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Genetic and Epigenetic Nature of Transgenerational Changes in Stressed Plants

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ABSTRACT

Plants, as sedentary organisms, are constantly exposed to a variety of abiotic and biotic factors. The ability for quick adaptation is perhaps the major reason for the diversity of plant species on our planet. The fact that plants are able to adapt within a single generation upon exposure to stress suggests the involvement of epigenetic mechanisms of inheritance. Changes in genome methylation and histone acetylation/methylation, being a part of epigenetic regulation, lead to a differential ability of certain areas of the genome to rearrange. If directed to a particular genomic region, epigenetic changes could result in an increased frequency of rearrangements leading to appearance of novel genes, thus new traits. We believe that epigenetic alterations are a general mechanism of plant adaptation to stress, and are the initial mechanism of permanent genomic changes leading to genome evolution. Here we present several examples of the influence of various abiotic and biotic factors on plant genome stability and plant tolerance to stress. We describe the influence of viral and bacterial pathogens on tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) genomes. Our experiments suggest that exposure of plants to pathogen stress triggers the production of a plant-derived signal, named the systemic recombination signal (SRS), that is capable of changing the stability of the plant genome. Infected plants "propagate" the information about pathogen infection by passing the signal to meiotic tissue. Progeny of infected plants show changes in global genome and loci-specific methylation patterns, indicating the epigenetic nature of the signal. This leads to multiple changes in plant physiology, including higher tolerance to stress and increased instability of resistance gene loci. Similar results were also obtained after exposure to several unrelated abiotic stresses. In this review we introduce a novel theory of stress-induced plant genome evolution and discuss the mechanisms behind such a phenomenon.

Keywords: abiotic and biotic stress, *Arabidopsis thaliana*, genetic and epigenetic response, genome stability, methylation pattern, *Nicotiana tabacum*, transgenerational effect

Abbreviations: Avr, avirulence; **HR**, homologous recombination; **LUC**, luciferase; **NHEJ**, non-homologous end joining; **"P_of_C"**, progeny of control; **"P_of_I"**, progeny of infected; **R**, resistance; **RFLP**, restriction fragment length polymorphism; **SAR**, systemic acquired resistance; **SRS**, systemic recombination signal; **TMV**, tobacco mosaic virus; **UVB**, ultraviolet radiation B

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INTRODUCTION

Living organisms are in a constant battle with their environment. Many of these external stimuli, commonly described as stresses, have an adverse effect on growth, development, and reproduction (Arnholdt-Schmitt 2004; Madlung and Comai 2004). Each organism has a set of "survival skills" that are employed for protection against the environment. These survival skills can be broadly divided into tolerance, resistance or avoidance mechanisms. One of the primary strategies against this overpowering influence is escape. Plants, as sedentary organisms, lack the ability of avoiding external stresses, and thus should posses the ability of interacting and exchanging signaling molecules with the

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environment (reviewed in Cronk 2001). The only two mechanisms that plants are able to mount are tolerance and resistance. Resistance allows plants to resist the changes in the environment, for example by closing stomata to prevent water loss or by utilizing the resistance gene-based mechanism of protection against pathogens, whereas tolerance only delays expression of stress symptoms (Hammerschmidt and Dann 1999).

Plants, unlike other higher eukaryotes, continue to develop throughout their entire life cycle and thus should have the ability to respond to the environment through multiple feedback mechanisms. This, and perhaps some other unknown reasons, allow plant-life to thrive on our planet. This fact can only be explained by the ability of plants to modify their genome upon the influence of stress. The control over gene expression in response to stress partially occurs at the level of selective methylation of various genome areas (Sha *et al*. 2005). Selective methylation also results in differential gene expression at different developmental stages and various tissues (Vanyushin 2006). Plants do not have a predetermined germ line and changes in somatic meristemic cells can potentially be passed to the next generation. This implies that the "memory" of stress exposure can be passed to the next generation via germ cells by way of differential methylation of various areas of the genome. It would be pleasurable to think that differenttial genome methylation upon stress exposure would result in a change of frequency of rearrangements of certain genomic areas. In fact, there is an inverse correlation between the level of methylation of certain areas of the genome and the rate of genome rearrangements, whereby the lower the methylation, the higher the rate of homologous recombination (Engler *et al*. 1993; Bender 1998; Bassing *et al*. 2002). The majority of these changes, however, would be neutral or deleterious, but in some rare cases they would be truly beneficial. This phenomenon could lead to the appearance of plants with a broad variety of modified traits.

