	Between Groups	0.18	1	0.177	4.06	**0.047**	4.0
	Within Groups	2.54	58	0.044			
Ct:#11	Between Groups	6.01E-05	1	6.01E-05	0.0009	0.977	4.0
	Within Groups	4.00	58	0.069			
Ct:#12	Between Groups	0.31	1	0.310	5.94	**0.018**	4.0
	Within Groups	3.02	58	0.052			
Ct:#13	Between Groups	0.02	1	0.017	0.41	0.526	4.0
	Within Groups	2.50	58	0.043			
Ct:#14	Between Groups	0.29	1	0.289	7.68	**0.007**	4.0
	Within Groups	2.18	58	0.038			
Ct:#15	Between Groups	0.61	1	0.611	15.98	**1.83E-04**	4.0
	Within Groups	2.22	58	0.038			
Ct:#16	Between Groups	0.13	1	0.132	2.85	**0.097**	4.0
	Within Groups	2.69	58	0.046			
Ct:#18	Between Groups	0.32	1	0.323	6.38	**0.014**	4.0
	Within Groups	2.94	58	0.051			
Ct:#19	Between Groups	0.002	1	0.002	0.04	0.838	4.0
	Within Groups	2.39	58	0.041			
Ct:#20	Between Groups	0.27	1	0.273	6.90	**0.011**	4.0
	Within Groups	2.30	58	0.040			

Appendix 6.8: Single factor ANOVA for the virus concentrations at 12 dpi in PofC and PofI plants infected with TMV

Statistical significance of the difference in the virus concentration between the PofC and PofI plants was analyzed with single factor ANOVA. In bold are lines with statistically significant difference.

Groups	*Count*	*Sum*	*Average*	*Variance*
Ct	40	8.80	0.220	0.013
#1	11	2.81	0.255	0.012
#2	20	8.70	0.435	0.020
#3	20	9.45	0.472	0.080
#4	20	7.51	0.375	0.034
#5	20	10.17	0.509	0.045
#7	20	6.76	0.338	0.014
#8	20	8.91	0.445	0.083
#9	20	7.59	0.380	0.048
#10	20	6.36	0.318	0.043
#11	20	4.97	0.248	0.032
#12	20	7.37	0.369	0.052
#13	20	8.44	0.422	0.143
#14	20	7.77	0.389	0.018
#15	20	7.95	0.398	0.024
#16	20	6.28	0.314	0.033
#18	20	7.74	0.387	0.044
#19	20	4.08	0.204	0.034
#20	20	7.25	0.362	0.028

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Ct:#1	Between Groups	0.01	1	0.011	0.85	0.360	4.04
	Within Groups	0.62	49	0.013			
Ct:#2	Between Groups	0.62	1	0.617	40.41	**3.49E-08**	4.0
	Within Groups	0.89	58	0.015			
Ct:#3	Between Groups	0.85	1	0.850	24.33	**7.17E-06**	4.0
	Within Groups	2.03	58	0.035			
Ct:#4	Between Groups	0.32	1	0.322	16.37	**1.56E-04**	4.0
	Within Groups	1.14	58	0.020			
Ct:#5	Between	1.11	1	1.112	47.18	**4.86E-09**	4.0

	Groups						
	Within Groups	1.37	58	0.024			
Ct:#7	Between Groups	0.19	1	0.186	13.85	**4.49E-04**	4.0
	Within Groups	0.78	58	0.013			
Ct:#8	Between Groups	0.68	1	0.678	18.87	**5.69E-05**	4.0
	Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
	Within Groups	2.08	58	0.036			
Ct:#9	Between Groups	0.34	1	0.340	13.99	**4.23E-04**	4.0
	Within Groups	1.41	58	0.024			
Ct:#10	Between Groups	0.13	1	0.128	5.61	**0.021**	4.0
	Within Groups	1.33	58	0.023			
Ct:#11	Between Groups	0.01	1	0.011	0.57	0.454	4.0
	Within Groups	1.11	58	0.019			
Ct:#12	Between Groups	0.29	1	0.295	11.38	**0.001**	4.0
	Within Groups	1.50	58	0.026			
Ct:#13	Between Groups	0.02	1	0.017	0.41	0.526	4.0
	Within Groups	2.49	58	0.0430			
Ct:#14	Between Groups	0.38	1	0.379	26.08	**3.81E-06**	4.0
	Within Groups	0.84	58	0.015			
Ct:#15	Between Groups	0.42	1	0.422	25.67	**4.41E-06**	4.0
	Within Groups	0.95	58	0.016			
Ct:#16	Between Groups	0.12	1	0.118	6.06	**0.017**	4.0
	Within Groups	1.13	58	0.019			
Ct:#18	Between Groups	0.37	1	0.372	16.02	**1.80E-04**	4.0
	Within Groups	1.35	58	0.023			
Ct:#19	Between Groups	0.003	1	0.003	0.16	0.688	4.0
	Within Groups	1.16	58	0.020			
Ct:#20	Between Groups	0.27	1	0.271	15.22	**2.51E-04**	4.0
	Within Groups	1.03	58	0.018			

Appendix 6.9: Single factor ANOVA for the methylation status in actin loci of PofC and PofI plants

Statistical significance of the difference in the methylation status of actin loci between the PofC and PofI plants was analyzed with single factor ANOVA.

Groups	*Count*	*Sum*	*Average*	*Variance*
P_of_C#3	14	211.4	15.1	658.8
P_of_I#3	14	824.3	58.9	336.6
P_of_C#4	14	36.9	2.6	19.5
P_of_I#4	14	248.5	17.7	136.1
P_of_C#7	14	443.0	31.6	321.9
P_of_I#7	14	766.3	54.7	484.9
P_of_C#8	14	472.5	33.7	358.6
P_of_I#8	14	615.3	43.9	274.8

	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
P_of_C#3/	Between Groups	13413.75	1	13413.75	26.95	2.03E-05	4.2
P_of_I#3	Within Groups	12940.45	26	497.71			
P_of_C#4/	Between Groups	1598.79	1	1598.79	20.55	0.0001	4.2
P_of_I#4	Within Groups	2022.77	26	77.80			
P_of_C#7/	Between Groups	3732.96	1	3732.96	9.254	0.005	4.2
P_of_I#7	Within Groups	10488.05	26	403.39			
P_of_C#8/	Between Groups	728.28	1	728.28	2.30	0.14	4.2
P_of_I#8	Within Groups	8234.49	26	316.71			