THE EFFECT OF PATHOGENS ON PLANT GENOME STABILITY

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Abstract

Resistance (R) genes, a key factor in determining the resistance of plants, have been shown often to be highly allelic entities existing in duplicated regions of the genome. This characteristic suggests that R-gene acquisition may have arisen through frequent genetic rearrangements as a result of transient, reduced genome stability. Tobacco plants transgenic for a recombination construct exhibited reduced genome stability upon infection with a virulent pathogen (tobacco mosaic virus). The reduced genome stability manifested as an increase in recombination events in the transgene. Such increases were observed following a virulent pathogen attack. This increase in recombination was shown to be systemic and was observed prior to systemic viral movement suggesting the presence of a systemic recombination signal. Further molecular analyses revealed that specific R-gene loci experience a large frequency of rearrangements following a virulent pathogen encounter. The possible targeting of instability to R-gene regions may be controlled through epigenetic processes, in particular, DNA methylation.

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LIST OF ABBREVIATIONS

Avr	avirulent determinants
cDNA	complimentary deoxyribonucleic acid
cv	cultivar
dpi	days post infection
dpg	days post germination
FMI	Friedrich Miescher Institute
GUS	β-glucuronidase
H_2O_2	peroxide
HPLC	high pressure liquid chromatography
HR	hypersensitive response
Ig	immunoglobulin
JA	jasmonic acid
LRR	leucine rich repeats
LUC	luciferase gene
MHC	major histocompatability complex
mRNA	messenger ribonucleic acid
NAC	N-acetyl-cysteine
NaCl	sodium chloride
ORMV	Oilseed rape mosaic virus
PCR	polymerase chain reaction
PofC	progeny of control plants
PofI	progeny of infected plants

PR	pathogenesis related gene
R-gene	resistance gene
RB	rose Bengal
RFLP	restriction fragment length polymorphism
ROS	radical oxygen species
RT	reverse transcription
SA	salicylic acid
SAR	systemic acquire resistance
SRS	systemic recombination signal
TMV	tobacco mosaic virus
tt4	flavonol mutant *tt4*
tt5	flavonol mutant *tt5*
UV	ultraviolet radiation
vtc	vitamin C mutant

1. INTRODUCTION

Gene-for gene disease resistance in plants is the result of sub-cellular interactions between plant host factors and pathogen virulence determinants (Flor, 1971). Resistant genes (R-genes) capable of recognizing the onset of a pathogen attack and initiating the steps necessary to combat and halt disease progression are conserved in numerous species. Although this interaction was discovered more than a century ago, the molecular basis of the process by which plants are capable of adapting to different pathogens has not been characterized. The existence of R-genes in highly duplicated regions of DNA suggests that R-genes have arisen from frequent duplication events (Baumgarten, *et al.*, 2003; Kroymann, *et al.*, 2003).

Genomic stability may be described as the potential of a genome to maintain its integrity by resisting, preventing or reversing changes in it's genetic sequence. More unstable genomes are prone to frequent DNA rearrangements resulting in point mutations, insertions, deletions, recombination, and transposon mobility. The occurrence of such rearrangements may affect the resistant phenotype of the plant to varying extents possibly resulting in the expression of novel traits. Organisms, specific tissues, or cells demonstrating a larger propensity to acquire various genomic changes are said to have reduced genome stability.

Reduced genome stability has previously been observed in plants following various abiotic stress conditions such as changes in light spectra (Ries, *et al.*, 2000b), radiation (Ries, *et al.*, 2000a; Kovalchuk, *et al.* 2003b), chemical mutagens (Brennan and Schiestl, 1998; Kovalchuk, *et al.*, 2003a), toxic substances such as herbicides (Besplug, *et al.*, 2003; Filkowski, *et al.*, 2003), temperature (Jiang, *et al.*, 2003; Boyko, personal

communication), nutrient availability (Boyko, personal communication) and water stress (Kalendar, *et al.,* 2000). Furthermore, genetic rearrangements have been induced following pathogen encounters. Lucht, *et al.* (2002) used a transgenic recombination assay to demonstrate that infection with an avirulent pathogen results in an increase in recombination events in *Arabidopsis*. In addition, work by Sudupak, *et al.*, (1993) and Richter, *et al.*, (1995) demonstrated that recombination events were specifically seen in a maize R-gene. Under the gene-for-gene model of resistance, a plant that possesses a larger number of R-genes has a higher probability of experiencing resistance to a wider range of pathogens (Flor, 1971). Therefore, in an attempt to achieve more durable plant species, it would be beneficial to understand the evolutionary mechanism by which novel R-genes may be acquired.

It may be hypothesized that susceptible plants reduce genome stability following pathogen encounter as a mechanism to acquire novel resistant phenotypes. This study investigated genome stability in susceptible tobacco plants inoculated with a virulent pathogen and their progeny. Genome stability was monitored by measuring recombination events in tobacco plants harboring a luciferase gene recombination construct, in an endogenous gene, as well as with restriction fragment length polymorphism analysis. Recombination was initially assayed in the treated and systemic tissues of plants challenged with tobacco mosaic virus (TMV). Leaf grafting experiments were performed and recombination was assayed in recipient plants to investigate the possibility of a systemic signal that was capable of affecting genetic stability in non-exposed tissues. If destabilization of the genome is a mechanism to acquire novel resistance traits, it was necessary to observe evidence of reduced genomic stability in the

progeny of TMV infected parents, specifically, in R-gene loci. Thus, progeny of infected plants were screened for genome rearrangements in R-gene-like regions that carry a significant degree of homology to the N-gene (resistance gene for TMV) by probing their restricted DNA with the 4th exon of a known R-gene (Whitman, *et al.*, 1994). The current study also examined the possibility that DNA methylation plays a role in altering the genetic stability of pathogen challenged plants. Finally, radical oxygen species were investigated as a possible trigger capable of inducing reduced genomic stability.

3. LITERATURE REVIEW

3.1. Disease resistance in plants

In plants, the presence of disease may be defined as any biotic disruption of the normal metabolism of the organism. Plant diseases are often caused by penetration of tissues by microorganisms such as bacteria and viruses or parasitic eukaryotes such as fungi and nematodes. These pathogens harm the host by absorbing necessary nutrients, blocking or redirecting the transport of food, nutrients and/or water, disturbing basal cellular metabolic processes or consuming the host upon contact (Jorgensen, 1994).

In order for a pathogen to successfully inhabit a host plant it must be able to enter the plant tissues, obtain nutrients and neutralize host defense strategies. In the absence of an "immune system", plants have evolved several strategies to cope with and resist the onset and progression of pathogen induced disease. These strategies fall into four categories: 1. Structural barriers; 2. Pre-existing chemical inhibitors; 3. Induced chemical inhibitors; and 4. Gene-for-gene interactions. These defense strategies may also be segregated into two broad groups: general defense that displays no specificity to any particular pathogen, and gene-for-gene defense that is launched only after a specific pathogen has been detected (Taiz and Zeiger, 2002). Disease resistance has been qualitatively understood for centuries. Selective breeding between different cultivars of particular plant species has occurred since the advent of agricultural innovation. However, specific mechanisms of resistance have only been understood for the last century. Most recently, revelations in genetic knowledge and techniques has provided an ability to dissect the molecular mechanisms involved in plant disease resistance, i.e., gene-for-gene resistance (Flor, 1971; Whitman, *et al.*, 1994; Glazebrook; 2001).

3.1.1. General defense mechanisms

General defense strategies include constitutive preformed physical (will be discussed in 2.1.1.1) and chemical components (will be discussed in 2.1.1.2). These are responsive, dynamic entities that may be altered or intensified under stressful times; yet, these barriers sustain a general capacity of defense as they still do not display specificity toward the attacker (Taiz and Zeiger, 2002).

3.1.1.1. Mechanical defense

A common facet to all plants is the presence of a cell wall: a critical structure in providing a high level of primary protection by acting as an initial, physical barrier to pathogen infection. The plant cell wall is a tough, impermeable structure that is constructed of several durable substances such as lignin, hemicellulose, cellulose, pectic substances, and cutin (Taiz and Zeiger, 2002). These substances require enzymatic degradation or mechanic disruption before a pathogen can infect the host, and therefore, limit the number of threatening pathogens to those that produce degradative enzymes or are capable of physical damage. In addition to the cell wall, plants possess an existing, unique, superficial defense structure in the cuticle; i.e., a wax-containing layer that coats virtually all plants. As of yet, no pathogen is known to possess enzymes capable of degrading the wax in the cuticle; therefore, only mechanical disruption permits the penetration of a pathogen leading to infection. In addition to providing protection by impeding penetration, the cuticle repels water creating a poor environment for pathogen germination and propagation, thereby deterring prolonged pathogen presence on plant

surfaces (Taiz and Zeiger, 2002). Collectively, the presence of these structures limits successful pathogen ingression to either sites of wounding or to pathogens capable of circumventing the cell wall components (via enzymatic degradation or through stomata).

In the instance that wounding (either physical or chemical) of the cell wall structure has permitted successful pathogen penetration, the existing complement of wall components may be reinforced so as to strengthen it for future attacks (Brisson, *et al.*, 1994; Jorgensen, 1994; McLusky, *et al.*, 1999). Further mechanical defense may be supplied by the formation of additional structures such as tyloses (over growth of protoplasts of adjacent parenchyma cells that protrude into xylem vessels through pits), or a suberized layer (formation of cork layers over wound sites) (Taiz and Zeiger, 2002). These structures are formed for the purpose of stopping the ingress of, flow of nutrients to and/or flow of possible toxins from the invading pathogen. Infected plants may also induce abscission by reducing auxin-to-ethylene ratios in abscission layers, causing the complete dissociation of infected plant parts from the remaining, healthy plant (Taiz and Zeiger, 2002).

3.1.1.2. Defense compounds

Preformed chemical inhibitors to pathogen attacks are formed via secondary metabolic pathways. These compounds are not required for the necessary metabolic processes of the plant, but they provide an added benefit by facilitating resistance of a host to pathogen infection. Plants lacking these preformed chemicals are compromised in disease resistance (Papadopoulou, *et al.*, 1999). Secondary metabolites such as phenolic compounds are active on several levels of plant resistance. They are involved in:

mechanical defenses such as cell wall composition (lignin), signaling (salicylic acid), and toxic effects on pathogens (isoflavones) (Taiz and Zeiger, 2002). Yao, *et al.*, (1995) demonstrated that plants compromised in their ability to produce phenolic compounds exhibited depressed levels of resistance as compared to their non-compromised counterparts. Conversely, plants altered to over-produce a phenolic compound also experienced enhanced disease resistance (Li and Steffens, 2002). Plant phenolics operate through a number of mechanisms. Specifically, these include degradation of integral parts of the invading entity, inactivation of pathogen enzymes, acting as toxic substances, or detoxification of pathogen toxins (Taiz and Zeiger, 2002; Williams, *et al.*, 2002). Similar to reinforcement of mechanical barriers, plants are also capable of producing secondary metabolites on demand.

3.1.2. Induced defense

The induced mechanisms of combating pathogen invasion likely require some notice or signal that superficial physical barriers (cuticle and cell wall) have been breached, thereby alluding to the existence of a cell surface detection system. Although a non-specific, surface receptor system detecting pathogen presence has not been explicitly identified, its existence is strongly implied by the mechanism of infection of pathogens such as *Agrobacterium tumefaciens* (Duban, *et al.*, 1993), identification of receptors with similarity to mammalian and insect domains frequently seen in innate immunity (Gomez-Gomez and Boller, 2000) as well as observation of early defense onset (Alan, *et al.*, 2001).

Genomic analyses have identified a close similarity in the response of a plant to abiotic stress factors and pathogen attack (Cheong, *et al.*, 2002). Many of the expressed characteristics such as gene induction, physical modifications, signaling molecules and secondary metabolite accumulation are observed following pathogen attack and are identified following acute exposure to abiotic factors such as increased ozone, wounding, UVC, UVB or chemical elicitors (Dat, *et al.*, 2000; Cheong, *et al.*, 2002; Mahalingam, *et al.*, 2003). These observations indicate that plants have evolved to rely on a few broad responses to resist any threat to their homeostatic state. Mahalingam, *et al.*, (2003) demonstrated that 1058 genes experience altered transcription following a stress event, either biotic or abiotic, and that 55% of these genes fall into the rarely transcribed category. These results imply that plant genomes include a number of genes that exist to help plants cope with a wide number of stresses encountered in their sedentary life style (Mahalingam, *et al.*, 2003); (e.g., drought, high salinity, wounding, and disease).

Plants exhibit two types of induced defense responses. The first is termed induced systemic resistance (ISR) and has been shown to be induced by a number of bacteria. For example, ISR arises as a result of wounding from root colonizing, non-pathogenic rhizobacteria (Pieterse, *et al.*, 1996). The second, systemic acquired resistance (SAR) occurs as a result of an incompatible pathogen reaction and is normally preceded by a hypersensitive response (Dangl, *et al.*, 1996; Ryals, *et al.*, 1996). Both pathways ultimately create a state of systemic immunity for the plant to a broad range of stress. Although they differ in the mode of induction (wounding-versus-pathogen recognition), signal transduction (jasmonic acid and ethylene as compared to salicylic acid (SA) and pathogenesis-related (PR) gene induction) (Pieterse, *et al.*, 1996) and may

at times act as inhibitors to one another, they also may act in concert providing cross-resistance between each system (Kunkel and Brooks, 2002). The HR/ SAR will be the focus of the remainder of this review.

A hypersensitive response and systemic acquired resistance characterize a resistant state following an avirulent pathogen attack. A virulence determinant is a necessary component of the pathogen's repertoire (usually manifested as a toxin, hydrolytic enzyme or inhibitor) for establishing disease in the host (Hawkesford and Buchner, 2001). Resistance is only achieved if the attacking pathogen expresses a determinant that is detected by a corresponding R-gene. In the instance that either the R-gene or *Avr* factor are absent, infection will progress. The plant becomes diseased and is said to be susceptible. Pathogen interactions with a susceptible plant are said to be the result of infection by a virulent pathogen and are termed a compatible reaction. Interactions that culminate in a resistance are affected through an avirulent pathogen or incompatible reaction (Flor, 1971).

Interaction with an incompatible pathogen may occur directly, with *Avr* factors from a particular pathogen being recognized by an R-protein, or indirectly with the R-gene product detecting alteration of one or more of the host's cellular components. The theory of indirect recognition (the guard model), stipulates that the R-protein acts as a "guard" of a particular endogenous entity, usually one on which *Avr* factors will act (van der Hoorn, *et al.,* 2002). The R-gene *Prf* in tomato plants is believed to act as a guard protein for a cellular kinase that regulates basal defense responses. The *AvrPto* of *Pseudomonas syringe* acts on this kinase. In plant mutants lacking the kinase but

possessing the *Prf* R-gene, the signal transduction leading resistance is not induced (van der Hoorn, *et al.*, 2002).

There is a higher level of efficiency and longevity in the guard model thesis, because rapid changes in *Avr* determinant type would allow a pathogen presence to go unnoticed if the interaction was direct; i.e., every adaptation in the *Avr* factor would necessitate an equivalent adaptation in the R-gene. Thus, a large number of differing R-genes would be required to provide effective resistance to the rapidly adapting pathogens. In the guard model, the R-gene product simply monitors changes in a native protein that is the target of *Avr* gene products. As long as the *Avr* target is not changed, the R-gene product is capable of recognizing the ensuing invasion of that pathogen and, hence, provides a more efficient model of gene-for-gene interaction (Tornero, *et al.,* 2002; van der Hoorn, *et al.,* 2002). When an R-protein recognizes a pathogen attack, it affects a number of signaling cascades and transcription factors culminating in a hypersensitive response in the immediately adjacent cells, and SAR in tissues distal to the site of infection.

The types of resistance that may result from such an R-gene – *Avr* recognition event, are either non-host or race-specific resistance. With non-host resistance all cultivars are resistant to infection by a specific pathogen. Race-specific resistance demonstrates a differing specificity of cultivars to various races of the pathogen (Hawkesford and Buchner, 2001). The phenomenon of race-specific versus non-host resistance provides empirical evidence of differing recognition strategies for R-genes. For example, multiple alleles are capable of conveying race-specific resistance to powdery mildew in barley; however, each allele seems specific to a particular *Avr* factor.

Consequently, as *Avr* factors are altered, a relatively frequent cyclic pattern of resistance to susceptibility and resistance is observed in the plant (Jorgensen, 1994). Race-specific resistance may be described as the result of directly reacting R-genes, whose capabilities are limited to recognition of a specific *Avr* factor, the narrow specificity of this reaction arising from the direct binding interaction between the R-gene product and the *Avr* factor. For example, powdery mildew resistance in barley has been shown to be a result of at least 85 R-genes existing in at least 10 loci throughout the genome, providing differing functions/recognitions in resistance (Jorgensen, 1994). On the other hand, non-host resistance may be (at least) partially, the result of guard-model type recognition. With indirect perception, a broader specificity of recognition is achieved, possibly rendering the plant capable of identifying the pathogen presence as opposed to merely perceiving the *Avr* particle. However, it is important to note that induction of the aforementioned constitutive defense characteristics (i.e., crosslinking of the cell wall and increase in phenolic compounds) is the usual reason for a non-host resistant state (Heath, 2000).

3.1.2.1. Hypersensitive response

The hypersensitive response has been shown to be a prudent method by which invading pathogens are sequestered and even killed. The characteristic event of mounting a HR is a large, rapid increase in free radical oxygen species (ROS), and is one of the earliest steps in the inducible defense response (Dangl, *et al.*, 1996). Although the high ROS levels stimulate apoptotic death processes in these local cells, there are two positive outcomes of the cell death. First, ROS are toxic species capable of killing all living cells, and therefore, are capable of killing the pathogen. Second, the ROS burst

produces a physical barrier of dead host cells, which in the event that the pathogen survives the ROS burst, prevents migration to new, healthy tissues (Dangl, *et al.,* 1996; Mittler, *et al., 1*999).

The radical burst includes a number of components with varying potential for cellular damage. Initially, superoxide anion (O_2^-) levels rapidly increase but, with a half life of less than a second; this species is short lived. The O_2^- is dismutated to hydrogen peroxide (H_2O_2) enzymatically via superoxide dismutase (SOD) or non-enzymatically in the presence of a metal catalyst. H_2O_2 may also be protonated yielding a hydroperoxyl radical (HO_2^-). The HO_2^- radical is capable of converting membrane lipids to toxic lipid peroxides (Grant and Loake, 2000). In addition, H_2O_2 may undergo the Fenton reaction in the presence of iron producing a hydroxyl radical; i.e., a self-propagating species capable of damaging lipids, proteins and nucleic acids (the only radical mentioned thus far capable of directly damaging DNA) (Finkl, 2001).

A number of possible mechanisms may generate an oxidative burst. Besides the omnipresent sources of radicals due to regular metabolism (Moller, 2001; Munne- Bosch and Alegre, 2002), an NADPH-dependent oxygenase has been proposed as a major source for O_2^- production. This oxygenase, a membrane bound, holoenzyme is responsible for producing the oxidative burst of O_2^- in human neutrophils. Evidence suggests that a plant protein with activity similar to the mammalian, NADPH-dependent oxygenase may exist. Antibodies raised against three human subunits ($p22^{phox}$, $p47^{phox}$ and $p67^{phox}$) react with proteins of comparable size in plants; known inhibitors of the neutrophil burst also suppress ROS accumulation in plants, and cDNA of one of the

human subunits (gp91^{phox}) displays high homology to rice and *Arabidopsis* homologs of the protein (Groom, *et al.*, 1996; Keller, *et al.*, 1998).

It has also been suggested that the radical burst fulfills the initiation of signaling events that ultimately will alter gene transcription and mount SAR in distal tissues (Chamnongpol, *et al.*, 1998; Dat, *et al.*, 2000). Interestingly, the H_2O_2 profile demonstrates a biphasic accumulation with an initial burst occurring in both an avirulent (incompatible) and virulent (compatible) attack within one hour of infection, and a second burst occurring only in the avirulent exposure by two hours post-infection. The purpose of the initial oxidative burst is of interest since SAR is not established until the second ROS accumulation has occurred (Grant and Loake, 2000), and it is seen in the compatible reactions where SAR is absent. Other possible purposes of this ROS burst in virulent attacks will be discussed later in 5.6.

3.1.2.2. Systemic acquired resistance

SAR may be described as the ability of previously challenged plants to detect and defend against a broad spectrum of future pathogen attacks—often referred to as plant immunity (Dong, 2001). Acquisition of a SAR state typically follows the initiation of a HR and is dependent upon accumulation of SA (Ryals, *et al.*, 1996).

The importance of SA in this mechanism is revealed though several observations. First, transgenic plants expressing bacterial enzymes that degrade SA are also incapable of mounting SAR (Gaffney, *et al.*, 1993); second, the SAR state is induced in plants following exogenous SA application (Dong, 2001); third, chemical analogs of SA are also capable of mounting an efficient SAR state (Gorlach, *et al.*, 1996) and fourth, mutant

plants which constitutively express SA, demonstrate enhanced resistance to both avirulent and virulent pathogens (Yu, *et al.*, 1998). During SAR, PR gene expression is up-regulated. The protein products of these genes typically act as antimicrobial proteins such as chitinases and glucanases (Hawkesford and Buchner, 2001). In addition, and as described above, SAR induction leads to a reinforcement of the general defense characteristics of plants including cell wall strengthening (i.e., production of lignin, callose, and cell wall proteins) and secondary metabolite production (phytoalexins). Furthermore, R-gene expression is increased in SAR tissues with the result being a more rapid response to future invasion in the unaffected tissues (Ryals, *et al.*, 1996; Dong, 2001). Interestingly, SAR is maintained for a prolonged period of time, up to several weeks, following the initial attack, demonstrating an attempt by plants to strengthen the probability of survival for the purpose of creating progeny (Dong, 2001).