Continuous exposure to a particular stress will ultimately lead to the evolutionary selection of adaptive traits beneficial to those conditions, albeit at a slow and gradual rate. This is not surprising, since retention and fixation of the necessary trait requires the selection for a number of neutral random changes in the genome. However, plants are also capable of acclimating on a reduced time scale by altering their homeostasis and, therefore, adjusting to a frequently changing environment (Shinozaki *et al*. 2003; Sung *et al*. 2003). Both types of changes, whether in genotype or phenotype, respectively, are the signs of adaptive genomic changes that enhance the environmental fitness of a population of organisms. The very fact that plants respond to unrelated physical, chemical, or temporal environmental factors suggests the existence of complex perception and response signaling pathways (Shinozaki *et al*. 2003; Chinnusamy *et al*. 2004; Ludwig *et al*. 2004).

In the current review, we discuss what is known about stress-induced epigenetic changes resulting in differential genome rearrangements and present the data from several experiments indicating the role of methylation changes and homologous recombination in the plant response to pathogen stress.

REGULATION OF GENOME STABILITY

Plant response to stress has been studied for more than a century, and the physiological effects of stress have been described for a long time. In contrast, the effect of stress on the genetic material, DNA, was not known until the early work of Barbara McClintock. She predicted that the plant response to stress involved a variety of genome changes, starting from unusual changes including transposon activation and "other structural modifications of the chromosomes" (McClintock 1984). McClintock predicted that there are at least four distinct examples of stress that could cause large genomic rearrangements facilitated by activation of transposon movement and chromosome breakage-fusion. These four stresses included tissue culture, exposure to pathogens, crosses between species, and the separation of the germline from somatic tissues during early development (McClintock 1984).

Genome instability generally refers to the susceptibility of the genome to rearrangements and activation of mobile elements, whereby a stable genome impedes these mechanisms. What are the mechanisms that are employed for genome protection? Excluding various mechanisms of mutagen detoxification and physiological protection based on the production of various protective pigments, plants have more direct mechanisms of genome protection. The genome of every plant species has a specific structure that depends on the different arrangement of methylated and unmethylated histones and attachment of chromatin loops to the nuclear matrix. Changes in chromatin structure are a part of the natural plant ability in the response to stress (Takeda *et al*. 2004; Buchanan *et al*. 2005). The latter paper describes one of the novel proteins, BRU1 that involved in the maintenance of both, genetic and epigenetic information and thus regulates structural and functional chromatin stability (Takeda *et al*. 2004). Due to inability of quick postreplicative modifications of chromatin structure, such as pericentromeric heterochromatin condensation and maintenance of transcriptional gene silencing, BRU1 mutant is extremely sensitive to stress (Takeda *et al*. 2004). Formation of specialized chromatin structure in response of facultative halophyte *Mesembryanthemum crystallinum* to salt stress allowed the control over the expression of multiple genes associated with adaptation to salt stress (Dyachenko *et al*. 2006). Another, perhaps more direct mechanism of genome protection, is the availability of a variety of DNA repair mechanisms, starting from simple, so-called direct repair and ending with the complex non-homologous end-joining (NHEJ) and homologous recombination (HR) repair (Jeggo 1998; Hays 2002). The latter two mechanisms are believed to be the two most important for genome changes and genome evolution (Puchta 2005; Schuermann *et al*. 2005). Whereas NHEJ is the error-prone mechanism resulting in frequent deletions and insertions of various sizes, the HR is a relatively error-free repair mechanism in somatic cells (Hays 2002). At the same time, HR when used in G1 can potentially lead to the loss of heterozygocity. HR is the mechanism responsible for crossing-over during meiosis. Unequal crossing-over events lead to generation of gene duplications, deletions and conversions (Meyers *et al*. 2005).

Plants are apparently able to regulate the stability of the genome by applying different DNA repair mechanisms at different developmental stages. Our recent experiments revealed that *Arabidopsis* plants use HR more frequently at early developmental stages when compared to the later developmental stages (Boyko *et al*. 2006c). In contrast, the frequency of employment of NHEJ does not change. The differential rate of HR and NHEJ repair in early and late developmental stages could be explained by genome endoreduplication, whereby younger cells contain a much smalller number of genomes per cell when compared to older cells. This process of endoreduplication occurs with plant maturity, and results in genome replication without cell division. This process results in a drastic increase in the number of genomes per cell. It is possible that older cells suppress the activity of HR in order to decrease the chances of deleterious rearrangements (Boyko *et al*. 2006c). On the other hand, it is possible that younger cells have an elevated frequency of HR in order to increase the chances of inheriting the rearrangements in certain genome areas. In this case, exposure to mutagen at an earlier developmental stage should result in a higher increase in HR frequency. Indeed, recent experiments in our laboratory revealed that the exposure of *Arabidopsis* and tobacco plants to UVB at early in development resulted in a higher increase in HR frequency when compared to the exposure at an older stage (Boyko *et al*. 2006a).

Homologous recombination as a mechanism supporting rearrangements

It appears that homologous recombination is not just a simple DNA repair mechanism that acts on single- and double-strand breaks. The fact that HR is the primary mechanism responsible for crossing over events during meiosis suggests that it is an immediate mechanism for creating diversity (Gerton and Hawley 2005). The homologous recombination mechanism can prove dangerous to cells, as it can be responsible for the induction of recessive genotypes from heterozygous loci. The recessive traits, however, are not necessarily deleterious, as they may appear useful under certain environmental conditions. Having a large proportion of traits in the heterozygous stage serves as a prerequisite for more "evolutionary flexible" genomes; thus, genome stability is closely monitored to the balance risks of negative events with the need for genome diversity.

Hypermethylation, the addition of methyl groups, together with specific histone modifications, stabilizes the genome and prevents recombination events (Madlung and Comai 2004). This stability is largely due to the addition of functional groups, most commonly methyl groups, to DNA and/or histones. The loss of methyl groups, termed hypomethylation, allows for rearrangement events, such as homologous recombination, to occur (Engler *et al*. 1993).

CHANGES IN DNA METHYLATION AND HISTONE MODIFICATIONS. EPIMUTATIONS

Gene expression in plants is regulated by several mechanisms including kinase-activated transcription factors, RNA turnover, posttranscriptional gene silencing as well as changes in protein half-life (Jover-Gil *et al*. 2005). Yet an additional, and perhaps more versatile mechanism, of the control of gene expression is based on changes in chromatin structure. A variety of small regulatory RNAs, named short interfering (si)RNAs are involved in targeting specific areas in the genome and establishing a new chromatin structure (Mathieu and Bender 2004; Steimer *et al*. 2004; Kapoor *et al*. 2005). This process starts by the generation of sequence specific siRNAs, via developmental regulation or a change in environmental conditions, that are capable of spreading to specific tissue or plant organs, and promoting changes in methylation status followed by changes in methylation/acetylation of specific histones (Steimer *et al*. 2004; Sunkar and Zhu 2004; Borsani *et al*. 2005). These changes lead to differential chromatin modifications in the cells that have or have not received the specific siRNA. Such changes in chromatin structure reinforced by small regulatory RNAs allow the establishment and maintenance of new patterns of chromatin modifications required for proper leaf development and transition to flowering (Fransz and de Jong 2002; Liu *et al*. 2004; Peragine *et al*. 2004; Grigg 2005; Kandasamy *et al*. 2005).

Changes in histone acetylation and histone methylation represents another level of chromatin modification associated with response to stress. Several recent works relate specific modifications in histone H3 lysine (K) 4 methylation with the response to stress (Seol *et al*. 2006; Tsuji *et al*. 2006). Although specific pattern of mono-, di- or trimethylation at K4 is different in yeasts or plants exposed to different stress, it represents a general chromatin surveillance mechanism. Tsuji *et al*. (2006) reported dynamic and reversible changes of histone H3-K4 methylation and H3 acetylation in stress-responsive submergence-inducible ADH1 and PDC1 genes in rice. Seol *et al*. (2006) showed that deletion of specific histone H3 methyltransferase SET1 resulted in the induction of a subset of stress responsive genes in yeasts. This changes was directly associated with loss of H3 lysine (K) 4 methylation.