3.2. R-genes

In plant disease resistance, immunity is achieved through the expression of a particular gene, which recognizes a specific race of pathogen in a process termed gene-for-gene interaction (Flor, 1971). R-genes are responsible for recognizing the presence of a pathogen and many have been characterized at the molecular level in higher plants including *Arabidopsis*, lettuce, barley, maize and rice (Sudupak, *et al.*, 1993; Jorgensen, 1994; Botella, *et al.*, 1997; Dangl and Jones, 2001; Li, *et al.*, 2001).

With the gene-for-gene model, it is logical to postulate that it would be beneficial for a plant to possess numerous R-genes, each recognizing a different pathogen or race, in order to widen the spectrum of avirulent pathogens (through increasing the likelihood of

an incompatible interaction). This theory is supported by the evidence that most existing R-genes occur in highly duplicated, clustered loci within numerous plant genomes including *Arabidopsis*, barley, rice, maize, flax, lettuce and tomato (Hulbert and Michelmore, 1985; Jorgensen, 1994; Botella, *et al.*, 1997; Leister, *et al.,* 1998; Tornero, *et al.,*). Furthermore, multiple gene sites may include genes with different alleles for resistance to the same pathogen (intragenic recombination) or genes present in different loci have identical specificity (intergenic recombination) (Hulbert and Michelmore, 1985; Jorgensen, 1994; Crute and Pink, 1996; Li, *et al.,* 2001).

The increased incidence of gene duplication as a result of recombination has, in some instances, produced numerous R-genes conferring some level of resistance to specific races of a pathogen. Although most R-genes are dominant, it has become clear that some R-gene phenotypes do not display simple, complete patterns of dominance but, rather exhibit epistatic patterns of dominance (Li, *et al.,* 2001). That is, a particular *R-Avr* pair provides a resistant state even though an expected incompatible reaction would occur as the result of another *r'-Avr* pair (where R and r' have identical pathogen specificities) (Li, *et al.,* 2001). In addition, R-gene activities have been shown to be strongly influenced by the presence of other genes. Collectively, this information suggests that the membership of most R-genes in a complex network of cellular functions will culminate in an incompatible interaction between host and pathogen—resistance (Glazebrook, 2001).

Furthermore, R-genes may display unexpected patterns of dominance due to interactions with environmental factors. For example, *N. tabacum* cv Havana contains a well characterized resistance gene towards TMV termed an N-gene. Under growth

conditions at 22°C, plants challenged with TMV display all the characteristics of disease resistance including the HR, oxidative burst and SAR. However, the same plants grown at 28°C or higher are susceptible to TMV (Boyko, *et al.,* 2000).

3.2.1. R-gene evolution

Evidence exists supporting the belief that gene duplication and recombination are the primary mechanisms for the production and accumulation of numerous R-genes in plants, each demonstrating resistance to different pathogens or races within a pathogen family (Sudupak, *et al.,* 1993; Whitham, *et al.,* 1994; Richter, *et al.,* 1995). These genes commonly exist in close proximity to one another, i.e., as tandem arrays of a number of gene copies. They are likely the result of duplication and rearrangement of a region of the chromosome (Baumgarten, *et al.,* 2003; Kroymann, *et al.,* 2003). Further compounded sequence differences are known as polymorphisms and indicate that mutations leading to amino acid changes are the mechanism through which novel R-gene functions arise (Dinesh-Kumar, *et al.,* 2000; Shirano, *et al.,* 2002). Obeying the tenets of Darwinian evolution, it can be suggested that R-genes arise and are maintained because of an external selection pressure. If an existing R-gene is present, able to detect the presence of a particular pathogen and inducing a defense response, then it may be suggested that it is in the lack of such a functional R-gene that a pressure favoring rearrangement and alteration occurs. The best scenario for this rearrangement is the production of a novel R-gene; one capable of recognizing the presence of an unfamiliar pathogen. Indeed, such novel resistances were previously reported by Richter, *et al.,* (1995). Of 176 rearrangement events that were observed in the maize progeny from

parents (of known resistance specificities), four events manifested as novel resistance to rust races to which both parents were susceptible.

As generations pass, a specific R-locus may demonstrate a high degree of allelic differences, i.e., mutations are accumulated in the sequences of duplicated gene copies and passed on vertically through the progeny. However, if these duplication events are frequent it would be expected that R-gene polymorphisms and alleles would be fewer, as the young age of such genes would prevent them from acquiring many alterations. However, some R-loci do display high allelic diversity indicating that they have been impervious to gross recombination events, and as a result a polymorphism has been maintained (Kroymann, *et al.,* 2003; Mauricio, *et al.,* 2003). Based on the previously mentioned theory of selection pressure favoring recombination, it would be expected that older R-gene regions are those that are frequently employed and, thus, are not required to undergo high levels of mutation (Tian, *et al.,* 2002). These alleles most likely represent non-host specific resistance, and a more durable phenotype, which may recognize an *Avr* determinant that cannot tolerate much change, and thus, has been unable to adapt and overcome the R-gene functions.

Evolution rates of a particular gene can be investigated by comparing the rate of synonymous amino acid changes with non-synonymous ones. If mutations occur randomly throughout a particular gene, then there is an equal expectation of synonymous or non-synonymous mutations occurring. However, if a non-synonymous mutation is somehow favored, then the ratio of the rate of synonymous alterations to the rate of non-synonymous ones should be greater than 1 (Bergelson, *et al.* 2001). This occurrence is known as adaptive evolution and has been observed in tomato, rice, lettuce, and

Arabidopsis as well as the MHC in mammals (Crute and Pink, 1996). In investigations of mutation rates in a particular class (TIR-NBS-LRR) of R-genes, the characteristic leucine rich region (LRR) was shown to be particularly unstable (Whitman, *et al.*, 1994). That is, the LRR in this class of R-genes displayed a high level of divergence from one another. These results, together with the belief that the LRR region often possesses a binding domain, support the hypothesis that the specificity of a particular R-gene is determined by this region (Shirano, *et al.*, 2002; Tornero, *et al.*, 2002; Mauricio, *et al.*, 2003). Thus, perhaps it is the LRR that may interact closely with *Avr* determinants (or the host factor they guard) as the recognition sequence for invading pathogens.

Early recombination assays focused on a known, particularly unstable maize R-gene (*Rp1*) and attempted to tie recombination events to unexpected, novel alterations in resistance phenotype (Richter, *et al.*, 1995). These assays relied on the alterations of different flanking regions, which had been introduced to either side of the *Rp1* locus, between parental and progeny generations to indicate interstrand exchange (crossover events). Sudupak, *et al.*, (1993) observed spontaneous meiotic recombination in two alleles of the *Rp1* gene locus by screening for susceptible progeny of resistant parents. In one allele, five individual progeny (of 9772 screened in total) experienced recombination events and exchange of the flanking regions. The second allele screened showed 19 progeny (of 5874 in total) that expressed flanking regions different from the parents. Richter, *et al.*, (1995) identified several recombination events (4/176 total events) that corresponded to unexpected resistance specificities in the progeny of parents with known *Rp1* susceptibility. All of the identified progeny had experienced recombination and, thus, the novel specificities were attributed to the occurrence of recombination.

3.2.1.1. Regulation of R-gene evolution

If novel R-gene production is the result of induced genome rearrangements, then it would be expected that these rearrangements are somehow targeted to R-gene loci. Cytosine methylation of DNA provides an important tool for gene regulation in higher eukaryotes (Brown, 1999). In plants, it may be presumed that a strong reliance on methylation exists as a large proportion of the genome contains highly repetitive sequence. Up to 30% of the genome is methylated suggesting that it may act as a stabilizing mechanism (Puchta and Hohn, 1996). Genomic methylation has multiple roles, including the suppression of genome rearrangements, i.e. transposable elements and recombination (Bender, 1998). Thus, the high level of cytosine methylation in plants is likely due in part to the frequent occurrence of repetitive sequences and gene duplication. The high frequency of repetitive sequences in plant genomes increases the possibility that strand breaks will be processed using the neighboring homologous sequences: the result being a higher number of rearrangements. The possible role of genome methylation in controlling DNA rearrangements has been suggested previously. Kovalchuk, *et al.* (2003c) have shown that methylation in higher plants exposed to extreme levels of ionizing radiation demonstrate hypermethylated genomes. Since this radiation has also been shown to induce strand breaks in DNA (Kovalchuk, *et al.,* 1998; 2003b), it was suggested that the excess methylation, acquired over multiple plant generations, might exist as a means to prevent massive, potentially deleterious DNA rearrangements. Similar results were observed in X-ray irradiated animal tissue (Kovalchuk, *et al.*, 2004)

3.3. Genome stability

Control of genome instability is an issue of paramount importance for all living organisms. The maintenance and integrity of an organism's existing/functioning genome ensures a continuity of information between both generations of somatic cells and the production of viable progeny. However, since the advent of life a lack of genome maintenance is the primary reason for the wide variation of species as well as traits between individuals of the same species (Brown, 1999).

It may be argued that the plasticity and flexibility within a genome is just as important in ensuring the survival of future generations, as the ability to accurately replicate one's genome. For example, under times of stress an organism that possesses a particular trait that favors survival is more likely to produce offspring. However, if individuals were capable of inducing mechanisms that increased the likelihood of acquiring a novel, more favorable phenotype, they would inevitably be better suited for survival.

The evolution of genomes and, therefore, of organisms occurs through acquisition of mutations. Phenotypic alterations (loss/gain-of-function) may arise through a number of mechanisms including nucleotide substitutions, insertions, or deletions. However, recombination events yield the most profound changes in phenotype as it is through this process that entire genes are duplicated and rearranged (Brown, 1999).

The process of recombination necessitates stand breakage of DNA molecules. These strand breaks may be repaired through homologous recombination (strand exchange that requires a large homologous DNA sequence) (Puchta, *et al.,* 1996) or non-homologous end joining (illegitimate recombination). The latter does not require high

sequence homology, but most likely involves a specific, short recognition sequence for the enzymes involved (Tsukamoto and Ikeda, 1998). Both methods have great potential to result in genetic variation (Brown, 1999).

Specific examples of such a rapid change in the expression of genetic information caused by recombination events include Ig and MHC in the mammalian immune system as well as the yeast mating type switch (Abbas, *et al.*, 1994; Brown, 1999). Ig and MHC molecules display a highly variable region of gene expression. This variation is the result of instability (enormous plasticity) at the V(D)J locus of the genome. The V(D)J locus undergoes rapid reshuffling of gene patterns providing the potential to display up to 10^8 different proteins (different disease phenotypes/specificities) within the host (Abbas, *et al.*, 1994; van den Bosch, *et al.*, 2002). Similarly, the mating type switch in yeast involves a genetic alteration between a and α sexual types (Brown, 1999; van den Bosch, *et al.*, 2002). A parallel situation may be drawn from SOS mutagenesis in *E. coli* cells exposed to harsh conditions or DNA damaging agents. The capability of the cells to "purposely" facilitate the ability to acquire and propagate mutations provides further support that genetic alterations are, under some stressful circumstances, tolerated (Bjedov, *et al.*, 2003). In addition, specific genes (*Umu*D' and *Umu*C) exist that permit such mutations (Walker, 1995; Hendrickson, *et al.*, 2002). Furthermore, the plastic nature and system of rearrangements and alterations appears to be somewhat controlled in all instances. That is, increased shuffling and output combinations of the Ig and MHC genes occurs during times of pathogen attack, while the mating type switch is induced when variation of mating type in a localized population is wanting. SOS mutagenesis follows exposure to strong mutagens or starvation conditions (Walker, G.C. 1995;

Hendrickson, *et al.,* 2002). Collectively, this evidence suggests a Lamarck-like theory where environment can govern the occurrence of genetic alterations and mutations.

3.3.1. Plant genome stability

In plants, genome stability has been shown to be influenced by numerous factors including abiotic and biotic stress. Abiotic influences known to affect rearrangements in plants include harsh environmental conditions such as changes in light spectra (Ries, *et al.,* 2000b), radiation (Ries, *et al.*, 2000a; Kovalchuk, *et al.* 2003b), chemical mutagens (Brennan and Schiestl, 1998; Kovalchuk, *et al.*, 2003a), toxic substances such as herbicides (Besplug, *et al.*, 2003; Filkowski, *et al.*, 2003), temperature (Jiang, *et al.*, 2003; Boyko, personal communication), nutrient availability (Boyko, personal communication) and water stress (Kalendar, *et al.*, 2000). It is likely that the higher incidence of rearrangements in some of these instances, namely radiation, mutagen, and toxin exposure, may be attributed to the damaging effects on DNA, either directly or indirectly, of the exogenous agent. However, other factors such as temperature, nutrient and water availability are not overtly suspected to cause DNA damage. Thus, it would appear that strand breaks and the ensuing recombination are perhaps the "purposeful" result of some mechanism induced by unfavorable living conditions. Likewise, many biotic factors also are not categorized as DNA damaging species, yet appear to cause an increase in rearrangement frequencies (Beguiristain, *et al.*, 2001; Lucht, *et al.*, 2002).

Plants experience a perpetual biotic assault in the form of pathogens and insects. Similar to abiotic stresses, biotic influence can induce rearrangements at the genetic level. Retrotransposon activity and transposition has been shown to tightly correlate with

pathogen presence. Tobacco cell extracts experienced a nine fold increase in mobility of the Tnt1 retrotransposon as compared to non-elicited cells (Melayah, *et al.*, 2001). Similarly, differential induction was observed between different subfamilies of the Tnt1 transposon. These subfamilies appear to have evolved alterations in the promoter region allowing for increased activity following a number of stressful events (Beguiristain, *et al.*, 2001). In addition, Lucht, *et al.* (2002) have previously demonstrated that recombination increased in *Arabidopsis* plants challenged with an avirulent oomycete pathogen—*Peronospora parasitica.* Furthermore, this increase was also observed in plants that were exposed to mimicking conditions of a pathogen assault including application of chemical analogs of SA, and a gain of function mutant, *cim3* (constitutive immunity), that constitutively expresses high levels of SA, and thus, SAR, but without incidence of HR (Lucht, *et al.*, 2002).

Somatic recombination levels also appear sensitive to developmental stage; i.e., recombination events are differently distributed in tissues of different ages (Boyko, personal communication), and differ between plant species, i.e., as demonstrated between tobacco and *Arabidopsis* plants (Filkowski, *et al.* 2004). Although these results strongly suggest an induced mechanism of genetic instability in plants under stress, it is necessary to explore whether this increase is localized to a particular genome region, and what mechanism is responsible for inducing rearrangements.

3.4. Practical applications

The ideal of universal plant immunity, which is durable and practically implemented, may be considered an apex goal in agro-economics. Although plant immunity together with R-gene function has been recognized for over 40 years, attempts at "creating" or facilitating the production of highly resistant crops has as of yet not provided a viable, long-term resistant plant. The same pressure of natural selection that yielded numerous R-genes capable of imparting at least a degree of resistance to various pathogens, also may have selected for pathogens with an altered *Avr* factor. Thus, the existing *R-Avr* interaction leading to incompatible states became moot as *r-Avr'* became prevalent. The result being that selectively bred, highly resistant crops only maintained this state for a finite period of time (Johnson, 1961). In addition, often the cost outweighed the benefit imparted on the plant. When creating a plant with resistant qualities towards a particular pathogen it frequently became more sensitive to other pathogens and abiotic stress (Heil, 2002). In creating an indefinite resistant state to a specific pathogen, it will be necessary to isolate or produce an R-gene whose affinity is for a mandatory *Avr*; i.e., an *Avr* that is incapable of sustaining genetic variation, as it would be lethal (Crute and Pink, 1996). Since it is a trial and error type of process for plants to achieve a durable R-gene, it is important to understand the mechanisms responsible for gene duplication and phenotype alteration that created novel R-genes. Additionally, comprehension of the systemic signals initiated by stress events may lead to the production of plants that are multi-stress resistant.

4. MATERIALS AND METHODS

4.1. Plant cultivation

4.1.1. Tobacco cultivars Havana 425, Petit Havana SR1, Xanthi NN (*Su/su*), and *Nah*G; *vtc, tt4, tt5* and 651 *Arabidopsis* mutants

In the current work, *Nicotiana tabacum* cv Havana 425 (Friedrich Miescher Institute; Basel, Switzerland), *Nicotiana tabacum* cv Petit Havana SR1 (Friedrich Miescher Institute; Basel, Switzerland) and *Nicotiana tabacum* cv. Havana *NahG* (Friedrich Miescher Institute; Basel, Switzerland) all transgenic for a luciferase gene recombination construct, as well as, *Nicotiana tabacum* cv. Xanthi NN (*Su/su*) (Friedrich Miescher Institute; Basel, Switzerland), heterozygous for the sulfur gene that controls chlorophyll content were used. The latter one provided an endogenous system to quantify recombination events. The Havana 425, Havana *Nah*G, and Xanthi *Su/su* plants all possess a function N-gene that conveys TMV resistance at temperatures below 28°C. The Petit Havana SR1 plants do not contain an N-gene and, thus, are TMV sensitive at all temperatures.

Arabidopsis mutants compromised in the ability to accumulate flavonols (*tt4*, *tt5*) or vitamin C (*vtc*) and that were crossed with the *uidA* recombination reporter line 651 were obtained from B. Hohn (Friedrich Miescher Institute; Basel, Switzerland).

4.1.2. Growth conditions

Tobacco seeds sown on sterile, all purpose potting soil (Plants Etc; Lethbridge, AB, Canada) were left to germinate and grow in a Conviron growth chamber (Winnipeg, MB, Canada) with constant 85% humidity. Plants were grown under high light (32.8

$\mu E \cdot m^{-2} \cdot s^{-1}$) provided by Cool White Fluorescent bulbs (Sylvania, Mississauga, ON, Canada) and Longlife Incandescent bulbs (Sylvania, Mississauga, ON, Canada) during 16 hours a day at either 22°C or 32°C. The night temperature was 18°C. Growth of tobacco under sterile conditions occurred under the same light, temperature and humidity conditions.

Arabidopsis grew under sterile conditions at 22°C and high light (32.8 $\mu E \cdot m^{-2} \cdot s^{-1}$) provided by Cool White Halogen bulbs (Sylvania, Mississauga, ON, Canada) and Longlife Incandescent bulbs (Sylvania, Mississauga, ON, Canada) for 16 hours and at 18°C during the darkness period for a total of 20-24 days.

4.1.3. Sterilization of *Arabidopsis* and tobacco

Arabidopsis seeds were rinsed in 70% ethanol for two minutes, sterilized with a 0.5% sodium hypochlorite and 0.05% Tween-80 solution for five minutes and, finally, rinsed twice with a large excess of sterile, distilled water for five minutes each. Following sterilization, seeds were plated on MS basic medium (Murashige and Skoog medium (Sigma; Oakville, ON, Canada) + Murashige and Skoog vitamins (Sigma; Oakville, ON, Canada) + 20g/liter sucrose + agar) contained in an 85mm Petri dish. Seed dormancy was broken by keeping seeds at 4°C in the dark for two days before being moved to growth chambers for germination and growth.

Progeny of infected (PofI) and control (PofC) SR1 tobacco lines were sterilized as indicated above excluding the ethanol step. Seeds were plated on sterile filter paper saturated with sterile water.

4.1.4. Plant infection

Tobacco plants were exposed to 300 ng of full-length TMV or ORMV RNA (M. Heinlein; Strasbourg, France) contained in a 100 mM phosphate buffer through topical application of the upper surface of the lowest, mature, healthy leaf at the 6-8 weeks old stage. To facilitate virus penetration, silicon carbide was utilized at the site of inoculation and rubbed onto the leaf. Mock-inoculated plants were treated with 100 mM phosphate buffer only. In order to assess whether the abrasive rubbing was responsible for recombination increases, control plants, that received no rubbing, were assayed as well. Plants were left to grow at 32°C (Havana 425, *Su/su*) or 22°C (Havana 425, Petit Havana SR1) under the previously mentioned (light and humidity) growth conditions for approximately 10 to 14 days. Recombination was scored after this time. Eight to twelve plants were treated for each group and recombination events were quantified in three to four leaves of each plant.

In order to assess the time frame of viral mobility throughout the plant, 50 Havana 425 plants were infected at 32°C. At this temperature, cv Havana 425 plants are not resistant to TMV or ORMV infection. The TMV treated leaves were removed at various intervals over a 72 hour period (0 min, 8, 24, 36, 48 and 72 hours). Disease progression in these plants was visually assessed 7-14 days post-infection (dpi) in the non-inoculated leaf tissue. Molecular analysis was carried out 14 dpi and included testing for the presence of viral RNA in tissue distal to the infection.

4.1.5. Grafting procedure

The healthiest, mature leaf was selected for grafting activities. This usually corresponded to the fourth or fifth leaf from the top. The graft donor leaf was excised by cutting parallel through the stem from approximately 2 cm above the petiole attachment to 2 cm below. This cut reached as deep as the vascular tissue at about the half way point of the lesion length. An identical cut was made on the graft recipient plant at a leaf of comparable position and size and the donor leaf was attached using a parafilm bandage (Figure 1). To enhance lateral shoot growth, the apex of grafted plants was cut, altering auxin to cytokinin ratios throughout the plant, thus, stimulating lateral shoot development.