Since methylated DNA mutates at a higher rate when compared to non-methylated DNA, changes in methylation pattern not only result in differential gene expression but also in a higher rate of mutations in somatic and, more importantly, in meiotic cells (Kapoor *et al*. 2005). In contrast to small mutations such as point mutations, deletions and insertions, gross chromosomal rearrangements such as translocations, duplications, and loss of chromosome arms are often the result of unequal crossing over (Perez-Ortin *et al*. 2002). Importantly, the crossing over mechanism, homologous recombination, is less frequently involved in areas with higher methylation status, and, *vice versa*, it is more frequent in the areas of less condensed chromatin, which are the areas with fewer methylations (Engler *et al*. 1993; Bassing *et al*. 2002). Thus, changes in DNA methylation represent a transition stage to more stable genetic mutations.

An important feature in plants is the ability to form gametes from meristemic cells. Since these cells are capable of "accumulating the information" from the growing plant, they are able to "incorporate" these epigenetic signals into a unique methylation pattern. The transition to the next generation will change the pattern of gene expression observed in the previous generation, leading to a substantial variation in the expression of differentially methylated genes. Such a natural gene variation is the result of a heritable epimutation, which are heritable changes in the phenotype due to the alteration in the methylation status of the coding gene. A broader term that describes the epimutations is "epialleles", which represent stable or reversible units of inheritance in plants (Finnegan 2002). Epialleles are a frequent phenomenon, associated with changes in plant growth conditions, including stress exposure. Epimutations and epialleles in plants are possible because there is no separation between germ line and soma in plants. This makes plants less efficient in resetting the methylation status of genes upon the transition through the germ line (Cronk 2001). One important advantage of epimutations is that they are often partially reversible, making the resulting phenotypes more variable and less severe as compared to those resulting from sequence changes (Cronk 2001).

Stress influence on methylation and on HR frequency

The link between stress exposure and hypomethylation has been established in cold stress, where it has been shown that cold treatment promotes tissue-specific hypomethylation of defined areas of the genome, including areas specific to retrotransposon sequences (Steward *et al*. 2002). Cold treatment of maize seedlings resulted in a global demethylation of root genomic DNA, particularly in nucleosome core regions (Steward *et al*. 2002).

Wada *et al*. (2004) showed that exposure of tobacco plants to tobacco mosaic virus induced demethylation of the *NtAlix1* stress-responsive gene as early as 12 h after pathogen exposure. This resulted in continuous (over 24 h), pathogen-induced accumulation of the gene transcript (Wada *et al*. 2004). In parallel, a number of other stress-responsive genes were activated (via hypomethylation) by the pathogen.

Several miRNAs were shown to be specifically regulated by abiotic stress (Sunkar and Zhu 2004). Moreover, there are several mechanical stress-induced miRNAs that exist in pine trees and do not exist in *Arabidopsis* (Lu *et al*. 2005). Despite the fact that the majority of miRNAs are conserved between Arabidopsis and pine tree, there are, apparently number of miRNAs that respond to stress specifically for a given plant species. Whereas miRNAs predominantly regulate the gene expression by mRNA degradation (more frequently in plants) or by translation inhibition (less frequently in plants), siRNAs are also capable of regulating the gene expression via sequence-specific TGS. The latter mechanism allows sequence-specific methylation of the defined genomic loci in response to environmental conditions. One of the good examples is the induction of *cis*-acting siRNA as part of the salt-tolerance response in Arabidopsis (Borsani *et al*. 2005).

A variety of external stresses including changes in growth conditions, such as light and temperature, exposure to salt, heavy metals, UVB, UVC and ionizing radiation,

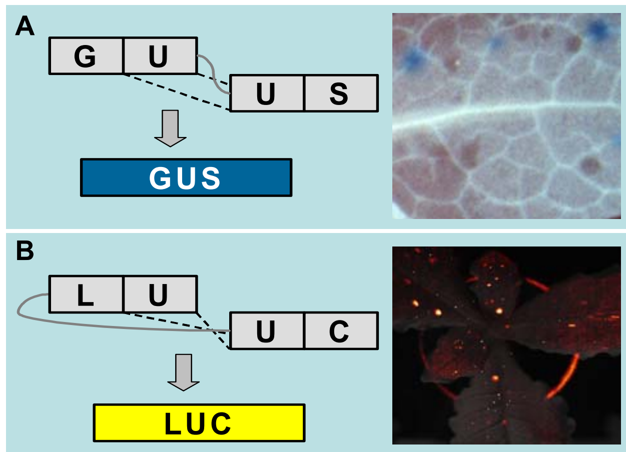

Fig. 1 Recombination reporter assay. Homologous recombination reporter lines carry in the genome two non-functional overlapping copies ("GU" and "US" or "LU" and "UC") of the transgene. The activity of the gene is restored via homologous recombination between the homology regions ("U"). The transgene activation is observed either as blue sectors (**A**) on the transparent background (chlorophyll washed with ethanol) or as shining sectors observed in luciferase camera (**B**).

and even pathogens and herbicides have been shown to influence the HR frequency in plants (Kovalchuk *et al.* 1998, 2000, 2003a, 2003b; Filkowski *et al.* 2003; Besplug *et al.* 2004; Kovalchuk *et al.* 2004; Molinier *et al.* 2005; Boyko *et al.* 2006b; unpublished data). Some of the stresses were also associated with changes in methylation pattern in the progeny of plants exposed to stress (Kovalchuk *et al.* 2003b, 2004). The association of other stresses with changes in methylation and genome instability remains to be established.

The primary assay that our group utilizes for the analysis of HR events in plants relies on two reporter genes, β-glucuronidase (*uid*A, or GUS) and luciferase (LUC) (Kovalchuk *et al.* 1998; Filkowski *et al.* 2004; Boyko *et al.* 2006c). The activity of either of these genes is readily visualized via either histochemical staining or via observation with a CCD camera, respectively. The recombination reporter constructs consist of two overlapping non-functional gene fragments. Strand breaks occurring in either region of homology could potentially be repaired via HR using the second intact region of homology as a template (**Fig. 1A, 1B**).

SYSTEMIC SIGNALING IN PLANTS

Response to a number of different stresses, as well as regulation of various developmental processes, requires sophisticated signaling mechanisms. Changes in the environment trigger a global response which includes the processes of systemic acquired resistance (SAR) (Verhagen *et al.* 2006; Xiao 2006), systemic wound signaling (Pearce *et al.* 1991), systemic acquired acclimation to light (Karpinski *et al.* 1999), systemic post-transcriptional RNA silencing (Waterhouse *et al.* 2001; Mlotshwa *et al.* 2002), and the photoperiodic induction of flowering (Colasanti and Sundaresan 2000). This is likely a minor portion of the variety of responses that plants are capable of. These responses, including those associated with pathogen infection, depend on the plant's ability of recognizing the stress and producing mobile signals that can activate specific responses in distant tissues.

Systemic recombination signal: mechanisms of appearance and possible nature of the signal

Much evidence suggests that chromosome instability can also occur in cells that were not irradiated but were in the radiation environment. These studies suggest that non-targeted or bystander-like effects may play a significant role in induced genomic instability (reviewed in Huang *et al.* 2003). The very fact that non-treated tissues are able to react to stress with an increased frequency of rearrangement suggests the presence of a systemic signal capable of traveling from exposed to non-exposed tissue and promoting changes in the genome.

Several experiments in our laboratory also suggested that plants are capable of bystander-like effects. We found that local (single leaf) exposure of plants to UVC, as well as local treatment with rose Bengal (a radical-producing chemical), increased the frequency of homologous recombination, not only in the treated leaves, but also throughout the whole plant (Filkowski *et al.* 2004). An important result that we have discussed in Filkowski *et al.* (2004) is the fact that pre-treatment with radical scavenging enzymes decreases, but not totally abolishes, the effect of the signal. Similar results have been observed with the treatment of plants with elevated concentrations of NaCl. A transgenerational effect of the stress has been observed in the progeny of plants exposed to NaCl, which have exhibited an increased recombination frequency (data not published).