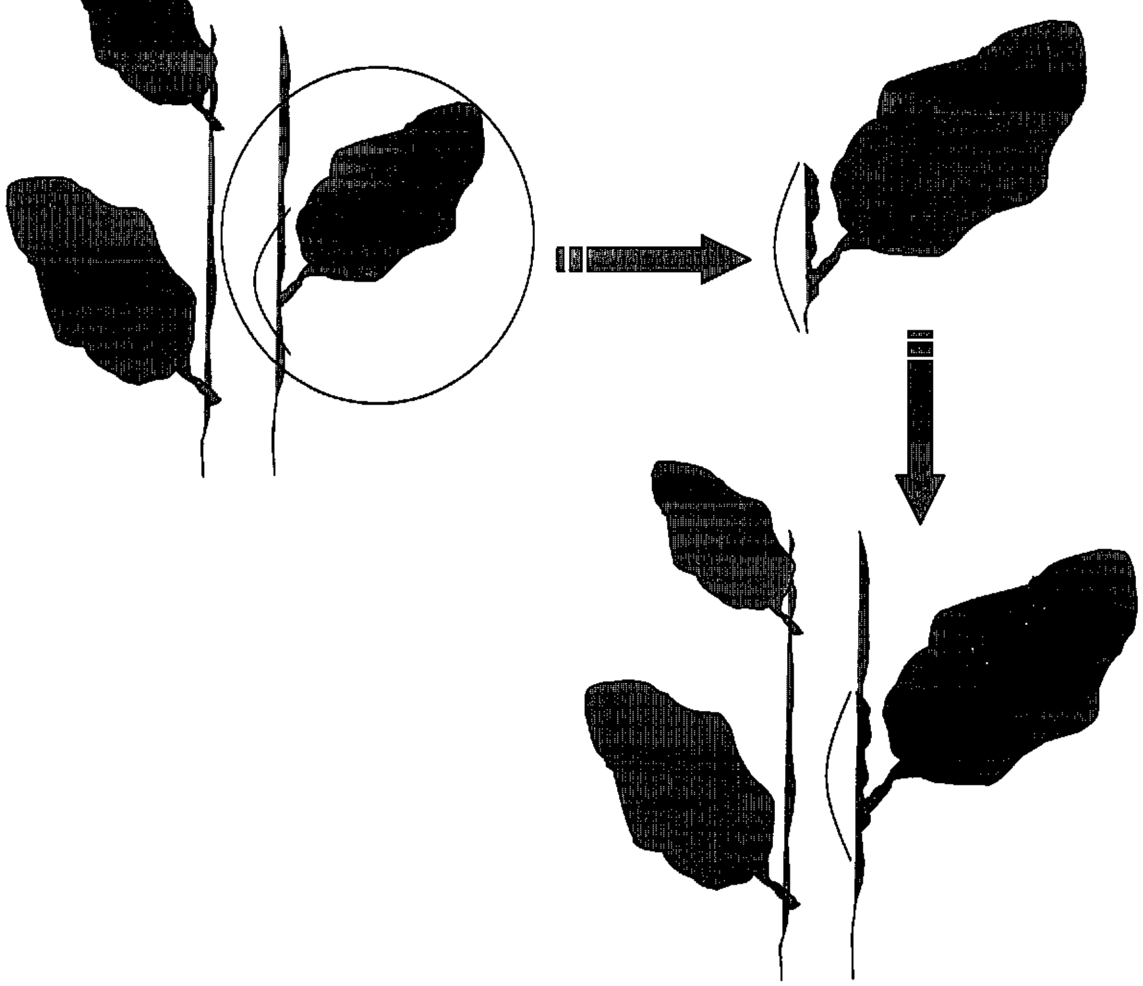


Figure 1. The grafting procedure. Mature, healthy leaves were excised from a plant by cutting from about 2 cm above the petiole, approximately ½ way into the stem, and finishing about 2cm below the petiole. The grafted leaf was attached to a 2nd plant at a site where a leaf had been excised in a similar fashion. Grafts were attached by wrapping parafilm around the wound site.

4.2. Measuring recombination rate

Recombination rate was measured using three different recombination reporter systems—one endogenous and two transgenic. *Nicotiana tabacum* cv Xanthi NN (*Su/su*) plants (TMV resistant) heterozygous for a gene that controls chlorophyll content in leaves (*Su/su*) provided the endogenous system. The *Su/su* phenotype expresses a pale green leaf color. Somatic recombination events may produce a phenotype switch to the *Su/Su* (dark green) or *su/su* (albino) (Shalev, *et al.*, 1999). Cells with recombination events affecting the *Su* locus are easily identified by the presence of a dual colored (dark green and albino) sector (Burk and Menser, 1964). Through growth and division the descendents of these recombined cells appear as contrasting sectors on a pale green background (Figure 2a).

Transgenic *Arabidopsis* plants utilized in the experiments contained two truncated, non-functional, inverted copies of the u*idA* gene (Swoboda, *et al.* 1994), while tobacco plants (cvs Havana 425 and Petit Havana SR1) harbored, truncated, but direct-orientated copies of a luciferase gene (Figure 2b,d). Following a somatic recombination event between the regions of homology each construct becomes intact and functional, thus, displaying full activity for either the β-glucuronidase (GUS, *uidA*) or luciferase (LUC). Both constructs, provided a visible, quantitative method for recombination detection.

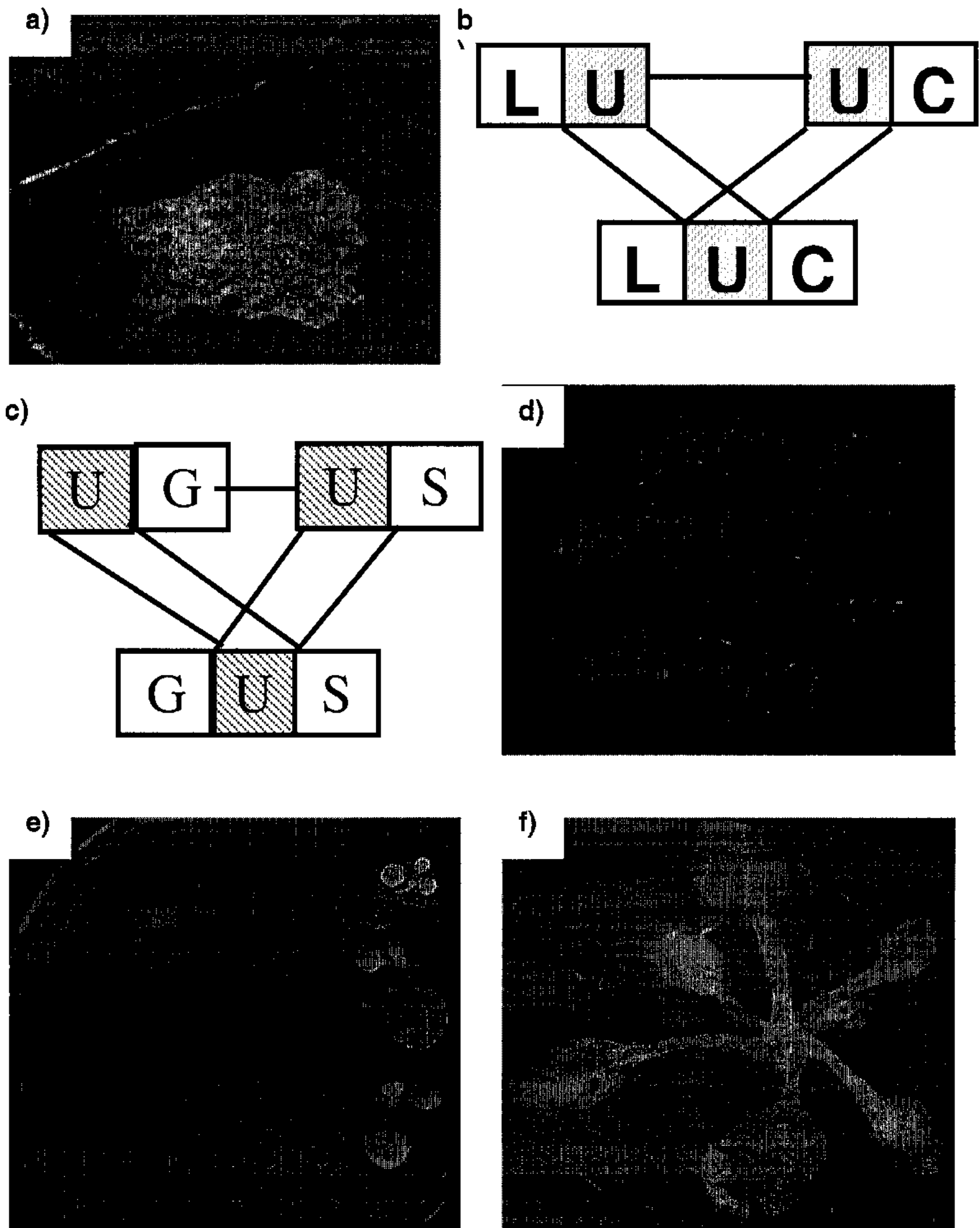


Figure 2. **The recombination assay utilized an endogenous recombination system and transgenic tobacco and *Arabidopsis* plants:** a) dual sectors on the Su/su leaves (4.5x), b)the endogenous recombination system luciferase recombination construct, c) the GUS recombination construct, d) somatic recombination events of luciferase as visualized with the aid of a CCD camera (0.1x), e) meiotic recombination results in LUC+, fully recombined plant (1.1x), f) recombination events in an *Arabidopsis* plant following GUS histochemical staining(6.0x).

4.2.1. Visualization of recombination events

4.2.1.1. Dual leaf sectors on *Su/su* plants

Dark green and albino (dual) sectors were counted on three to four newly emerged leaves of eight graft recipient plants for each treatment group. Plants were grafted at about six to seven weeks old and possessed five to six mature leaves at this age.

4.2.1.2. GUS

Recombination events that yielded an intact, functional copy of the GUS gene were visualized following a histochemical staining procedure. Plants at the full rosette stage were cut down and suspended in a GUS staining buffer containing: 500 μg/mL of X-glu (Rose Scientific Ltd.; Edmonton, AB, Canada) (initially dissolved in dimethylformamide (100 mg/mL); 0.1% NaN_3; in 100 mM sodium-phosphate buffer, pH 7.0. Plants were vacuum infiltrated using an aspirator device for 5 minutes and kept in an incubator (Heraeus; Geneva, Switzerland) at 37°C for 48 hours to allow cells containing an active GUS gene to cleave the X-glu substrate. The result of the cleavage reaction was an insoluble, indigo colored product. Following 48 hours, chlorophyll was removed by placing the plants in 70% ethanol at room temperature for 2-4 days. With the aid of a dissecting microscope, GUS stained plants appeared as translucent white with blue spots indicating the location of GUS activity (Figure 2f). The number of blue spots (recombination events) for each plant was counted.

4.2.1.3. LUC

In the transgenic tobacco plants harboring a luciferase gene based recombination construct, recombination events were visualized using a CCD camera (Gloor Instruments; Basel, Switzerland). Plants were topically treated with a 0.5 mM beetle luciferine (Promega; Madison, WI, USA), 0.05% Tween-80 solution. Following 45-60 minute incubation in the dark, plants were photographed with the CCD camera. Cells containing a recombined, functional LUC gene were able to cleave the luciferine resulting in ATP dependent production of light. Superimposing a light-exposed image (5-10 second exposure under white light) on a dark-exposed image (20 minute exposure with no background light to expose auto fluorescent, luciferase expressing cells) through the analiSIS program (Soft Imaging Systems; Muntser; Germany) allowed for the quantification of recombination events (Figure 2d,e). Recombination events were counted on the single, inoculated leaves in 12 treated cv Havana 425 for each treatment in the temperature experiment. In the grafting experiments, events were quantified on three to four new leaves of 10 to 12 graft recipient plants per treatment.

4.3. Systemic recombination signal elucidation

4.3.1. *Arabidopsis* mutants

These transgenic mutants were grown under sterile conditions and recombination was scored in 150 – 200 plants per line at approximately 3 weeks of age.

4.3.2. Detection of viral mRNA

4.3.2.1. Tissue

Roughly 100 mg of leaf tissue was used for RNA extraction. Tissue was collected from new growth on previously TMV inoculated plants.

4.3.2.2. RNA and cDNA

RNA was extracted using the Trizol protocol (Invitrogen, Burlington, ON, Canada). Briefly, this included thoroughly grinding tissue in the Trizol extraction solution, performing a chloroform separation, and isopropanol precipitation step all according to the manufacturer's protocol. cDNA was produced with 5 μg of total RNA with Ready-To-Go You Prime First Strand PCR beads from Amersham Biosciences (Piscataway, NJ, USA). Once again this was carried out according the product guidelines by adding the RNA suspension, oligonucleotide primers (poly-TG) and water to the provided reaction bead. Subsequent PCR was performed.

Primer pairs with the sequence:

(forward) 5'-CTGGTGAAGTTATTTGTCTGA-3',

and (reverse) 5'-ACCCGCTGACATCTTCACAT-3'

were used for amplification of the TMV cDNA via polymerase chain reaction. Reaction parameters included a 30 second denaturation at 95°C, 30 second annealing at 60°C, and a 30 second elongation at 72°C. The expected fragment size was ~550bp.

4.3.3. UVC and Rose Bengal treatment

Tobacco plants (Petit Havana SR1 and Havana *Nah*G) were exposed to 6000 ergs UVC, the highest does possible that did not cause visual damage to the plant. Entire plants were exposed for the systemic treatment, while a single leaf was exposed for local treatments. Aluminum foil protected the systemic tissues of the plant during the localized exposure.

Systemic RB exposure was achieved by supplying a 4 μM solution to the growth medium of the plant. Single, 100 μM (50 μL) applications of an RB solution was applied to single leaves in the local treatment.

4.3.4. Progeny of infected and control plants

N. tabacum cv Petit Havana SR1 plants (transgenic for the luciferase based recombination construct) of approximately the same size (four to five leaves) and between six and eight weeks old were rub-inoculated with TMV as described in section 3.1.3. After 24 hours, a leaf superior to the inoculated leaf was grafted onto another healthy SR1 plant as depicted in Figure 3. These plants were left to complete their life cycle. Seeds were collected from the new lateral growth of 22 individual graft-recipient plants and termed progeny of infected lines (PofI). The same procedure was carried out with SR1 plants originally mock-infected with 100 mM phosphate buffer only. These seeds were collected and served as the control plants and were identified as progeny of control lines (PofC).

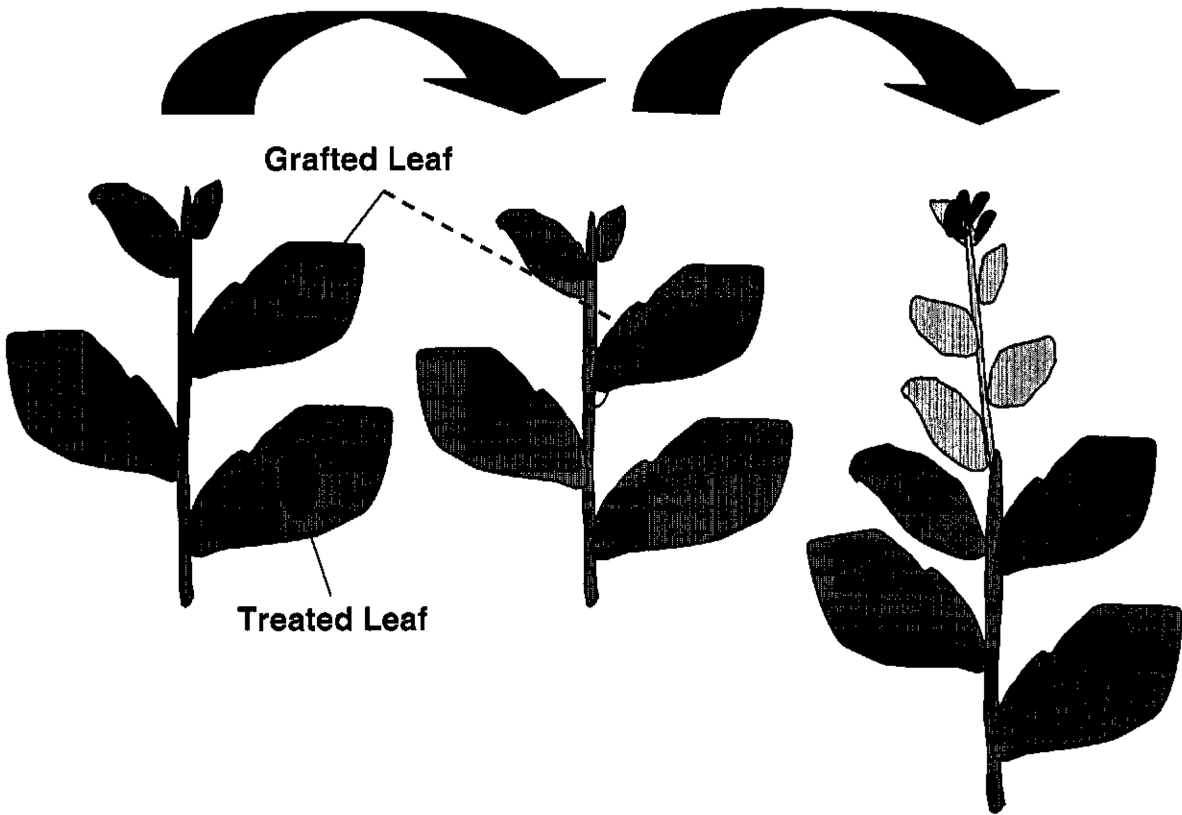


Figure 3. Grafting procedure for obtaining progeny of infected and progeny of control plants. Plants were treated 16 hours prior to the grafting procedure with either TMV or mock-inoculated with phosphate buffer. A superior, non-treated leaf was grafted to a healthy plant, the tops of the plants were cut to promote development of lateral buds, and seeds were subsequently collected from the new growth. Seeds were collected from 22 TMV infected plants (PofI), and 21 mock-inoculated plants (PofC).

4.3.4.1. Evaluation of DNA recombination rate

Spontaneous somatic and meiotic recombination events were measured in tobacco plants belonging to nine PofI lines #2, #4, #9, #10, #11, #13, #19, #21, and #22, and 21 PofC lines. Approximately 1000 - 2000 seeds were plated on 85mm diameter sterile, Whatman #1 filter paper with approximately 5 mL of sterile water. In total, 63,000 PofI and 34,000 PofC seeds were screened. Plants were left to germinate and grow in plant growth chambers under conditions described in section 3.1.2. At 10 -12 days post-germination, each plate of plants was screened for luciferase activity using the CCD camera (section 3.2.1.3). The average number of recombination events was calculated for each plate and used in the statistical analysis. Entire glowing plants revealed meiotic recombination events (Figure 2e), however, it was not possible to tell whether these events were true meiotic recombination or early somatic events.

4.3.4.2. Rose Bengal and NaCl treatment

In order to assess the specificity of recombination increases, nine PofI lines (#2, #4, #9, #10, #11, #13, #19, #21, and #22) were exposed to a chemical mutagen known to increase radical species; i.e., RB (Kim, *et al.*, 2001), and sodium chloride, a common environmental stress factor (Puchta, *et al.* 1995). These lines were germinated in sterile conditions on MS basic medium. At 10 dpg, plants were transferred to 24 well (2.5 cm diameter) multi-well plates (Falcon; Franklin lakes, NJ, USA) containing 1.5 mL of liquid germination medium (MS basic medium + 10g sucrose/liter) and various concentrations of either RB (4, 10, or 40 μM) or NaCl (25, 50, or 100 mM). Control plants were transferred to wells containing germination media only. Seven days later,

somatic recombination was examined in the plants (18 – 22 plants per treatment concentration).

4.3.4.3. Symptom progression

Eight to ten plants from 21 PofI lines (#1 - #22, excluding #17 as these seeds never germinated) were infected with TMV at 6-8 weeks of age. Disease progression was monitored daily in these plants via visual confirmation of symptoms (curling and mosaic patterning on leaf tissue).

4.3.4.4. RFLP Analysis

Genomic DNA was extracted from approximately 100 mg of leaf tissue collected from 149 PofI (from 21 different lines) and 147 PofC (from 21 different lines) plants using the Nucleon Phytopure DNA extraction kit (Amersham Biosciences, Piscataway, NJ). Briefly, plants were homogenized on liquid nitrogen and lysis buffer, the resulting homogenate was phase separated through a chloroform and resin extraction, DNA was precipitated (using isopropanol) from the aqueous phase and finally re-suspended in sterile water. For each sample, 5 μg of DNA was digested with 400 units (added to the digest volume in two 200 unit aliquots) *Hind*III endonuclease (Fermentas; Burlington, ON, Canada). The digests were separated on a 0.8% agarose gel via electrophoresis (1X TBE). DNA was transferred to a membrane and probed with the 4th exon of the N-gene: the coding sequence that corresponds to the LRR of the N-gene. A DIG-labeled probed of about 400 bp was obtained using the following primers:

N-gene 4th exon forward 5'-CATTTGCCGTCTCTACGGAGG-3' and

N-gene reverse 5'-GCTGCTTGGAAGAGCTACAAGG-3'

according to the manufacturers protocol (Roche; Laval, QC, Canada). The probe was created through a basic PCR process (94°C denaturation, 62°C annealing and 72°C elongation) using the supplied reagents, namely, radio-labeled dNTPS. Visualization was accomplished with the use of a CPD star fluorescence kit (Roche; Laval, QC, Canada). Thirty fragments of known size (between 50 bp and 10 kbp) were obtained. Size deviations from these known fragments were noted and the frequency of rearrangements in this N-gene-like R-gene regions was calculated according to the following formula:

$$f = n_f / p \times 30;$$

where n_f represents the number of rearranged loci; p, the number of plants screened; and 30, the number of loci that carry homology to the N-gene. It was possible to identify differences of ~5 bp for fragments up to 1 kbp in size. For fragments >1 kbp – 10 kbp in size, differences of 20 – 50 bp were detectable while shifts as small as 50 bp were detectable in the >10 kbp fragments.