The nature of this signal, named the systemic recombination signal (SRS), remains unclear. It is possible that the signal is generated by local stress and that signal production depends on the presence of short life reactive oxygen species (ROS). The role of ROS in signaling is well known from mammalian experimental systems (Finkel 1998). What are the other stresses that are capable of generating SRS in plants? Do plants respond to biotic stress in a similar manner? What is the role that SRS plays? How stable and long lasting is the effect of SRS in plants? We will now attempt to address these questions.

PATHOGEN-INDUCED GENOME REARRANGEMENTS

The plant systemic reaction to pathogens is a well-described mechanism involving the recognition of pathogen avirulence (*Avr*) genes by the plant resistance (*R*) genes (Grant and Lamb 2006; Xiao 2006). This interaction leads to a local hypersensitive response, immediately followed by SAR to potential future attacks by similar and even unrelated pathogens (Grant and Lamb 2006; Xiao 2006). Being a systemic process, SAR is directly dependent on the spread of a signal throughout the plant. The nature of this signal remains enigmatic. Such an *R/Avr* gene interaction, qualified of incompatible, prevents the pathogen from systemically infecting the plant. Should one of the partners, either *R* or *Avr*, disappear (mutate), the interaction becomes compatible. Plants that lack the *R* gene are not able to resist the virus and local infection quickly becomes systematic (Grant and Lamb 2006).

Inability to resist the pathogen is dangerous to plants as it can result in an inability to produce the next generation. Plants are able to use at least two strategies in their battle against compatible pathogens. The first is to increase the level of unspecific resistance, and the second is to acquire a "correct" *R* gene capable of imparting the resistance to a specific pathogen producing an incompatible interaction. It is logical to think that plants that are not able to protect themselves with the help of *R* genes would be forced to initiate mechanisms that would allow the creation of such genes. Such a process would surely require that a warning signal systematically spread throughout the plant tissue. As plants do not have a predetermined germline, it is probable that the changes triggered by this signal can be transmitted to the next generation, better preparing progeny plants to similar pathogen infections. This signal may well be of a similar nature to the one observed upon exposure to abiotic stress.

Generation of SRS in virus-infected plants

Previous work in our laboratory has shown that a compatible interaction between the pathogen Tobacco mosaic

virus (TMV) and the plant *Nicotiana tabacum* (tobacco) results in the production of a signal that leads to systemic changes in the frequency of somatic recombination (Kovalchuk *et al*. 2003b). This signal, or the SRS, was locally generated at the site of infection and was capable of altering genome stability in non-infected tissue (Kovalchuk *et al*. 2003b). The important feature of the signal is its ability of moving to non-infected leaves faster than the virus. The experiments in which the infected leaves had been cut off after certain time intervals (0-72 h) revealed that cutting the infected leaves as early as 8 h after infection still results in an increase in HR. In our experiments, TMV requires at least 30 h to move from infected leaf systemically throughout the plant (Kovalchuk *et al*. 2003b). Homologous recombination was measured in two different genes, the luciferase transgene and an endogenous sulfur gene involved in chlorophyll synthesis (Kovalchuk *et al*. 2003b). In both cases, a 2-3-fold increase in HR frequency was observed. It is important to mention that the SRS was gene-rated not only in plants that did not possess the resistance gene to TMV ("SR1" cultivar), the "N" gene, but also in plants that possessed an inactive form of this gene ("Havana" cultivar). The growth of "Havana" plants that carry the N gene at 32°C inactivates the N gene and plants become sensitive to TMV. Infection of "Havana" plants at 32°C also resulted in production of the SRS (Kovalchuk *et al*. 2003b). This suggests that SRS is generated as soon as the resistance gene is either absent (SR1) or inactive ("Havana" at 32°C).