4.3.4.5. Global versus R-gene locus methylation

4.3.4.5.1. Global

A 1 μg aliquot of tobacco DNA was digested with 10 units of methylation-sensitive *Hpa*II restriction endonuclease (Fermentas; Burlington, ON, Canada) and 1X enzyme buffer in a total volume of 20 uL overnight at 37°C. This enzyme leaves a 5' G overhang at the cleavage site (C↓CGG), which provides an opportunity for radio-labeled nucleotide incorporation; i.e., specifically [^{3}H]-dCTP (PerkinElmer; Boston, MA, USA). Following digestion, 10 uL of each sample underwent a cytosine extension assay

(Pogribny, *et al.*, 1999) by incubation in a total volume of 25 uL containing 1.5 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Fisher Scientific, Mississauga, ON, Canada), and 0.1 uL of $[^3H]$-dCTP at 56°C for 1 hour. Following extension, the 25 uL reaction volume of each sample was applied to 25 mm DE-81 ion-exchange filter paper (Whatman), washed with 500 mM sodium phosphate buffer for 10 minutes, repeated three times. Subsequently, filters were thoroughly dried, transferred to a vial containing 5 mL of scintillation cocktail and radiation levels (DPM) were detected by a scintillation counter (Beckman LS 5000CE; Fullerton, CA, Canada). As a control, each DNA sample also underwent the initial incubation in the absence of *Hpa*II enzyme and was subjected to identical extension conditions.

4.3.4.5.2. R-gene locus

Investigation of methylation patterns at specific R-gene-like loci was achieved through RFLP analysis. Following separate digestions with two different enzymes that were identical in recognition sequence (C↓CGG) but differed in cleavage capacity: *Hpa*II (methylation sensitive) would not cut if sequence was methylated; and *Msp*I (methylation insensitive) cut at recognition sequence regardless of methylation (Fermentas; Burlington, ON, Canada), 10 μg of digested DNA was electrophoresed on a 0.8% agarose gel in 1X TBE buffer, blotted to a nylon, positively charged membrane (Roche, Laval, QC, Canada) via ionic buffer (sodium citrate, sodium chloride) exchange, UV cross-linked and probed with a fluorescent-labeled 4th exon of the N-gene. Band intensities at 24 specific loci between differing samples were indicative of relative methylation levels. Hypomethylated samples experienced more cuts resulting in fragments with higher

intensity when digested with *Hpa*II, while the opposite was true for hypermethylated samples. Quantification of band intensities was done using the NIH Image 1.63 computer analysis program (United States Institute of Health; http://rsb.info.nih.gov/nih-image). Since *Msp*I cuts regardless of methylation sensitive, it was used as an internal control as identical intensities were observed at all 24 loci between the PofI and PofC plants.

4.3.5. NAC treatment

In order to assess recombination levels in the absence of ROS, a known inhibitor of free radical accumulation was used. N-Acetyl cysteine (NAC) fulfilled this capacity. NAC is a small, amino acid derivative that possesses strong antioxidant abilities (He and Harder, 2002). Pilot experiments revealed that an optimal concentration was achieved at 0.05 mM. Application was achieved through a topical application of 0.05 mM NAC (Sigma; Oakville, ON, Canada), 0.05% Tween-80 solution. Mock-NAC treatments involved the application of a 0.05% Tween-80 only solution. NAC was applied in all instances one to two hours prior to radical inducing treatments (RB or UVC).

4.3.6. Peroxide determination

Both aqueous and organic peroxide levels were measured in leaf tissue using a colorimetric assay kit (Peroxidetect kit#PD1) from Sigma (Oakville, ON, Canada). Leaves were homogenized in liquid nitrogen and incubated in approximately 10 mL of a 2:1 chloroform:methanol solution on a shaker for one to two hours at room temperature. The resulting suspension was then centrifuged at 11,300g for five minutes and the

supernatant filtered through a 1 cm cotton-packed Pasteur pipette. Two mL of 0.9% NaCl solution was added to the filtrate. The solution was mixed well and centrifuged at 11,300g for 5 minutes. Following centrifugation, the top, aqueous phase was separated from the organic, bottom layer. These two volumes (the aqueous volume containing aqueous peroxide, and the organic, the lipid peroxides) were evaporated under vacuum in a rotovap. Once dry, the organic part was re-dissolved in two to four mL of 90% methanol while the aqueous samples were suspended in two to four mL of distilled water. The peroxidetect kit (#PD-1) from Sigma (Oakville, ON, Canada) was used according to the manufacturer's protocol to assay the peroxide levels present in the samples. This involved adding a volume of extract to a volume of kit detection solution (containing indicator dye xylenol orange). In an acidic environment, the presence of peroxides facilitates the conversion of Fe^{+2} ions to Fe^{+3}. This conversion manifests as a color change.

4.4. Statistical analyses

Statistical tests performed were either two-tailed, paired t-tests (comparing data from two treatments) or single factor ANOVA (comparing data from three or more treatments). Significant results were defined where the probability for rejection of the null hypothesis was 0.05 or less. All statistical analyses were performed using the Data Analysis Tools package available on Microsoft Excel.

4. RESULTS

4.1 Somatic recombination in TMV infected Havana 425 and Petit Havana SR1 plants

To measure somatic recombination frequency, single leaves on 18-20 *N. tabacum* cv Havana 425 plants were rub inoculated for each treatment group. Following seven to ten days, the somatic recombination events were scored in the leaves of the plants. The experiment was performed three times and average data is presented. Recombination in leaves of mock-treated Havana 425 plants experienced 8.4 ± 3.1 spots per plant while those exposed to TMV at 32°C displayed an average of 28.5 ± 7.2 spots for a 3.4-fold induction ($p<0.05$, 1.4×10^{-5}, paired, two-tailed t-test). In addition, a 1.8-fold increase was observed in non-inoculated tissues of the treated plants. Recombination did not significantly differ between mock-treated and TMV exposed Havana 425 plants at 22°C ($p>0.05$, 0.1, paired, two-tailed, t-test).

Petit Havana SR1 plants (18-20 individuals per group) were inoculated in the same fashion as the Havana 425 plants. Recombination induction in these plants was not different between the two temperatures in local, 3.1- and 4.3-fold at 22°C and 32°C, respectively ($p>0.05$, -2.01, paired, two-tailed t-test); or systemic tissues, 1.9- and 2.1-fold at 22°C and 32°C, respectively ($p>0.05$, -0.55, paired, two-tailed t-test) (Table 1). Furthermore, the fold inductions in the Petit Havana SR1 and Havana 425 plants were about the same. The increase in systemic unexposed tissues was interesting as the recombination increases were thought to be due to presence of the pathogen. Therefore, it was necessary to determine whether the virus movement to untreated tissues was increasing recombination levels.

Table 1. Recombination events in TMV treated (local) and untreated (systemic) tissues of Petit Havana SR1 plants at 22°Cand 32°C. Single leaves of 18 – 20 Petit Havana SR1 tobacco plants were inoculated. Recombination was assayed 10 – 14 days later in the treated and untreated tissues (3 – 4 per plant). Average±SD represents average spots/plant ± standard deviation; Fold Induction, the relative induction rate of treated plants (Virus) as compared to the control (Buffer) plant for each temperature.

Treatment, Temperature (°C)	**Tissue Assayed**	**Average±SD**	**Fold Induction**
Buffer, 22°C	Local	3.5±1.1	N/A
Virus, 22°C	Local	10.8±2.9	3.1
Buffer, 32°C	Local	4.2±1.7	N/A
Virus, 32°C	Local	17.9±4.6	4.3
Buffer, 22°C	Systemic	16.5±3.7	N/A
Virus, 22°C	Systemic	31.4±8.0	1.9
Buffer, 32°C	Systemic	24.8±6.0	N/A
Virus, 32°C	Systemic	51.6±13.3	2.1

4.2 Viral movement

Single leaves on 54 Havana 425 plants were inoculated with TMV at 32°C. The inoculated leaf was excised from nine plants at various intervals post-inoculation (0, 8, 24, 36, or 72 hours). Seven to 14 days later, presence of viral RNA (via PCR), disease progression via visual assessment of symptoms and somatic recombination were assayed in three to four newly emerged leaves. The experiment was repeated three times, and values represent an average of the combined data. Both viral RNA presence (Figure 4) and visual symptoms (Table 2) were only detected in distal tissues of plants whose inoculated leaf was excised at least by the 36-hour time point. Thus, viral movement from the initial site of infection systemically occurred at some point between 24 and 36 hours. Average recombination events per plant were 11.2±1.1, 21.3±1.5, 25.8±1.1, 24.7±1.6 and 31.7±3.9 in plants with the inoculated leaf removed at the 0, 8, 24, 36, and 72 hour time point, respectively. An increase in recombination events was observed as early as the 8-hour time point (i.e., a 1.8-2.2-fold increase over the frequency of events and the zero time point) and the recombination increase plateaued by the 24h time point (Table 2). This earlier induction of recombination events in tissue where the virus was absent implied that perhaps a signal was propagated resulting in the systemic destabilization of the genome. To provide further support for the existence of such a signal, an attempt to induce recombination in plants via leaf grafting was performed.

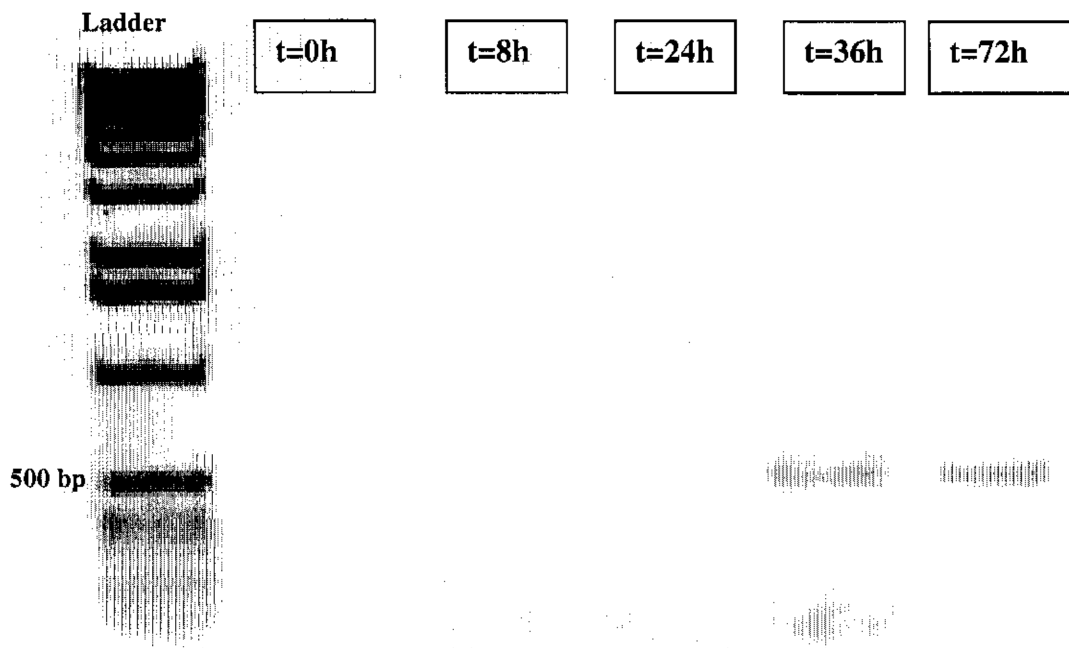


Figure 4 – Agarose gel containing PCR products of amplification of TMV viral RNA from challenged Havana 425 plants. Lanes represent the time following inoculation that that the infected leaf was removed. Bands at ~55 0bp in the t=36h and t=72h columns indicate viral RNA was present in the leaf extracts.

Table 2. Recombination events and viral presence measured in systemic tissues following local inoculation. Single leaves of 50 Havana 425 plants were inoculated with TMV at 32°C. Inoculated leaves were removed from 10 plants at various times following inoculation. Symptoms, viral RNA presence and recombination rates were assessed seven to fourteen days after inoculation on 3 – 4 leaves per plant. Average±SE represents the average recombination events per plant ± standard error; recombination fold, the relative induction between the zero time and other time intervals.

Time (hours)	Symptoms Visible?	PCR results	Average±SE	Recombination Fold Increase
0	No	(-)	11.2±1.1	1.0
8	No	(-)	21.3±1.5	1.8-2.2
24	No	(-)	25.8±1.1	2.0-3.0
36	Yes	(+)	24.7±1.6	2.0-3.0
72	Yes	(+)	31.7±3.9	2.0-3.0

4.3 Grafting experiments

4.3.1 Havana 425 and Petit Havana SR1 plants

Single leaves of six to eight *N. tabacum* cvs Havana 425 and Petit Havana SR1 plants were rub inoculated for each treatment group. The treated plants subsequently had leaves grafted onto 10-12 healthy, untreated plants. The recombination events were scored in the newly emerged tissue (three to four leaves per plant) of the graft recipients. The experiment was replicated three times. Average results between the three trials are presented. Graft recipient plants of SR1 inoculated with TMV at both 22°C and 32°C demonstrated a 1.8- ($p<0.05$, 6.7×10^{-5}, paired, two-tailed t-test) and 2.1-fold increase in somatic recombination frequency ($p<0.05$, 1.4×10^{-5}, paired, two-tailed t-test), respectively, and Havana 425 at 32°C; 2.3-fold increase ($p<0.05$, 1.9×10^{-5}, paired, two-tailed test) (Table 3). To determine whether the increased recombination was a property of the luciferase transgene specifically and not representative of the entire genome, it was necessary to observe recombination frequencies in an endogenous reporter gene.

Table 3 – Recombination events in graft recipient Petit Havana SR1 and Havana 425 plants. Recombination was assayed in 3 – 4 leaves of 10 to 12 graft recipient plants. Average ±SD represents the average spots per plant ± standard deviation; Fold induction, the relative induction of the treated group as compared to the buffer 32°C plants; and, t-test, the significance as determined by a paired, two tailed student's t-test.

Plant, Treatment and Temperature (°C)	Average ±SD	Fold Induction	T-test
SR1, Virus, 32°C	60.1±11.6	2.1	$1.4x10^{-5}$
SR1, Virus, 22°C	52.0±6.8	1.8	$6.7x10^{-6}$
SR1, Buffer, 32°C	29.2±6.5	N/A	N/A
425, Virus, 32°C	74.4±20.2	2.3	$1.4x10^{-5}$
425, Virus, 22°C	36.5±6.7	1.1	0.1
425, Buffer, 32°C	32.0±4.9	N/A	N/A

4.3.2 *Su/su* plants

Tobacco Xanthi NN *Su/su* plants were infected with 300 ng of oilseed rape mosaic virus (ORMV), a TMV related virus that was also inhibited by the presence of the N-gene. Untreated leaves of ORMV-inoculated or mock-inoculated *Su/su* plants were grafted to untreated recipient plants. Grafts were received by eight plants per treatment. Recombination events were scored in two to three newly emerged leaves. The experiment was repeated three times and average data between the three are presented (Table 4).

Plants that received leaves from ORMV treated plants (Virus 32°C, 0.62±0.42 spots/leaf) had a 3.4 times higher incidence of recombination events than those that received leaves from the mock-treated plants (Buffer 32°C, 0.18±0.19 spots/leaf) ($p<0.05$, 0.017 paired, two-tailed t-test) (Table 4).

Results thus far exhibited an increase in DNA recombination in somatic cells, however, the frequency of rearrangements during meiosis was not apparent. In an attempt to obtain data on meiotic recombination, rearrangements were quantified in the progeny of infected tobacco plants.

Table 4 – Recombination events in graft recipient *Su/su* plants. Recombination was assayed in two to three leaves from eight graft recipient plants (per treatment group). Average ±SD represents the average spots per leaf ± standard deviation; Fold induction, the relative induction of the treated group as compared to the buffer 32°C plants; and, t-test, the significance as determined by a paired, two tailed student's t-test.

Treatment and Temperature (°C)	**Average ±SD**	**Fold Induction**	**T-test**
Virus, 32°C	0.62±0.42	3.4	$1.7x10^{-2}$
Buffer 32°C	0.18±0.19	N/A	N/A

4.4 Recombination in progeny of infected and control plants

4.4.1 Meiotic recombination

Recombination events during meiosis would lead to a fully recombined LUC+ plant. Every cell in a LUC+ plant contains the active luciferase gene and, thus, the entire plant appears to glow (Figure 2e). Progeny from the infected and control Petit Havana SR1 plants were screened for such meiotic events. In total, 63,000 seeds from 15 of the PofI lines and 34,000 of the PofC lines gave rise to 60 and 10 LUC+ plants, respectively. This translated to a 3.2-fold higher incidence ($p<0.05$, $2.3x10^{-4}$ paired, two-tailed t-test) of fully recombined plants (meiotic events) when the average data from the progeny of infected were compared to data from progeny of control plants (Figure 5).

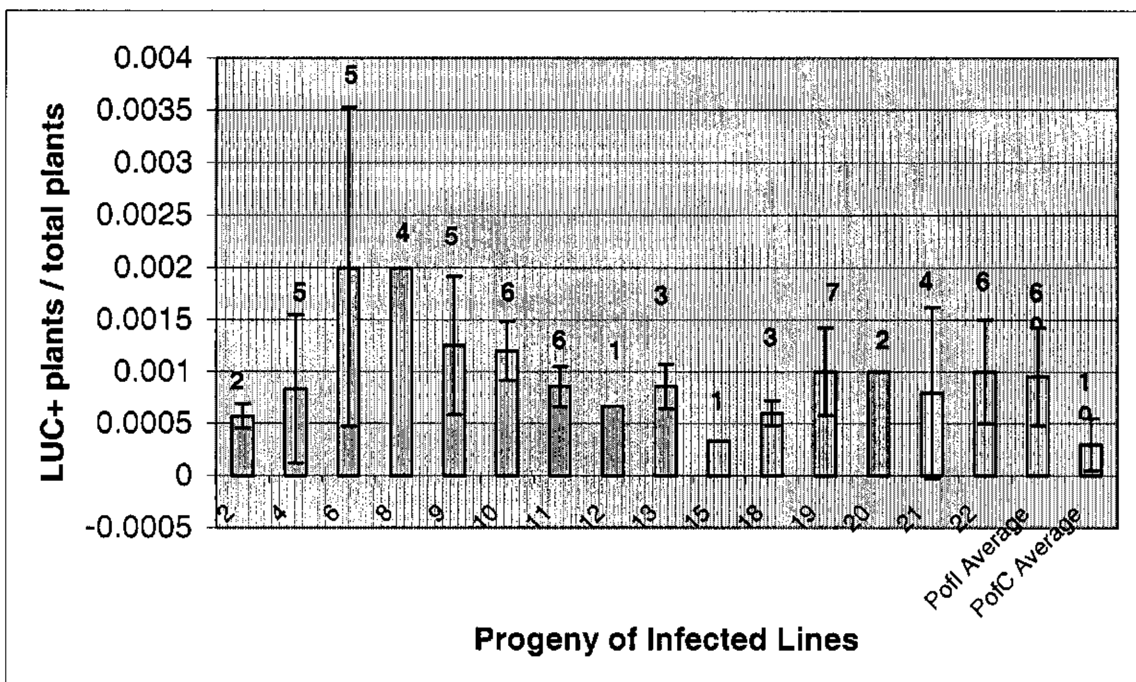


Figure 5 – Frequency of meiotic recombination events in the PofI and PofC plants. Plants from 15 different PofI lines (numbers under bars represent the PofI lines, 4000 – 7000 plants per line, 63,000 in total) and 21 different PofC lines (34,000 in total) were screened at 10-14dpg. Bars represent the average meiotic recombination events ± standard errors. Numbers above bars represent the number of LUC+ plants observed in that PofI line.

4.4.2 Somatic recombination

Somatic recombination events were also higher in the PofI lines relative to the PofC. Somatic recombination was scored in 63,000 progeny of infected plants from 15 different lines, while 34,000 progeny of control plants from 21 lines were used. On average, PofI plants displayed 0.03±0.015 somatic recombination events per plant, while the PofC plants demonstrated 0.02±0.004 events per plant. Somatic recombination events in the PofI plants were 1.5-fold higher than the PofC plants ($p<0.05$, -2.87, two-tailed t-test) (Figure 6). It is important to note that the recombination events were observed in small tobacco plants (approximately 2 weeks old) and, thus, recombination frequencies are lower than what was described earlier in mature leaves.

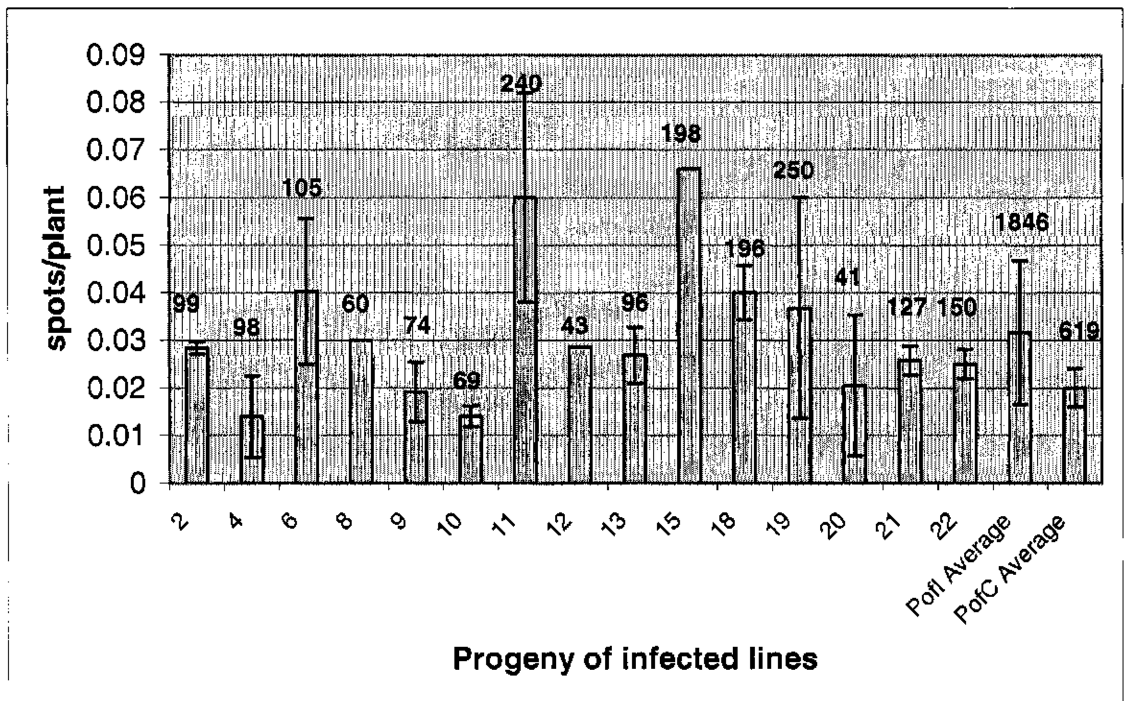
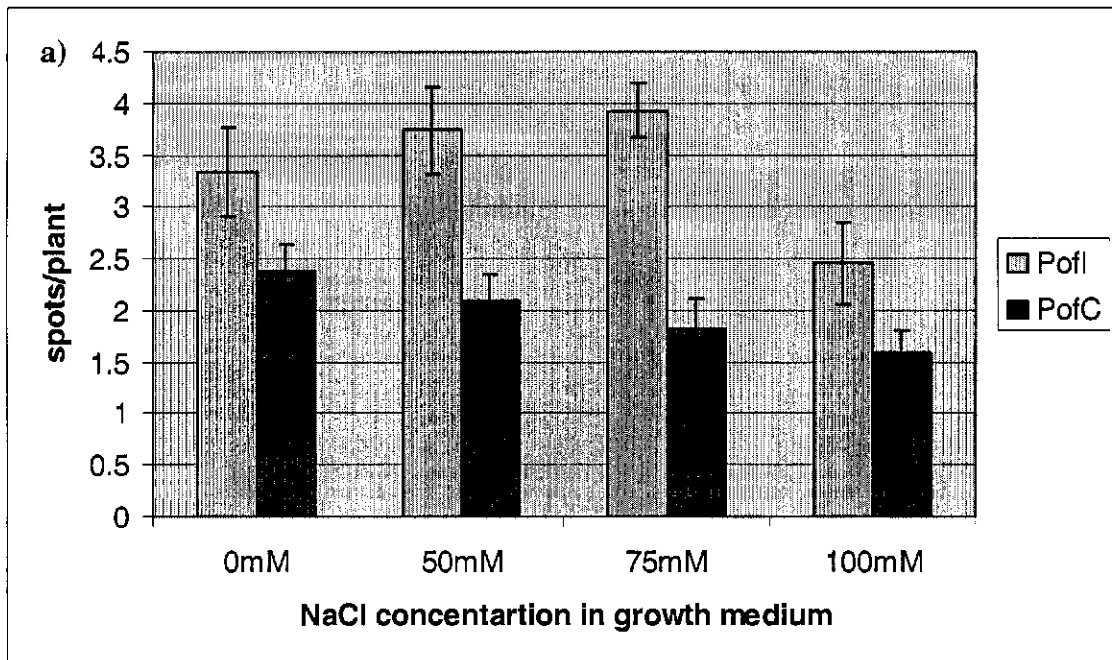


Figure 6. Somatic recombination frequency in PofI and PofC plants. Recombination events were scored 10-14 dpg in 4000 – 7000 plants per line (numbers under bars represent the 15 PofI lines screened), 63,000 PofI and 34,000 PofC plants in total. Bars represent averages ± standard error. Numbers above bars represent the total number of spots seen in that PofI line.

4.4.3 Recombination following NaCl and RB exposure

PofI and PofC plants were exposed to both sodium chloride and RB (a chemical mutagen) to assess their response (with respect to genome stability) to general stress. Plants were exposed to either the NaCl or RB in the growth medium over a seven day period. Recombination events were subsequently scored in 16 – 18 PofI plants per line and 120 – 130 PofC plants (at the four leaf stage). Two independent experiments were performed and the data presented represents the average between the two.

PofI plants tended to display an increased frequency of recombination relative to the PofC plants (Figure7a). The average recombination events in PofI plants were 3.3±0.4, 3.7±0.4, 3.9±0.3 and 2.5±0.4 in the 0, 50, 75 and 10 mM concentrations of NaCl, respectively. PofC plants exhibited average recombination events of 2.4±0.3, 2.1±0.2, 1.8±0.2 and 1.6±0.2 at 0, 25, 50 and 75 mM NaCl, respectively. In particular, PofI lines #11 and #22 demonstrated a significantly large increase (3- and 1.6-fold, respectively) in the recombination events between 0 and 50 mM NaCl. Average recombination events in #11 were 1.57±0.2 4.9±0.46 respectively in the 0 and 50 mM NaCl treatments. Line #22 displayed an average of 2.9±0.31 and 4.8±0.62 recombination events per plant at the same concentrations. Each line also maintained this higher recombination rate when NaCl concentrations were raised to 75 mM; 4.5±0.5 in #11 and 4.5±0.6 in #22. When related to the control line, these increases in recombination represent a 2-fold increase at the 50 mM and 1.9-fold increase at the 75 mM concentration (Figure 8a).



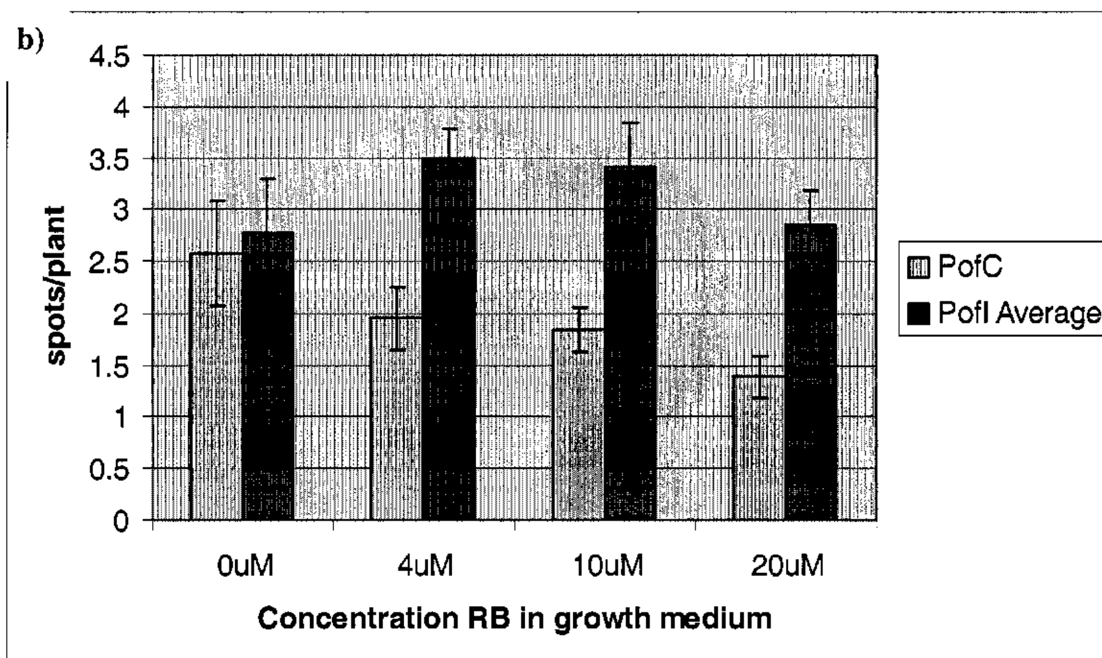
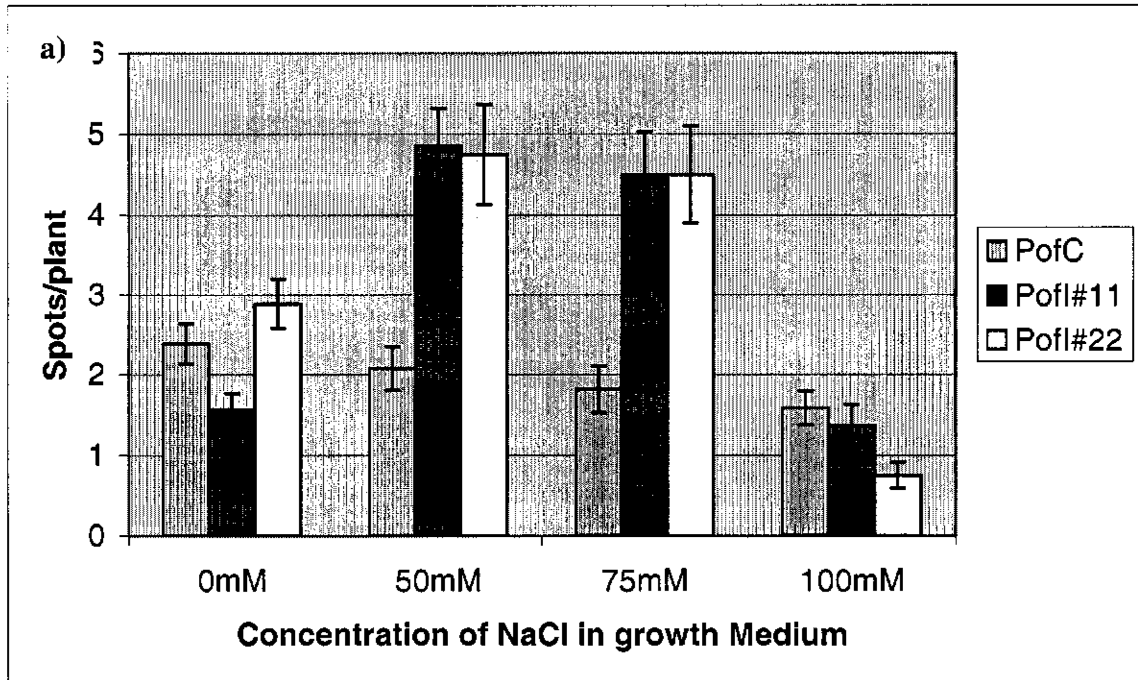


Figure 7 - Effect of NaCl and RB in the growth medium of PofI and PofC plants. General trend in combined PofI and PofC plants germinated under sterile conditions on MS agar. At 7-10dpg, plants were transferred to sterile liquid medium containing various concentrations of NaCl or RB. Recombination events were scored in 18-21 day old plants. Values represent the average recombination events per plant ± standard error; events were scored in 140 – 150 PofI (from 9 lines) and 120 – 130 PofC plants. a) Plants moved to NaCl medium; b) Plants moved to RB medium.



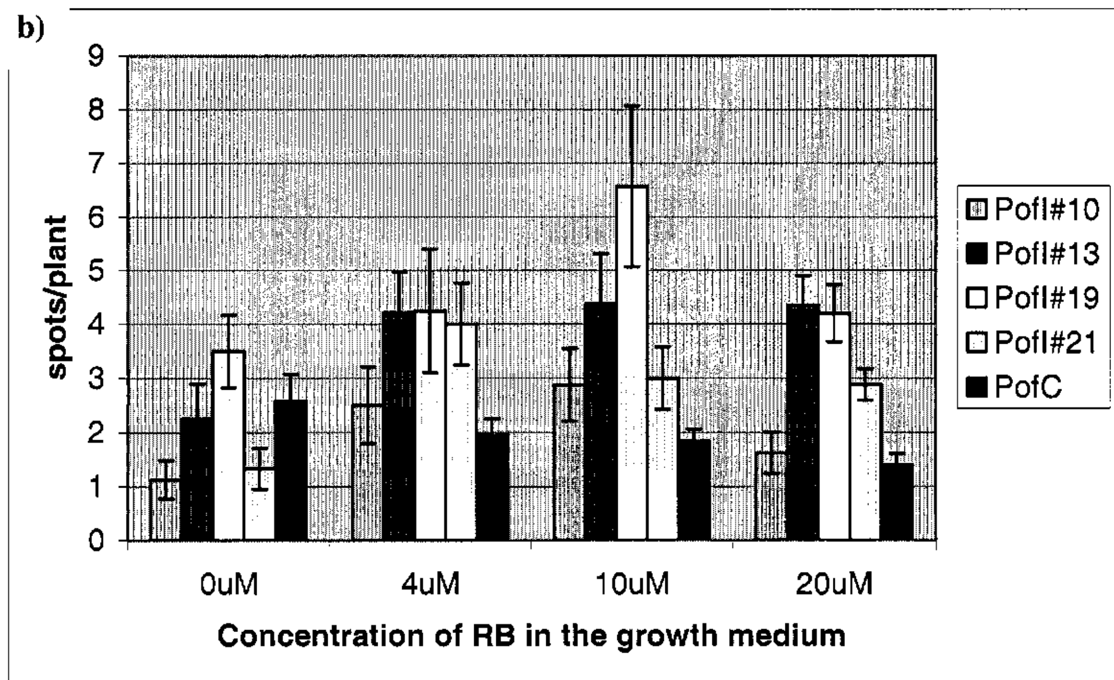


Figure 8. The PofI lines that demonstrated the largest fold induction of recombination events following systemic exposure to either NaCl or RB. Recombination events were scored 18 – 21 dpg in 16 – 18 plants per line. Values represent the average recombination events per plant ± standard error. a) Plants moved to NaCl medium; b) Plants moved to RB.

Results for the RB treatment were similar, however, the increase in recombination events was more pronounced. Once again the PofI lines, on average, displayed higher recombination rates in response to the RB than the PofC plants (Figure 7b). Average recombination events in the PofI plants were 3.5±0.3, 3.4±0.4, and 2.9±0.3 at 4, 10 and 20 μM RB concentrations, respectively. The PofC plants displayed average recombination events of 2.0±0.3, 1.8±0.2, and 1.4±0.2 at the same RB concentrations. This corresponds to a 1.8-, 1.9-, and 2.1-fold induction in recombination events in PofI, as compared to PofC plants. The largest increases in recombination were observed in lines: #10, 2.2- and 2.5-fold (at 4 and 10 μM, respectively); #13, 1.9-fold (at all three concentrations); #19 1.9-fold (at 10 μM) and, #21, 3.0-, 2.25-, and 2.17-fold (all three concentrations). Line #10 displayed an average of 1.1±0.4, 2.5±0.7, and 2.9±0.7 events at 0, 4, and 10 μM RB, respectively. Line #13 displayed an average of 2.3±0.6, 4.2±0.8, 4.4±1.1 and 4.4±0.8 events at 0, 4, 10 and 20 μM RB, respectively. Line #19 displayed average events of 3.5±0.8 and 6.6±0.5 at 0 and 10 μM RB concentrations, respectively. Line #21 displayed 1.3±0.4, 4.0±0.6, 3.0±0.5 and 2.9±0.3 events at 0, 4, 10 and 20 μM RB, respectively. Once again, when compared to the control line, the relative increases in recombination were higher: approximately 1.7-fold in line #13, between 1.7- and 2.6-fold for line #19, and between 1.2- and 1.6-fold in line #21 (Figure 8b).

4.4.4 Symptom progression in TMV challenged progeny

If genome rearrangements were induced as a mechanism to potentially acquire novel resistance, it was important to screen for progeny with a higher resistance to TMV than the parents. This increased resistance was determined by monitoring symptom

progression in PofI and PofC plants that were locally infected with TMV. Seven to ten plants from 21 PofI and 21 PofC lines were screened for a total of 159 PofI and 125 PofC plants. PofC plants demonstrated an average of 26±0.5 days to symptom appearance while the average time in the PofI was 27.2±1.0 days. Two PofI lines showed an earlier onset of disease, however, this reduction was not significant (20±3.0 days in PofI#1 and 21±2.4 days in PofI#11). Four of the PofI lines did not display symptoms until 34±2.0 (#5), 30±1.0 (#13), 30±1.8 (#19) or 31±4.0 (#20) days post infection (Figure 9). This represented a significant increase in time elapsed to disease onset for: #5, ($p<0.05$, -3.76, two-tailed, paired, students t-test); # 13, ($p<0.05$, -3.17, two-tailed, paired, students t-test); and #19, ($p<0.05$, -2.06, two-tailed, paired, students t-test).

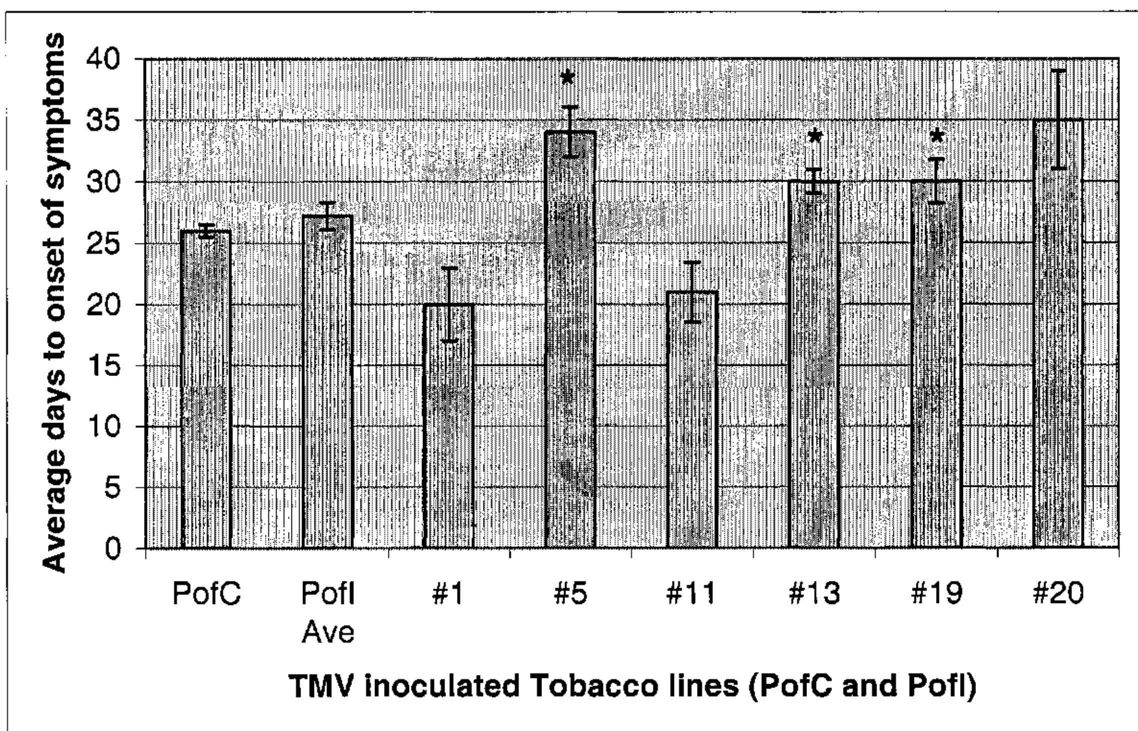


Figure 9. Time elapsed to onset of symptoms in PofI and PofC plants. Single leaves on eight to ten PofI plants from 6 representative lines (numbers under bars represent PofI line) were inoculated with TMV and left at 32°C. Disease progression was measured by monitoring for the symptom appearance of mosaic pattern and leaf curling on new leaves. PofI Ave represents the average days to symptom appearance from all plants of the 21 PofI lines; bars represent standard errors; and percentages relate the time to symptoms in PofI to the PofC plants. Lines demonstrating significant increases are marked with a (*).

4.4.5 RFLP of N-gene-like loci

Again, if genome stability is reduced as a mechanism to acquire novel R-gene specificities, it was important to observe changes in a R-gene locus specifically. RFLP analysis was conducted using the 4th exon of the N-gene as a probe. Following *Hind*III digestion of genomic DNA from PofI and PofC and southern analysis, 30 loci with homology to the N-gene consistently appeared in the tobacco plants (Figure 10a). Deviations in band size from these fragments were indicative of genome rearrangements and were detected by an unexpected band size in one of the 30 predictable fragments (Figure 10b).

Altogether, 33 and 4 fragments of different size from the expected fragment were observed in 149 PofI and 147 PofC plants screened, respectively. This translated to an 8.2-fold increase in rearrangements in the PofI plants (see materials and methods for calculation data). All lines exhibiting identified "shifts" in band size were confirmed by a 2nd digestion and blotting process.

The data indicated that R-gene loci were less stable following elicitation of recombination, however, it was not clear as to how this instability was affected at the genome level. Changes in DNA methylation was investigated as a possible cause of the instability.