Virus-induced changes in HR can be inherited

Whatever the purpose of the SRS, the increase in recombination only seems logical if it happens in somatic cells extremely early in plant development or if it happens in meiotic cells, and thus gets inherited. To understand the role of SRS upon pathogen infection, we have designed a complex approach allowing the analysis of the genome stability and methylation changes (**Fig. 2**). We have collected the seeds from the infected plants and plants treated with buffer (providing a control for mechanical damage) and analyzed the number of plants with a completely recombined transgene (**Fig. 3**). These plants express luciferase in all cells (**Fig. 4A**). These experiments showed a more than 2-fold increase in the number of plants with a completely recombined transgene (**Fig. 4B**). This experiment suggests that changes in somatic cells promoted by SRS can potentially be transmitted to the next generation (Kovalchuk *et al*. 2003b). It is also possible that the SRS is transmitted to meiotic tissue and promotes the increase in meiotic recombination. The increase in the frequency of meiotic recombination observed in the transgene suggests that there could be a similar increase in other loci. However,

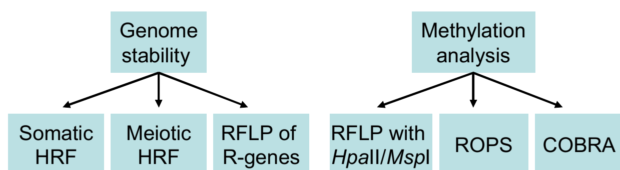

Fig. 2 Schematic representation of the complex approach to the analysis of genetic and epigenetic changes in pathogen treated plants. Main emphasis was given to the analysis of the genome stability and methylation pattern. Genome stability was analyzed by detecting somatic and meiotic homologous recombination frequency (HRF) and by detecting changes in *N*-gene like *R*-gene loci (RFLP). Methylation pattern was analyzed by ROPS, random oligonucleotide-primed synthesis assay and by COBRA, combined bisulfite restriction analysis. Whereas former assay allows the analysis of global genome methylation, the latter allows the analysis of locus-specific changes in methylation. Alternative method for locus-specific analysis of methylation pattern is a RFLP (using locus-specific probe) of the genomic DNA digested with *Hpa*II or *Msp*I.

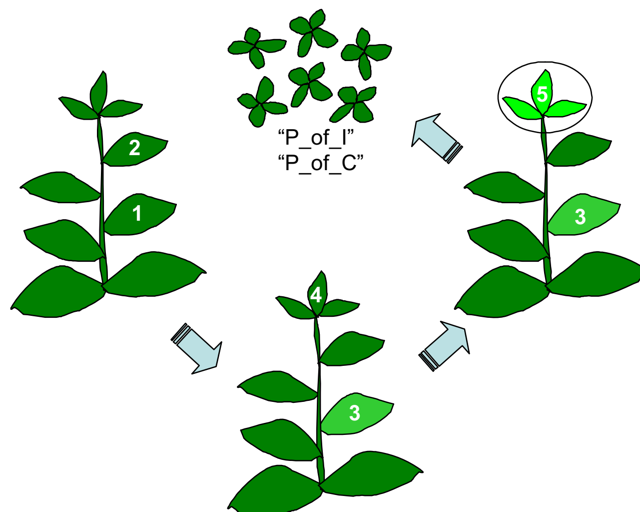

Fig. 3 Experimental set up: analysis of the progeny of infected and progeny of control plants. Schematic representation of the experimental set up. Briefly, in a previous experiment, single leaves (leaf numbered as "1") of 10-week old SR1 tobacco plants were inoculated with 300 ng of TMV RNA (21 plants) or mock treated (20 plants). 24 hours after inoculation, the upper, non-treated leaves (leaf numbered as "2"; virus-free) from these plants were grafted onto 10 week-old healthy plants (leaf numbered as "3"; 21 plants with leaves from virus-treated and 20 plants with leaves from mock-treated plants), from which the tops were previously removed (leaf numbered as "4"). The seeds derived from the newly emerged tissue (encircled; leaf numbered as "5") were collected and named "progeny of infected" ("P_of_I") or "progeny of control" ("P_of_C"). These seeds were used to analyze global and loci-specific methylation and RFLP of various loci.

as these rearrangements can prove harmful, we hypothesized that important housekeeping genes essential to proper plant function would remain stable, while the loci of genes that could prove beneficial, such as the loci carrying the homology to *R* genes, would become unstable. The instability of the transgene could reflect the fact that plants possess the mechanisms of recognition of the "neutrality" or "importance" of the gene. On the other hand, it is possible that newly formed loci (such as a transgene) do not establish similar levels of chromatin structure, facilitating the control of rearrangements. If our hypothesis is correct, an increase in rearrangements in *R* gene loci due to a compatible infection could be seen as an attempt to formulate novel *R* genes for the next generation (Richter *et al*. 1995; Tornero *et al*. 2002).