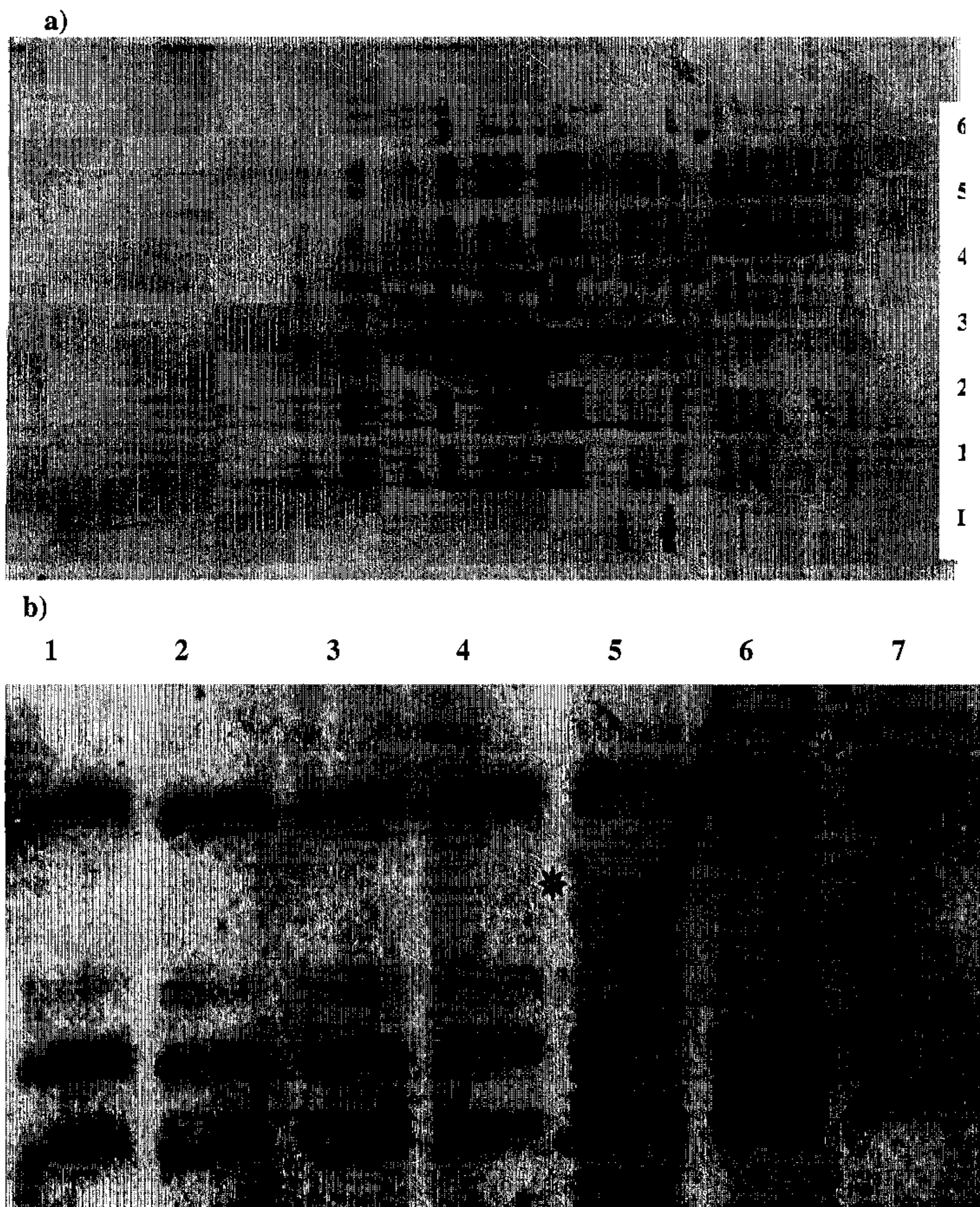
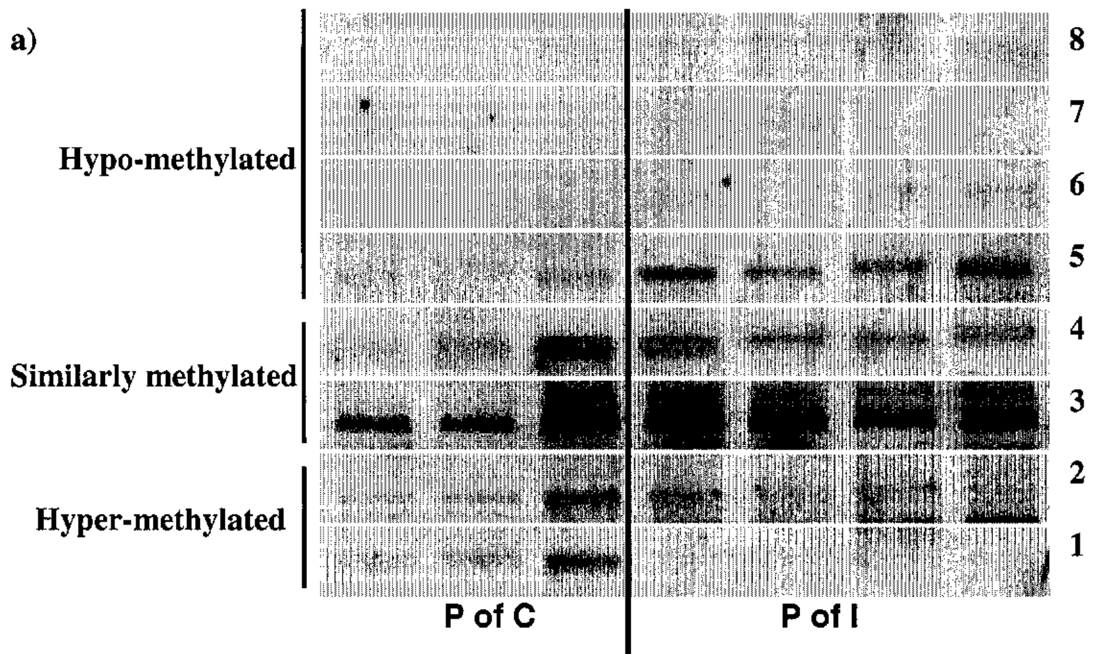


Figure 10. RFLP anaylsis of N-gene-like loci. Total genomic extracts were digested thoroughly with *Hind*III endonuclease. Digested DNA was electrophoresed on a 0.8% gel and blotted to a positively charged, nitrocellulose membrane. Blots were probed with the 4th-exon of the N-gene. a) Restriction resulted in 30 predictable loci as seen from six plants (lanes 1 – 6). b) Shifts in expected band size are indicative of a rearrangement event (indicated by the stars) in plant individuals represented in lane 5 and 6.

4.4.6 Methylation analysis

To assess methylation status of the N-gene-like loci, RFLP analysis was conducted again. In this instance DNA was restricted with the methylation sensitive *Hpa*II endonuclease. Increased methylation would diminish the frequency of cuts by the *Hpa*II enzyme rendering fewer fragments of a given size to be detected through the probing process, while decreased methylation would increase the number of cut sites, thus, increasing the number of fragments. Since *Msp*I cut the sequence regardless of methylation, equal numbers of bands should have been observed between DNA samples of both the PofI and PofC plants and was used as an internal control. Comparison of band intensities following *Hpa*II restriction, blotting and probing determined the frequency of cuts and, thus, the relative methylation status of the samples (Figure 11a). Three loci showed a statistically insignificant, 20-50% decrease in band intensity ($p>0.05$, 0.17, 0.47, 0.39, single factor ANOVA), whereas five loci displayed between 4- and 8-fold more intense bands, and, thus were 4 to 8 times hypomethylated ($p<0.05$, $1.0x10^{-3}$, $9.0x10^{-3}$, $6.0x10^{-3}$, and $1.3x10^{-5}$, single factor ANOVA) (Figure 11a, Table 5).



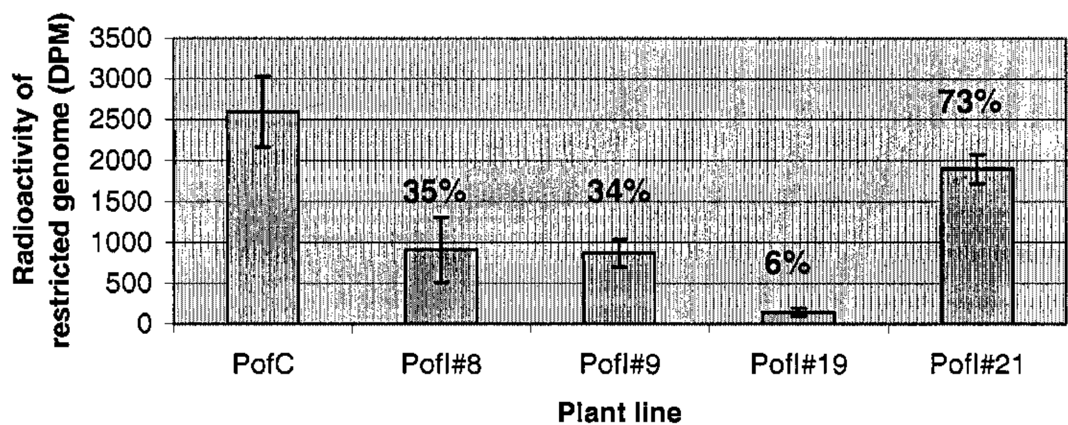


Figure 11. Methylation levels in an R-gene specific locus and globally. a) Methylation levels were assessed by measuring different band intensities of several fragments created following digestion with a methylation sensitive endonuclease—*Hpa*II. Rows 1 – 8 represent different loci that display different intensities between PofC and PofI. Rows 3 & 4 display similar methylation; 1 & 2, hypermethylated; and 5 – 6 hypomethylated. b) Global methylation was measured through a radio-labeled nucleotide incorporation event. Once again genetic material was digested with the methylation sensitive endonuclease *Hpa*II. Values graphed are the average radioactivity incorporation with standard error. Percentages represent the difference between the methylation levels of control and PofI lines.

Table 5. Methylation analysis of N-gene-like resistance gene loci. Genomic DNA was digested with the methylation sensitive endonuclease *Hpa*II were probed with the 4th exon of the N-gene. Rows 1 – 8 represent different loci that demonstrated differing band intensities indicating different levels of methylation. Rows 1 – 3 demonstrate insignificant hyperemthylation, while 4 – 7 indicate hypomethylation. PofI and PofC columns represent average intensities ± standard deviation as calculated from eight to ten plants in 12 lines; fold represents the ratio between the PofI and PofC intensity for a given locus; and P-value, the significance of the change in intensities.

Locus	PofC	PofI	Fold	P-value
1	12.5±2.0	7.6±2.6	-1.6	0.17
2	7.3±1.9	4.7±2.9	-1.5	0.47
3	26.5±7.1	17.8±6.7	-1.5	0.39
4	8.4±2.5	34.3±5.7	4.1	1.0×10^{-3}
5	2.5±0.7	21.2±5.9	8.4	9.0×10^{-3}
6	4.4±1.3	17.7±3.8	4.1	6.0×10^{-3}
7	10.4±4.4	84.3±9.5	8.1	1.3×10^{-5}

To examine the extent of global genome methylation a cytosine reaction was employed. Genomic DNA from PofC and four lines of PofI plants were restricted with the methylation sensitive endonuclease *Hpa*II. Fifteen PofC and eight to ten plants per PofI line were used. Following cleavage, it was possible to incorporate [^{3}H]-dCTP. The amount of incorporation was inversely proportional to the amount of methylation. Following radiation quantification, the PofI plants were found to be between 20 and 210% hypermethylated as compared to the PofC plants ($p<0.05$, 4.32, single factor ANOVA). Average radioactivity (DPM) for each PofI line was: #8, 910±396; #9, 871±168; #19, 150±50; and #21 1896±181. Average radioactivity for the control line was 2596±433 DPM (Figure 11b).

4.5 Influence of ROS on DNA recombination events

4.5.1 Recombination in Petit Havana SR1 systemically treated with UVC and RB

Recombination events were quantified in transgenic Petit Havana SR1 plants that were systemically treated with UVC, RB or nothing 10 days earlier. Spots were counted in four to five leaves on 10 plants per treatment. The experiment was replicated three times and the results presented represent an average of the three replicates. Plants exposed to the UVC experienced a 3.1-fold increase of recombination events as compared to the control plants ($p<0.05$, $8.3x10^{-6}$, two-tailed, paired t-test). While plants exposed to RB exhibited a 3.7-fold increase ($p<0.05$, $1.7x10^{-7}$, two-tailed, paired t-test) (Table 6). Once again, it was important to observe whether local treatments of the ROS-inducing agent could alter systemic recombination rates.

Table 6 – Induction of recombination by systemic UVC or RB exposure. Tobacco SR1 plants transgenic for the LUC recombination substrate were either exposed to 6000 ergs of UVC at age 7 days, or grown on an RB containing medium. Recombination events were scored in four to five leaves per plant. Ten plants per treatment. Average±SE represents the average recombination frequency in 10 plants ± standard error; fold, represents the fold induction of the treated plants relative to the control group; t-test represents the significance of the data as determined by a paired, two-tailed students t-test.

Treatment	Average±SE	Fold	t-test
Control	10.5±3.7	N/A	N/A
UVC	32.2±6.2	3.1	8.3×10^{-6}
RB	39.2±7.1	3.7	1.7×10^{-7}

4.5.2 Systemic recombination increases following localized UVC and RB treatment

To assess systemic effects of a local increase in ROS, the activating agents were applied to single leaves and recombination events measured in distal tissues. Growing plants on an RB containing medium achieved the systemic exposure to RB. Since the relationship between plants and their environment is more acutely experienced through the root and phloem system, only minute doses were required as larger doses caused visible damage to plant tissues. The dose chosen, 4 μM, in the systemic exposure represented the highest dose that did not cause visible lesions. Plants were capable of tolerating a higher dose of RB with the local, single leaf application before displaying physical damage. Thus, it was believed that absorption through leaf tissue layers was more difficult and a higher dose of RB was necessary to achieve a comparable level of radical production. Therefore, the highest dose that did not cause lesions was chosen, i.e., 100 μM.

Recombination events were measured in three to four newly emerged leaves on each plant. Ten plants per treatment were initially exposed. The experiment was replicated three times and data presented represents the average of the three. Plants locally treated with either RB or UVC exhibited higher recombination in both treated and systemic tissues. As compared to the control plants, recombination increased 3.9- and 3.1-fold in UVC ($p<0.05$, 6.7×10^{-5}, two-tailed, paired t-test) and RB ($p<0.05$, 2.8×10^{-6}, two-tailed, paired t-test) treated plants, respectively. Although recombination was not induced to as high an extent in the systemic (non-treated) tissues it, nevertheless, was a

significant increase. Systemic recombination was increased 2.5- and 1.9-fold in the UVC ($p<0.05$, 2.5×10^{-6}, two-tailed, paired t-test) and RB ($p<0.05$, 1.9×10^{-6}, two-tailed, paired t-test) treated plants (Table 7). To confirm the systemic effect of the SRS observed from this data, grafting experiments were required.

Table 7. Induction of recombination throughout tobacco plants experiencing a localized treatment of UVC or RB. Single leaves of 10 Petit Havana SR1 plants were treated with either Tween-80, UVC or RB. Recombination was scored in 3 – 4 upper, non-treated leaves. Treated represents data collected from the treated leaf, while non-treated is data from systemic leaves. Total leaves screened for each group was comparable. Average±SD, is the average recombination frequency ± the standard deviation; fold, the fold induction as compared to the control group of each respective treatment; and, t-test, the significance as determined by a paired, two-tailed students t-test.

Treatment	Average±SD	Fold	t-test
Control – Treated	1.9±1.0	N/A	N/A
Control – Non-treated	6.9±1.7	N/A	N/A
UVC – Treated	7.5±2.3	3.9	6.7×10^{-5}
UVC – Non-treated	17.1±2.9	2.5	2.5×10^{-6}
RB – Treated	5.8±1.9	3.1	2.8×10^{-6}
RB – Non-treated	12.8±1.9	1.9	2.6×10^{-6}

4.5.3 Grafting between UVC/RB treated and non-treated Petit Havana SR1 or *Nah*G plants

To confirm the role of a signal in induction of recombination in systemic tissues, grafting experiments were performed. In addition to the Petit Havana SR1 plants, *Nah*G (Havana) plants were used as well. *Nah*G plants are compromised in their ability to accumulate SA due to the presence of the bacterial gene salicylate hydroxylase. SA is an important signaling molecule in SAR (Ryals, *et al.*, 1996) and, therefore, its importance in the SRS was investigated. Single leaves on Petit Havana SR1 or *Nah*G plants were locally exposed to either UVC or RB. Superior, unexposed leaves were grafted to healthy, unexposed plants. Recombination events were scored in three to four newly emerged leaves on the graft recipient plants 10 – 14 days later. Seven to nine Petit Havana SR1 and six *NahG* plants received grafts. The experiment was replicated twice and data presented is an average of the two experiments. Once again, recombination was significantly increased: 1.4-fold in UVC ($p<0.05$, 0.027, paired, two-tailed t-test) and 1.8-fold in RB ($p<0.05$, 0.024, two-tailed, paired t-test), as compared to non-treated grafts (Table 8).

The *Nah*G graft recipient plants, from UVC treated plants, also demonstrated a significant increase, 1.5-fold ($p<0.05$, 0.025, paired, two-tailed t-test).

Table 8 - Increase in recombination in graft recipients of SR1 and *Nah*G plants locally treated with UVC or RB. Healthy SR1 or *Nah*G plants transgenic for the LUC recombination substrate received grafts from upper non-treated leaves of donor plants. Recombination was scored in the newly emerged tissue of seven to nine SR1 plants and six *Nah*G plants (three to four leaves per plant). Average±SD represents the average recombination frequency ± standard deviation; Fold, the fold induction as compared to the control group; and t-test the significance of the data as determined by a paired, two-tailed students t-test.

Treatment	**Sum Spots**	**Average±SE**	**Fold**	**t-test**
SR1 Control	119	13.2±3.3	N/A	N/A
SR1 UVC	151	18.9±4.5	1.4	0.027
SR1 RB	163	23.3±4.1	1.8	0.024
NahG Control	110	18.3±1.3	N/A	N/A
NahG UVC	63	27.2±2.5	1.5	0.025

4.5.4 DNA recombination events in Petit Havana SR1 and *NahG* plants pre-treated with NAC and exposed to UVC or RB

NAC was topically applied to the tobacco plants using the highest concentration that had previously been shown not to alter expected recombination frequencies (data not shown). Application locations included the to-be-treated leaf and to-be-grafted leaves, one hour prior to the respective activity (treating or grafting). Control groups represented those plants that received a mock-NAC treatment consisting of a Tween-80 only solution. *Nah*G plants did not receive an RB treatment.

Leaves were grafted to seven or eight (Petit Havana SR1) and six (*Nah*G) plants per treatment group. Recombination was assayed in three to four newly emerged leaves per treatment. The experiment was replicated twice and data presented represents average between the two replicates. Grafted plants that were not treated with NAC (Tween-80 only, no ROS inhibition) showed a 1.9- and 1.5-fold increase following UVC and RB exposure, respectively, as compared to the Tween-80 treated control. When recombination events in the NAC treated (inhibited ROS production) UVC and RB plants were compared to the no-NAC control plants, no significant increase in recombination was noted; 0.94- and 0.59- fold induction (UVC and RB, respectively). However, when the results from NAC treated (all groups, ct, UVC, and RB) were compiled, significant increases of 2.1- and 1.3-fold for the UVC and RB treated plants, respectively, was observed (Table 9).

Table 9. Effect of NAC treatment on systemic recombination in graft recipient Petit Havana SR1 and *Nah*G plants. To-be-treated leaves were pre-treated with NAC(+) or NAC(-) one hour prior to inducing treatments—UVC or RB. To-be-grafted leaves were also pre-treated one hour before grafting with NAC(+) or NAC(-). Leaves were grafted to 7 – 8 Petit Havana SR1 plants and 6 *NahG* plants. Recombination events were scored in new tissue (3 – 4 leaves/plant) of either SR1 or *NahG* plants. Fold NAC(-):NAC(-) refers to the induction of recombination in UVC and RB treated plants of the NAC(-) group as compared to the control; NAC(+):NAC(-), is the fold induction in NAC(+) UVC and RB as compared to the NAC(-) control; while fold NAC(+):NAC(+) is the induction in NAC(+) UVC and RB as compared to the NAC(+) control.

Treatment	**Recomb. NAC(-)**	**Fold Induction** NAC(-):NAC(-)	**Recomb. NAC(+)**	**Fold Induction** NAC(+):NAC(-)	**Fold Induction** NAC(+): NAC(+)
Control	34.5±4.1	N/A	15.4±1.8	N/A	N/A
UVC	66.9±8.7	1.9	32.6±5.4	0.94	2.1
RB	53.1±7.2	1.5	20.3±3.5	0.59	1.3
*Nah*G CT	18.3±1.3	N/A	8.1±0.7	N/A	
*Nah*G UVC	27.2±2.5	1.5	16.2±2.4	0.89	2.0

Recombination was induced 1.5-fold in *Nah*G plants treated with Tween-80 only and UVC, while recombination did not change significantly between the NAC(-)-control group and NAC(+)-UVC treatment; 0.89-fold. Again, the trend in recombination induction from the NAC(+)-control to NAC(+)-UVC treated group was maintained as shown by the fold induction, 2.0-fold.

4.5.5 Aqueous and organic peroxide content of graft recipient Petit Havana SR1 plants

Since peroxide is a radical species, the amount of peroxide was measured in the systemic leaves of locally treated Petit Havana SR1 plants. Content was determined in two leaves of five to six UVC treated and control plants. The experiment was replicated twice, and the data presented represents an average of the two. Both control and UVC induced plants did not experience a significant change in the aqueous peroxide content, however, following NAC treatment, organic peroxides fell to about 60% and 74%, respectively, of the levels seen in the Tween-80 only treated plants (11.3±4.2 to 6.6±0.9 for the controls; and, 10.0±3.4 to 7.4±1.2 for the UVC induced plants) (Table 10).

Table 10. Levels of peroxides produced in systemic tissues of locally treated Petit Havana SR1 plants. Leaves superior to the local NAC (+/-) treated leaf were assayed for peroxide content. Both aqueous and organic peroxide content was analyzed. Numbers represent average peroxide content (nmol/mL/g) ± standard error from three to four plants per group.

	NAC(-)		NAC(+)	
	Aqueous	*Organic*	*Aqueous*	*Organic*
Control	2.5±1.0	11.3±4.2	2.6±1.0	6.6±0.9
UVC	3.5±1.2	10±3.4	5.0±2.6	7.4±1.2

4.5.6 DNA recombination events in *Arabidopsis* mutants

Spontaneous DNA recombination was assayed in transgenic *uidA Arabidopsis* mutant plants (*vtc*, *tt4* and *tt5*) compromised in their ability to detoxify ROS. Recombination events were scored in 150 – 200 *Arabidopsis* at the full rosette stage grown on MS medium under sterile conditions. The experiment was replicated three times and data presented represents the average of the three replicates. The 651 line (*uidA* transgenic plant in a Columbia wild type background) acted as the control plants. All mutants displayed higher levels of spontaneous recombination events. Specifically, *vtc* mutants showed an average of 2.88-fold increase (0.73±0.15 events per plant as compared to 2.1±0.51 events per plant in the 651 line; $p<0.05$, $4.3x10^{-10}$, single factor ANOVA), while the *tt4* (2.2±0.32 events per plant) and *tt5* (1.7±0.37 events per plant) flavonoid mutants displayed a 3.01-fold ($p<0.05$, $6.2x10^{-17}$, single factor ANOVA) and 2.33-fold ($p<0.05$, $2.4x10^{-5}$, single factor ANOVA) increase, respectively (Table 11).

Table 11. Recombination events in various *Arabidopsis* mutants compromised in their ability to detoxify ROS. Recombination is listed as average spots per plant ± standard deviation as determined from 200-250, three week old plants per line; Fold induction to 651_{spont}, is the increase in mutant lines as compared to the control line (651).

Mutant	Spont. Recomb.	Fold Induction to 651_{spont}
651	0.73±0.15	N/A
Vtc	2.10±0.51	2.88
tt4	2.20±0.32	3.01
tt5	1.70±0.37	2.33

5. DISCUSSION

5.1 Local and systemic recombination rates measured using transgenic tobacco plants

The current work showed the existence of a systemic signal that is triggered in plants upon compatible interactions with a pathogen. The signal promotes rearrangements in non-infected tissues, leads to meiotic rearrangements and results in a higher frequency of rearrangements in the next generation. More specifically, the signal triggers rearrangements in R-gene loci, results in global genome hypermethylation followed by R-gene specific hypomethylation in the progeny of infected plants. What is the meaning of such rearrangement events? Are there any indications that R-genes rearrange under pathogen infections? Do other systemic signals exist in plants? All these and other questions will be discussed.

5.1.1 Recombination rates in the incompatible and compatible reactions

In mammals immunity is perpetually acquired over the life of the organism. The state referred to as immunity is achieved in susceptible hosts following encounters with virulent pathogens. In this system, identifiable, molecular traits of the invading, virulent pathogen are "remembered" by the mammalian antibody cells (Abbas, *et al.*, 1994). It is possible that an analogous mechanism exists in plants. Plants encountering a virulent pathogen may make an attempt to acquire novel R-genes (analogous to mammalian antibodies) by destabilizing their genome, thus, facilitating genetic rearrangements. If genome stability is reduced as a mechanism to increase the probability of acquiring novel R-gene function, then it was expected that DNA recombination would not be induced in

plants experiencing an incompatible reaction with an avirulent pathogen. The difference in recombination frequency following a pathogen attack was studied in plants that are able (incompatible reaction) and unable (compatible reaction) to mount the protective response, SAR.

An incompatible reaction occurs following infection of tobacco plants carrying in their genome an active TMV resistance gene (N-gene). Havana 425 tobacco plants contain a functional N-gene for TMV resistance. At temperatures below 28°C (i.e., 22°C, as was used in the current experiments) the N-gene plants confer resistance and, therefore, it was hypothesized that genome rearrangements would not be induced. In contrast, plants in which the N-gene function for TMV resistance was lost (i.e., temperatures above 28°C) might promote the occurrence of such rearrangements.

Lucht, *et al.*, (2002) have previously observed increased DNA recombination events in *Arabidopsis* plants challenged with an avirulent (incompatible reaction) pathogen. However, this pathogen exposure was systemic as entire plants were sprayed with a suspension of *Peronospora parasitica* causing a systemic HR. Thus, it was impossible to separate recombination events that were incited by the pathogen presence from those induced by the chemical characteristics of a HR. Here pathogen exposure was limited to a local treatment on a single leaf. Thus, it was easy to account for differences in directly treated and systemic tissues.

The temperature dependent susceptibility of the Havana 425 plants triggered a series of events leading to an increase of recombination events. The frequency of recombination increased both locally (3.4-fold) and systemically (1.8-fold) indicating that reduced genome stability was experienced in plants challenged with a virulent pathogen.

Importantly, the induction of recombination at the incompatible temperature was insignificant. Once again, this differs from Lucht, *et al.*, (2002) who observed systemic increases in recombination in plants systemically challenged with an avirulent pathogen. Such exposure would cause HR throughout the plant, and, therefore, it was impossible to rule out the chemical nature of the HR as an elicitor of the higher recombination.

The data corresponds with reduced genome stability (as measured by retrotransposon mobility) seen in pathogen elicited tobacco cell suspensions (Beguiristain, *et al.*, 2001). The Tnt1 retrotransposon activity was induced in tobacco leaf discs following exogenous elicitation with a pathogen protein as well as stress-associated signaling molecules (SA and methyl jasmonate) and the plant hormone auxin (Beguiristain, *et al.*, 2001). Furthermore, these results are similar to data on reduced genome stability in plants following other stressful abiotic events such as change of light spectra (Ries, *et al.*, 2000b), radiation (Ries, *et al.*, 2000a ; Kovalchuk, *et al.* 2003b), temperature (Jiang, *et al.*, 2003; Boyko, personal communication) and drought (Kalendar, *et al.*, 2000). This implies that some common feature exists between biotic and abiotic induction of genome rearrangements. The increase in recombination events in *Arabidopsis* following SA (and its chemical analogs) application (Lucht, *et al.*, 2002) and transposon activity following SA and methyl jasmonate exposure (Beguirstain, *et al.*, 2001), suggests that signaling pathways or individual members of such pathways may be the common feature.

To eliminate the possibility that recombination increases were caused by the different temperatures and not the N-gene functionality, *N. tabacum* cv Petit Havana SR1 plants, which do not contain a functional N-gene, and, thus experience disease

progression of TMV at any temperature, were assayed for recombination events following inoculation. The resulting somatic recombination was comparable to that seen in the Havana 425 plants inoculated at 32°C, a compatible reaction, and did not differ between the two temperatures (22°C and 32°C), indicating that compatibility governed the induction of somatic recombination.

Once again, these results supported the initial hypothesis that recombination events would increase in plants that did not possess a functional R-gene. The ability for an organism to tolerate genome instability is seen in other systems. Both SOS mutagenesis and mating type switch in yeast cells are examples of reduced genome stability. With SOS mutagenesis, mutations as a result of DNA damage are tolerated (Walker, 1995; van den Bosch, *et al.*,2002). Yeast mating type switch genetic rearrangements are induced in populations experiencing an undesirable ratio between the two mating types (α and a). This "purposeful" adaptation in the yeast seems to be induced as a mechanism to increase the likelihood of survival in yeast populations as equal numbers of a and α genotypes leads to the formation of stable a/α diploids (Brown, 1999).

5.1.2 Recombination rates triggered by a systemic signal

Besides an increase in recombination events of the directly infected tissues, systemic tissues also exhibited a recombination increase. In an attempt to either suggest or negate the physical viral presence as a cause of the recombination events, the time frame of viral mobility throughout the plant was assessed. Interestingly, recombination events were significantly induced in the systemic, non-inoculated tissues as early as eight

hours post-viral treatment while the physical viral presence was not detected in these tissues until the 36 hour interval. This suggests the recombination increase may have been induced by a signal.

The ability of a pathogen encounter to initiate such a signal coincides with previously observed events. Plant-pathogen interactions, both compatible and incompatible, affect numerous signaling pathways. Viruses typically affect changes in uninfected, plant cells distant from their physical location (Maule, *et al.*, 2002), possibly to alter the future host cell environment to one more favorable for the virus. mRNA expression in healthy cells of a cucumber mosaic virus infected plant showed that host genes not required by the virus were "shut-off," certain heat shock proteins were up-regulated, NADPH supplies increased, and antioxidants were accumulated (Maule, *et al.*, 2002). Furthermore, incompatible reactions that result in systemic immunity (SAR and ISR) require accumulation of signaling molecules such as SA in SAR and jasmonic acid (JA) in ISR. Both these pathways involve molecular and cellular changes distant from the site of infection including strengthening of the cell wall, increase in secondary metabolites toxic to pathogens and increased expression of R-genes and PR-genes (Dong, 2001; Heil and Bostock, 2002; Kunkel and Brooks, 2002).

Although the recombination signal observed thus far and SAR are not related (incompatible reactions that result in SAR do not result in recombination increases), the SAR related signaling molecule (SA) was capable of inducing recombination in *Arabidopsis* and transposon activity in tobacco (Beguirstain, *et al.*, 2001). Therefore, it is possible that the systemic increase of recombination events observed earlier than a systemic pathogen presence, may be the result of a systemic signal. Small peptides have

previously been observed in plant defense signaling. Systemin, a small polypeptide, is detected in insect-induced wounding in tomato plants (Lindsey, *et al.*, 2002), and exogenous applications of systemin activates numerous defense genes (Bergey, *et al.*, 1996).

5.1.3 The recombination rates of graft recipient plants

The aforementioned results demonstrated that the signal responsible for initiating the increase in DNA recombination events was mobile as it produced a systemic increase throughout challenged plants. However, its strength and persistence had not been fully evaluated. To further elucidate the potential of a systemic signal, recombination in graft recipient plants (Figure 2) of both cvs Havana 425 and Petit Havana SR1 at different temperatures were measured. Non-inoculated leaves were grafted from inoculated plants within 20 hours (of inoculation), thus, ensuring that the virus was not present in the grafts. Therefore, the somatic recombination induction observed in the Petit Havana SR1 at 22°C (1.8-fold) and 32°C (2.1-fold), and the Havana 425 plants at 32°C (1.5-fold), was not the result of physical viral contact. As expected, Havana 425 recipients at 22°C did not experience any significant increase again supporting the hypothesis that recombination is not induced following an incompatible pathogen attack.

Plants respond to stressful environments through acclamatory processes (Taiz and Zeiger, 2002). Acclimatization requires systemic signaling as immediate encounters ultimately affect constitutive changes. For example, high light conditions initiate changes such as chloroplast migration, reduction of photosynthetic centers, increase in photoprotective pigments and thickening of the cuticle throughout the entire plant

(Mullineaux and Karpinski, 2002). Likewise, high salt levels and low water can initiate senescence patterns and alter plant hormone concentrations level (Vieira-Santos, *et al.* 2001; Jiang and Zhang, 2002). This process requires systemic changes in plant hormone levels and, thus, is triggered and maintained by signaling molecules (Taiz and Zeiger, 2002).

Since pathogen infection is also a stressful event it is probable that various systemic signals may be initiated (Kunkel and Brooks, 2002). The current work, suggests that a systemic signal capable of reducing genetic stability does exist. Such a signal implies that reduced genome stability may be beneficial to plants following compatible pathogen encounters. This has been previously proposed by retrotransposon activity observed in stressful situations (Kalendar, *et al.*, 2000; Beguirstain, *et al.*, 2001; Jiang, *et al.*, 2003). In the event of a virulent pathogen attack, perhaps reduced stability increases the probability that novel R-genes will be acquired, increasing resistance in future generations. Additional conclusions about the relative time of induction provided cursory indications that recombination events may be more likely to be inherited by progeny. All groups experiencing a significant increase in recombination exhibited larger spots (at least twice as big) indicating that the events occurred earlier than in the plants with smaller spots. These larger spots demonstrate a potential for inheritance of early somatic events and their importance will be discussed in section 5.2.

5.1.4 Detection of recombination events using an endogenous recombination system

Since the experiments described have relied heavily on transgenic plants that harbor a non-endogenous reporter gene under the control of a constitutive promoter, it is possible that the results observed were influenced by these unnatural entities. This would not be surprising as *Agrobacterium* mediated transformation delivers T-DNA to transcriptionally active genomic regions. High gene expression may result in more frequent recombination processes. Therefore, it was necessary to employ an endogenous system capable of detecting recombination events. *N. tabacum* cv Xanthi NN plants provided such a system. First, these plants contain a functional N-gene within their genome and, are subject to the same temperature constraints on resistance as the Havana 425 plants. Thus, it was possible to challenge the plants in such an environment that a susceptible disease reaction may be obtained. Second, these plants were heterozygous for the sulfur gene and displayed a pale green phenotype. Recombination events may lead to dual color (dark green and white) spots on the leaf, allowing for easy quantification of recombination events (Shalev, *et al.*, 1999).

Grafting experiments were performed with the *Su/su* plants to further demonstrate the existence of an SRS that incites recombination in tissues not directly exposed to a pathogen. Somatic recombination data collected following a grafting experiment was in accordance with data collected from the transgenic LUC reporter system: recombination was induced 3.5-fold in graft recipients from ORMV-treated *Su/su* plants and 3.4-fold in the transgenic Havana 425 plants (under compatible conditions). These results suggested that there was no reason to suspect that the transgenic plants exhibit abnormal

recombination levels. Shalev, *et al.*, 1999, also observed similar results in recombination of tobacco plants containing a transgenic GUS reporter system and the *Su/su* endogenous system. Specifically, the transgenic GUS reporter system displayed a 11.4-fold increase in recombination events as compared to a 12 fold increase in the *Su/su* plants.

This signal was termed a systemic recombination signal (SRS).

5.2 Transmission of rearrangements to subsequent generations

Up to this point, recombination had only been identified to exist in somatic tissues. Although such a mutational event was interesting, the significance of it in an evolutionary sense was unimportant. Somatic events occurred within individual cells of a plant. Through mitotic cell divisions, daughter cells expressing the intact reporter gene accumulated and were identified as shining spots on the leaf. Typically, organisms are capable of sustaining these mutation events in their somatic tissue with no effect on variation or evolution of the genome (Brown, 1999). Only mutations in germ cells or early somatic recombination events are passed on to future generations. Therefore, if an increase in recombination was a mechanism by which novel R-gene functions arise, than it was necessary to identify that these events were more likely to occur during meiosis in plants exposed to a pathogen. Meiotic recombination events would be inherited and constitutively expressed by the subsequent generation.

5.2.1 Meiotic recombination rates in progeny of infected and control plants

Meiotic recombination events were 3.5-fold higher in the PofI as compared to the PofC plants. The increase in meiotic recombination frequency of the PofI plants indicated a higher probability that challenged plants will pass on genome rearrangements to their progeny. If these rearrangements are induced as a mechanism for acquiring novel resistance genes then it was important that such rearrangements be identified in the progeny of infected plants. Sudupak, *et al.*, and Richter, *et al.*, (1995) had previously observed meiotic recombination events between markers of a maize R-gene locus—*Rp1*. Using a similar system to ours to measure recombination, Ries, *et al.*, (2000a) observed that reduced genome stability in plants chronically exposed to UVB led to increased frequency of meiotic recombination. In the UVB experiments, meiotic recombination frequencies increased four to ten fold as the UVB dose increased. Again, the result of the abiotic exposure in these plants parallels the data presented here as a result of biotic stress.

5.2.2 Somatic recombination rates in progeny of infected and control plants

Another interesting characteristic was noticed when the somatic recombination events of PofI and PofC plants were quantified. Although neither plant type had been challenged, biotically or abiotically, in this generation (F_1), the spontaneous, somatic recombination rates were almost twice as much in the PofI plants as in the PofC. This suggests that the persistence and longevity of the SRS lasts over multiple plant generations. Ries, *et al.*, (2000a) also noted an increase of about 2.3 – 2.5 in the fold induction of recombination events between generations of transgenic *Arabidopsis* plants

exposed to elevated UVB levels. This increase in levels of induction suggests that exposed plants may pass on a reduced stability to their progeny, thus, causing the progeny to experience a greater number of rearrangements following stress. Our data complies with such a belief as the spontaneous (non-induced) recombination rates of the PofI were on average 1.8-fold higher than PofC plants. The reduced stability of the progeny was caused by a virulent pathogen attack in the parental generation while progeny in the Ries, *et al.*, (2000) data were the result of abiotic UVB stress. It appeared possible that the reduced stability may have been the result of a general stress pathway. Exposure of PofI plants to sodium chloride and rose Bengal displayed an average higher rate of recombination (~2-fold) than in the PofC plants. Sodium chloride stress was previously seen to induce recombination in *Arabidopsis* plants using a similar reporter system (Puchta, *et al.* 1995). Similarly, the chemical mutagen rose Bengal also was shown to induce recombination in the transgenic *Arabidopsis* (Kovalchuk, *et al.*, 2003a).

Induction of genetic rearrangements under stress conditions has been observed in other organisms including bacteria (Bjedov, *et al.*, 2003) and yeast (Brown, 1999). Molecular analysis of numerous stress-related genes in *E. coli* suggested that these genes may have evolved with a predisposition for rearrangement. Relative to other non-stress response genes, the stress genes display an over representation of sequence repeats (Rocha, *et al.*, 2002). These repeats increase the ability for recombination, which may be induced under stress conditions.

Plant genetic stability also was shown to be affected by other stressful events. Kalendar, *et al.*, (2000) identified an increase in both retrotransposon number and activity in water stressed barley. This activity invariably led to larger genome sizes containing

more repetitive sequences (i.e., the BARE-1 retrotransposon). Similar to Rocha, *et al.* (2002), it was suggested that the higher incidence of repeats may render the plant more genomically flexible and, thus, better suited to survive stressful situations. Similar data has recently been presented by a transposon family in rice (Jiang, *et al.*, 2003). Rice cultivars adapted to harsher environmental conditions (cool, temperate zones with short growing seasons) display more extensive amplification of the *mPing* transposon element as compared to cultivars from more favorable (tropical zones) environments (Jiang, *et al.*, 2003). Thus, it may be suggested that the stressful conditions encountered during acclamatory and adaptive responses induce genomic rearrangements.

5.3 TMV tolerance in the progeny of infected and control plants

Since the postulated purpose of the increased genomic instability is to potentially acquire novel R-gene functions, which may impart a beneficial phenotype to future generations, PofI plants were screened for an increase in resistance to TMV. Although it was impossible to conclusively attribute the later disease onset to acquisition of a novel R-gene that imparted TMV resistance, it may be speculated that the progeny of challenged plants were likely to experience a heightened resistant state. Richter, *et al.*, (1995) had previously screened 4200 progeny of maize plants looking for interchromosomal exchange with an R-gene region. These recombination events were subsequently screened for novel resistance to several races of a rust fungus. Of the 176 rearrangement events noted, most (150 / 176 total) were not attributed to novel resistance, and some (9 / 176) displayed reduced resistance as compared to the parental generations, four recombination events did give rise to novel resistances that were not present in the

parents. Our results showed a similar result in that most lines did not differ from the parental susceptibility, some appeared more sensitive (albeit insignificant), and a few displayed a higher resistance. However, our experiment only screened about 150 plants. It is possible that with a larger sample size, the possibility of obtaining a completely resistant plant would be more likely.

5.4 Rearrangements detected specifically in R-gene-like loci

The meiotic and somatic recombination in PofI and PofC plants suggested that the reduced genome stability was maintained through at least two generations. It was also clear that the induced instability yielded an increased number of heritable, meiotic recombination events. However, the response of specific, individual regions or loci of the genome was unknown. The employment of the transgenic based recombination system only conveyed information on a fraction of recombination events, namely those that occur between the overlapping regions of the construct. This, coupled with the fact that the reporter system had randomly integrated itself into the genome, meant that data collected thus far could not specifically be attributed to a given region of the genome. Since it was hypothesized that recombination was induced as a mechanism to increase the production of novel R-genes, it was important to demonstrate some specificity of the rearrangements to an R-gene locus. Petit Havana SR1 plants lack a functional R-gene but, however, do possess a number of loci with significant homology to the 4th exon of the N-gene (Whitman, *et al.*, 1994). The Petit Havana SR1 plants were challenged with TMV and N-gene-like loci were evaluated for instability in the progeny of these challenged plants.

Using RFLP analysis, 30 fragments of different lengths containing homology to the N-gene locus were found to be common between plants. Genetic rearrangements altered the sequence causing a shift in the expected size of restriction fragments (as visualized on the fluorescence tagged film exposures). The PofI plants experienced an 8.2-fold increase in the number of rearrangement events in their N-gene-loci. It is important to note that this is a larger increase as compared to the increase measured with the luciferase recombination substrate. The RFLP analysis did not discriminate between types of rearrangements and, therefore, encompasses all other events besides recombination. Nevertheless, this result strongly demonstrates a trend of a higher degree of genomic instability in progeny of infected plants. The rate of rearrangement presented here (~1 / 1100) was in the same range as the spontaneous rate of rearrangement (~1 / 2000) previously reported by Whitman, *et al.*, (1994).

The rearrangements in an R-gene locus of progeny plants had been explored previously. Sudupak, *et al.*, (1993) determined interchromosomal recombination of two alleles of a maize R-gene to occur in the order of ~0.5 events / 1000 progeny and ~3 events / 1000 progeny. Similar to our results, the Sudupak results demonstrate that rearrangements do occur specifically in R-gene loci. Furthermore, they showed that differing degrees of stability are experienced by different R-gene alleles. These results combined with the present findings suggest that instability maybe differentially targeted between regions of the genome (i.e., N-gene-like loci and *Rp1* region) and even differentially controlled at various alleles within a given region (Sudupak, *et al.*, 1993). This theory will be explored and further discussed in section 5.5.

5.5 Global and R-gene-like loci DNA methylation patterns

Since the digested DNA was probed with the 4th exon of the N-gene, all the alterations were catalogued as shifts within an R-gene-like locus. Thus, it was plausible that the increased events seen in pathogen challenged plants were targeted to R-gene loci and, therefore, increased the potential to produce novel R-genes and/or functions. If further RFLP examinations of non-R-gene loci demonstrate that these regions did not have a propensity to acquire sequence alterations in pathogen stressed and non-stressed plants, then it could be further suggested that instability is specifically targeted to the R-gene loci.

In an attempt to provide cursory support for the targeted instability hypothesis, epigenetic characteristics of the plant genomes were observed. Methylation patterns have been shown to play an integral role in maintenance of recombination activities of loci in the mammalian immune system (Engler, *et al.*, 1993). Furthermore, plant genomes are known to be highly repetitive and highly methylated, lending to the belief that methylation may act to stabilize the genome preventing rearrangements (Puchta and Hohn, 1996).

RFLP analysis using methylation sensitive endonucleases and probing blots with the 4th exon of the N-gene (corresponds to the LRR) provided data on the methylation status of these N-gene-like loci. While most of the predictable 24 loci were similarly methylated, four of the predictable 24 loci, were hypomethylated. Specifically, the degree of hypomethylation was between four and 16 fold.

Since methylation is a well explored process of genome maintenance (methyl groups tend to make chromatin less accessible to various remodeling processes), this

hypomethylation could be suggested as a mechanism facilitating the rearrangement of the genome. Studies have also shown that DNA regions of highly duplicated genes also contain a high level of methylation. Removal of gene copies reduces methylation density of the remaining copies (Bender, 1998). Conversely, the high rate of recombination in the V(D)J region of immunoglobulin and T-cell receptors in the mammalian immune system are regulated by DNA hypomethylation (Bassing, *et al.,* 2002; Inlay and Xu, 2003). Engler, *et al.* (1993) quantified rearrangements in the V(D)J locus of immune cells in mice and demonstrated that demethylated loci experienced a higher frequency of rearrangement events. Methylated loci were highly stable and the methylation patterning was facultative as hypomethylation was experienced during temporal downstream expression of both rearranged and stable loci (Engler, *et al.*, 1993).

Once again, this data did not elucidate the specificity or extent of the hypomethylation. Did the pattern of hypomethylation extend throughout the genome, or was it also localized to the N-gene-like loci? To examine the extent of global methylation a cytosine extension reaction was employed. Once again, the genome of numerous samples was restricted with the endonuclease *Hpa*II. The resulting 5' G overhangs allowed for the extension reaction to incorporate a radio-labeled C nucleotide. Global methylation status of four of the PofI lines was shown to be between 20 to 210% hypermethylated relative to PofI plants.

Hypermethylation is associated with increased genome stability. Although genome plasticity may impart a benefit to organisms under stress (Kalendar, *et al.*, 2000; Rocha, *et al.*, 2002; Bjedov, *et al.*, 2003; Jiang, *et al.*, 2003), gross rearrangement events may be detrimental to the survival of the organism (Kovalchuk, *et al.*, 1998). Global

hypermethylation following exposure to stressful environments was previously demonstrated by Kovalchuk, *et al.,* (2003c). The data collected previously indicated a trend of decreased stability in the transgene and R-gene loci of progeny of infected plants. When the global hypermethylation was taken together with the previous N-loci specific data, it suggested that besides plants reducing methylation at the R-gene locus, they increased methylation elsewhere. Understanding the mechanism of genomic methylation as a control of genome stability, this result suggests that methylation is differentially distributed so as to decrease stability in a particular region, while perhaps increasing stability in others. This may in part explain why some loci seem to be less duplicated with more conserved characteristics than others (Sudupak, *et al.*, 1993; Mauricio, *et al.,* 2003; Kroymann, *et al.,* 2003). Perhaps in these instances, methylation patterns are not reduced or are even more heavily methylated and, thus, genome rearrangements are not facilitated and a higher level of stability is exhibited. Unfortunately, the data here does not provide the temporal characteristics of the events culminating in the differential methylation expression. The impact of methylation patterns on the theory of balancing selection would be better understood following protein expression and activity assays with respect to those enzymes known to act in *de novo* and maintenance of DNA and histone methylation events.

5.6 Role of reactive oxygen species in the SRS

Elucidating the signal that stimulated recombination events in systemic tissues and in separate plants (transmitted via grafting) is a daunting, important task to be completed. Here, a possible member of the local events that result in increased

recombination within a given tissue was investigated—ROS. Upon profiling H_2O_2 presence in a plant, Grant and Loake (2000) noted a biphasic presence, i.e., an initial burst: detected in both compatible and incompatible reactions, and a second burst, noted only in the incompatible reaction and closely associated with the onset of SAR. This initial burst is of interest as it was present in plants that do not possess a functional R-gene and, therefore, do not mount a HR. Since ROS function is thought to be tightly associated with the HR, and no HR response was expected in these plants, the ROS presence might fulfill the capacity of another function in plant defense (i.e., signaling). Perhaps the initial, lower dose of ROS experienced by plants during the compatible reaction stimulates the signaling events that result in reduced genome stability. The larger burst subsequently experienced in the incompatible reaction may act as a suppressor or negative regulator to the recombination signal explaining why recombination is not seen systemically in locally inoculated plants. The suggestion that the level of intracellular radicals defines what cell response occurs has been previously suggested (Finkl, 1998). The antagonistic relationship between various plant signals has also been identified. The two types of systemic immunity (SAR and ISR), although cooperative in some facets, also have inhibitory effects on one another. Accumulation of salicylic acid, a major signaling molecule in SAR, experienced following an avirulent pathogen encounter displays antagonistic effects on jasmonic acid (JA), the major signaling molecule in ISR, following wounding (Doares, *et al.*, 1995). Likewise, the constitutive expressing SA *Arabidopsis* mutant, *mpk4*, blocks expression of particular JA-induced defense genes (Petersen, *et al.*, 2000). Concomitant excess UV light and pathogen encounter results in suppression of the UV protection pathway but not the

defense pathway, suggesting that initiation of the signal leading to SAR also inhibits the signal responsible for up-regulation of UV protection (Logemann and Hahlbrock, 2001).

Due to their instability and highly damaging nature, it is unlikely that ROS are the transmittable signal responsible for propagation of the SRS. However, it is possible that ROS initiate the SRS in tissue experiencing recombination events.

5.6.1 Effect of exogenous agents, known to increase ROS, on SRS induction

To investigate the possible role of ROS agents, transgenic plants were treated with agents known to induce radical activity. Both rose Bengal (RB) and UVC radiation are known to elicit radical formation (Brit, 1995; Kim, *et al.,* 2001; Hollosy, 2002). Rose Bengal is photoactivated to produce singlet oxygen ($1O_2$) and superoxide anion—a ROS. Exposure to RB through the growth medium or topically, affected an increase in radical presence within the cell (Kim, *et al.*, 2001). In addition, UVC is known to generate ROS species through the splitting of water molecules (Brit, 1995; Hollosy, 2002). ROS presence has been correlated with other types of genome rearrangements such as transposon activity. Ikeda, *et al.*, (2001) have shown that retrotransposon MAGGY is activated under oxidative stress in a plant fungus. This activity increased transient strand breaks providing an opportunity for further genome rearrangements. Furthermore, free radical presence following chemical exposure has been cited as a potential inducer of intracellular recombination events (Brennan and Schiestl, 1998; Kovalchuk, *et al.*, 2003a). Corresponding data was obtained when the transgenic LUC tobacco plants were systemically treated with UVC and RB. As compared to the control group, UVC irradiated and RB exposed plants experienced a 3.1- and 3.7-fold increase, respectively.

However, with the systemic treatment it was not known whether the increase was due to direct exposure to the radical inducing agents, or whether a local radical induction could induce the systemic signal of recombination.

Systemic recombination was scored in plants that had received a local (on a single leaf) application of the radical inducing agents. Once again, recombination was significantly increased 2.5- and 1.9-fold, in UVC and RB treated plants, respectively. HPLC analysis of distal tissues (of locally RB treated plants) at various times following the RB exposure (up to 48 hours later) have shown that RB migration does not occur (data not included). Thus, it is safe to assume that systemic increases were not due to the migration of the RB itself. This data indicates that local increases in radicals are capable of reducing genome stability in distal tissues, further suggesting that ROS possess some role in the SRS pathway. Treatment of single leaves with UVC further supported this assumption as locally applied UVC would not travel throughout the plant.

Further grafting experiments supported the theory of ROS inciting the SRS. The purpose of the graft was to assess whether the signal could be dissociated from the radical increase and still affect recombination in separate, individual plants or not. Grafting experiments were performed after treating a leaf on one plant locally with UVC or RB. Following 24 hours, a superior leaf (non-treated) was excised and transferred to another untreated plant. Graft recipient plants demonstrated a higher increase in recombination events. Recipients from UVC treated plants increased 1.4-fold while the recipients from RB treated plants increased 1.8-fold.

The propagation of the SRS in absence of a pathogen, but in the presence of increased ROS supported the hypothesis that radicals fulfill some role in the induction of

genomic instability. The grafting experiments further suggested that localized ROS induces systemic signals. The role of ROS in signal transduction pathways has been described previously. ROS are known to participate in signal transduction within the mammalian immune system, neurons, endothelial cells and fibroblasts (Finkl, 1998), as intracellular messengers in receptor signaling (Junn, *et al.*, 2000; Ding, *et al.*, 2002), in plant response to wounding (Leon, *et al.*, 2001), incompatible pathogen presence (Alvarez, *et al.*, 1998; Dat, *et al.*, 2000; Kunkel and Brooks, 2002) and excess excitation energy (Karpinska, *et al.*, 2000). In addition, different sets of genes are stimulated by H_2O_2 and O_2^- during redox regulation of transcription in prokaryotes (Demple, 1997; Rosner and Storz, 1997).

5.6.2 Effect of NAC, a ROS inhibitor, on DNA recombination events

To assess the validity and importance of the ROS specifically and to dismiss the suggestion that the UVC and RB treatments were directly responsible (i.e. directly caused DNA damage) for the recombination, the presence of a known inhibitor of ROS accumulation was incorporated into the grafting experiments. N-acetyl-L-cysteine (NAC) is a powerful antioxidant capable of scavenging ROS (He and Harder, 2002). Therefore, it was believed that the application of NAC prior to a radical inducing treatment of a plant would reduce ROS accumulation and, this reduction in radical presence would inhibit recombination events. As suspected, the NAC treatment significantly reduced the number of recombination events in the plants receiving grafts from treated individuals. This indicates that the exogenous application of a ROS inhibitor prior to radical inducing treatment does indeed decrease the frequency of

recombination, but that all plant groups experienced the NAC effect equally. That is, although total recombination events were lower in all three treatments, the trend of increased recombination in the UVC and RB groups was maintained. This indicates that although NAC is able to decrease the effect of ROS on recombination, it is not able to block the signal causing recombination increases in systemic tissues. This further supports the suggestion that although ROS act in triggering the signal, they are not the signal themselves.

Similar results were reported by Brennan and Schiestl (1998) when the recombinogenic effects of RB, thiourea and cadmium were suppressed following an NAC pre-treatment in yeast cells. Kush and Sabapathy (2001) provide further evidence for antioxidants reducing DNA rearrangements. Over-expression of the *Arabidopsis* antioxidant gene Oxy5 in mammalian cancer cells protected then against the damaging activity of hydrogen peroxide (Kush and Sabapathy, 2001).

5.6.3 Role of salicylic acid in SRS production

The role of SA was further investigated in the present study. *Nah*G plants incapable of accumulating SA due to a bacterial transgene (salicylate hydroxylase) that degrades SA were treated with the ROS eliciting treatment—UVC. Grafting procedures (as described above) were performed. *Nah*G graft recipients displayed a significant increase in recombination events of 1.5-fold. Importantly, this increase was about the same as the graft recipients of UVC and RB treated Havana 425 plants (1.4- and 1.8-fold, respectively), demonstrating an equal potential for initiation of an SRS in the SA(-) *Nah*G and SA(+) Havana 425 plants. Likewise, grafting experiments using the *Nah*G plants

pre-treated with NAC(+) or NAC(-), also demonstrated responses similar to the non-*Nah*G plants.

SA fulfills an important role in SAR in plants (Ryals, *et al.,* 1996). Its accumulation is necessary for achieving SAR as *Nah*G plants compromised in their ability to accumulate SA also are incapable of achieving SAR (Gaffney, *et al.*, 1993); exogenous SA application induces SAR in plants (Dong, 2001); exogenous application of chemical analogues are also capable of mounting SAR (Gorlach, *et al.* 1996); and mutant plants that constitutively express SA, experience heightened resistance to pathogen attacks (Yu, *et al.* 1998). However, its purpose, if any, with respect to the SRS are not fully known. Lucht, *et al.*, (2002) demonstrated that recombination events were increased in plants treated with SA, SA-analogs, and in a constitutive SA expressing *Arabidopsis* mutant, however, it was not clear whether SA was necessary for the recombination increases or not. Thus, although SA can induce an SRS (Lucht, *et al.* 2002), it can be concluded that the absence of SA does not interfere with the production of a SRS. Further analysis of recombination rates in *Arabidopsis* defense gene mutants that experience abnormally high and low levels of SA (*cpr*5 and *pad*4, respectively (Glazebrook, 2001)), is currently underway and should provide additional insight into the role of SA in the SRS.

5.6.4 Quantification of H_2O_2 levels in the distal tissues of non-induced, graft-recipient plants

To demonstrate that recombination events may be linked to a localized increase in radical species, quantitative assays of peroxide were conducted. Since "radical" is a

broad term applied to any species with oxidative capabilities, data so far was assumed to be the result of numerous oxidative species. However, peroxides are thought to be a major contributor to oxidative stress in plants (Willekens, *et al.,* 1997; Chamnongpol, *et al.,* 1998; Grant and Loake, 2000).

Peroxides were extracted, and assayed from new leaves on graft recipient plants that received their graft from NAC(+/-) and control or UVC treated plants. Interestingly, no difference was detected in aqueous peroxide content between the NAC(-) and NAC(+) treatments for both the control and UVC treated groups. However, organic peroxides fell to about 60% and 74% in the NAC(+) treated groups of control and UVC-exposed plants, respectively. Importantly, recombination was not induced in these leaves (0.94-fold induction, table 6) and, thus, the lower levels of organic peroxides detected in the NAC(+) plants (as opposed to NAC(-) plants) suggest that the ROS inhibition prevented an SRS.

It is interesting to speculate why recombination decreased (as expected) but aqueous peroxide levels did not change. There are two possible explanations for why aqueous peroxide levels do not change significantly but recombination does. First, the scavenger NAC may be specific as to what oxidative species it scavenges. For example, perhaps NAC is effective at reducing organic peroxides, but is not effective in blocking aqueous peroxide accumulation. Second, it may be possible that oxidized, cellular, organic entities (i.e., lipids) are more intimately related with the SRS than other ROS. An increase in ROS would also affect an increase in oxidized cellular components and, thus, the data presented so far may be representative of a removed, indirect effect of ROS on production of a SRS.

5.6.5 Frequency of DNA recombination events in *Arabidopsis* mutants

The indication that radicals were involved in the induction of recombination was further supported following analysis of the response of *Arabidopsis* mutants impaired in their ability to detoxify ROS. Plants are perpetually exposed to a wide range of environmental factors, including high light, which facilitate the production of radical species. In an attempt to combat such an occurrence, an antioxidant defense system comprised of quencher molecules exists. These antioxidants often act by absorbing excess energy, i.e., from radical species, in a non-harmful manner (Jansen, *et al.*, 1998; Smirnoff and Wheeler, 2000). Antioxidants such as ascorbic acid (or vitamin C) and flavonols are examples of cellular species that act to reduce the presence of intracellular radical species. Vitamin C is a small molecule that serves dual purposes. Besides displaying antioxidant activities in the reduction of ROS, it possesses functions in growth and mitosis. The *vtc* mutant is the result of a dysfunctional enzyme (GDP-mannose pyrophosphorylase) in the ascorbic acid biosynthetic pathway (Conklin, *et al.*, 1996; 1999). Flavonoids are non-photosynthetic pigments that are manufactured to act as protectors from excess light energy when the photosynthetic machinery is saturated (Hollosy, 2002). The flavonoid mutants, *tt4* and *tt5*, both are inept in producing non-photosynthetic pigments. However, their expressed phenotype arises from different points in the biosynthetic pathway. Specifically, *tt4* is deficient in chalcone synthase while *tt5* lacks chalcone isomerase activity. The absence of these enzymes caused an increase in sinapate esters in the *tt4* mutant relative to *tt5* that renders *tt4* more resistant to UV light damage than *tt5* (Li *et al.* 1993). Eliminating the presence of either of these

antioxidants would inevitably affect an increase in the intracellular ROS. For this reason, *Arabidopsis* mutants lacking the ability to produce ascorbic acid (*vtc*), or accumulate flavonoids (*tt4* and *tt5)* were observed for recombination frequencies both spontaneously and following UVC induction.

The observation of all mutants experiencing higher levels of spontaneous recombination was predictable. Mutants would experience a higher incidence of radical species and radical presence has been shown to induce recombination (Brennan and Schiestl, 1998; Ries, *et al.*, 2000a ; Kovalchuk, *et al.*, 2003a). Radicals are known to lead to DNA strand breaks (Brennan and Schiestl, 1998), and recombination is a repair mechanism for such breaks (Puchta, *et al.*, 1996). The results further support the idea that radicals may be involved in triggering production of strand breaks leading to the increase in DNA recombination frequency.

6. SUMMARY

The data presented here provides initial support for the theory of adaptive evolution in R-gene loci of plants. Initially, the observation that recombination, and ergo, genome stability, is adjusted differently between plants possessing a functional R-gene (resistant) and those lacking such a gene (susceptible) allowed one to postulate that perhaps instability is a purposeful mechanism. Perhaps plants employ destabilizing processes in an attempt to increase the likelihood of acquiring novel R-gene functions. Since evolution is achieved through heritable traits and characteristics, it was important to observe an increase in the number of meiotic recombination events in the progeny of infected plants. These recombination events represented alterations and rearrangements in the germ cells of the plants, and, thus, represented changes that were passed between generations. Direct support of the adaptive evolution process was achieved through RFLP analysis of polymorphisms in R-gene-like loci in the progeny of infected plants. Although the recombination substrate assay was capable of conveying information on a global genomic level, it lacked the capability to provide a direct measure of rearrangements in a specific locus. With the RFLP analysis, proof of more destabilized R-gene loci (relative to the progeny of control plants) was observed in the progeny of infected plants. Additional, RFLP analysis using methylation sensitive endonucleases in concert with global methylation studies provided data suggesting that destabilization was perhaps targeted specifically to R-gene loci, and that this targeting may be achieved through epigenetic processes.

In addition to the evidence linking reduced stability and increased genetic rearrangements, empirical evidence for the acquisition of heightened resistance was

presented. Some lines of the PofI plants displayed a stronger response to stressful environments than did the PofC plants. In addition, a number of the PofI plants experienced a higher resistance to TMV infection than their control counterparts. Although, the plants did not show an absolute resistant state, they did display a larger time lag to the appearance of symptoms and, thus, were "more" resistant, suggesting that reduced genome stability may lead to acquisition of novel resistance traits.

Besides compiling data on the rearrangements resulting from reduced genome stability, the project also provided information on how reduced stability may be achieved systemically in plants. Collectively, this data demonstrates that recombination is achieved systemically through the transmittance of a systemic signal. Although this signal is initiated by virulent pathogens, it is produced earlier than the actual pathogen colonization and is persistent in the complete absence of pathogen contact (grafting experiments). ROS levels likely affect either the initiation or propagation of this signal. It is elicited in plants where ROS have been induced; known radical inhibitors reduce the occurrence of recombination events, and mutant plants incapable of ROS scavenging display high levels of recombination. Although it is not likely that ROS represent the only entity involved in the induction of reduced genomic stability, current evidence strongly suggests it likely fulfills a major role in the signaling pathway. In addition, data suggesting that SA is not necessary in the initiation of a SRS was presented. Plants incapable of accumulating SA (*Nah*G) did not display any difference in recombination frequency following radical eliciting and inhibiting treatments.

7. FUTURE DIRECTIONS

Many voids still exist in the knowledge of the processes and mechanisms of plant immunity. Of primary interest, would be to carry the PofI plants through an additional generation to observe the longevity of the destabilizing effect. Also in the future, it would be important to further assess the different mechanisms contributing to the increase in DNA recombination events in pathogen challenged plants. Although, non-homologous end joining has been suggested here as a major contributor to genetic variability, it is necessary to develop an assay capable of measuring such events. In the presented data, strong support was presented for the targeting of genomic stability to a particular region within the genome. To further this belief, it would be interesting to investigate the occurrence or lack of similar polymorphic entities at a non-R-gene region of the genome. In addition, DNA methylation was presented as a potential regulatory mechanism in the destabilization process. However, data on the enzymes involved in *de novo* DNA methylation and their temporal expression involved in this process would provide a more intimate picture of the epigenetic changes that ensue. Another point of interest arose with the differential response of PofI, relative to PofC plants, to the presence of chemical stresses—RB and NaCl. How general or specific is the destabilizing mechanism? Are plants capable of perceiving the difference between an invading virulent pathogen and a general, abiotic stressful environment? Finally, elucidating and identifying the phloem mobile signal entity and genetic pathway that propagates the systemic recombination signal is necessary.

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