This form of adaptive response to biotic stress through genomic alterations resembles the original model proposed by Barbara McClintock entailing the activation of transposable elements in response to environmental changes (McClintock 1984). Extensive research has supported this model showing that salicylic acid, methyl jasmonate, oxidative stress, wounding, pathogen attack, $CuCl_2$, cell subculture, and protoplast isolation activate transposons (reviewed in Arnholdt-Schmitt 2004). Activation of transposons is associated with a substantial decrease of genome stability (Dennis and Brettell 1990; Miura *et al*. 2001). Since plant genomes contain a great number of transposons, regulation of their activity is a very important task. Transposon activation is directly associated with hypomethylation (Wang 1996; Neidhart *et al*. 2000; Cui and Fedoroff 2002), hence one of the primary mechanisms for controlling transposon movement is through hypermethylation. It was previously shown that loss of methylation at the heterochromatic areas in *ddm1* was the direct cause of the activation of transposon *CACTA1* (Kato *et al.* 2004). It remains to be established, however, whether there is a link between biotic stress, loci-specific hypomethylation, and changes in the genome stability of pathogen-infected plants.

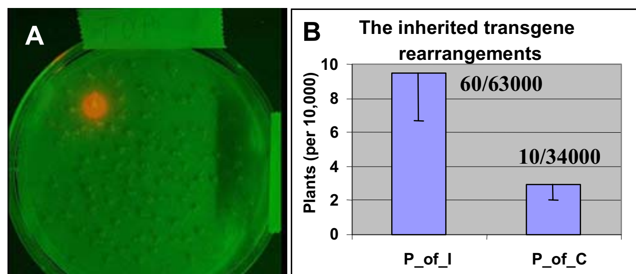

Fig. 4 Increase in the number of LUC+ plants. Seeds of the "P_of_I" and "P_of_C" plants were screened for the HR events in the luciferase transgene. Appearance of totally recombined (brightly shining seedlings) plants was analyzed at 3-5 days post germination (A) and then rechecked at an older plant stage. Part "B" shows the frequency of the occurrence of the fully recombined plants as shown per 10,000 seedlings screened. First number at the top of each bar shows the total number of LUC+ plants and second number shows total number of plants profiled. Bars show the SD.

Progeny of infected plants exhibits changes in global and locus-specific methylation and the increased instability of the *R*-gene loci

To analyze the changes in the progeny of infected plants, we performed several experiments. Single leaves of *N. tabacum* cultivar 'SR1' were inoculated with 300 ng of TMV RNA or treated with phosphate buffer for a mechanical damage control (Kovalchuk *et al.* 2003b). The buffer control was important since it was shown to produce a similar effect on genome stability (data unpublished). At 24 hours after the treatment, the upper, non-treated leaves were cut off and grafted onto 10 week-old healthy plants from which the tops were previously removed. These leaves did not contain the virus. To analyze the next generation, we collected seeds that were derived from the newly emerged tissue of the donor and grafted plants and named them "progeny of infected" ("P_of_I") and "progeny of control" ("P_of_C") (**Fig. 3**; Boyko *et al.* 2007). All of the following experiments described in this review were done on the 21 independent "P_of_I" lines ("P_of_I"#1, "P_of_I"#2 etc) and 20 independent "P_of_C" lines (Boyko *et al.* 2007).

Global methylation changes in the progeny of plants infected with virus

Gene expression is regulated on genetic and epigenetic levels. Whereas the former requires permanent changes in the coding sequence or in the sequence of the promoter area, the latter relies on reversible activation/inactivation via differential methylation patterns. The role of epigenetic control in the adaptation and acclimation process is hard to underestimate, as transgenerational changes in methylation patterns are a powerful tool to reversibly modify the expression of a number of genes. We have shown that the progeny of plants constantly exposed to ionizing radiation had hypermethylated genomes (Kovalchuk *et al.* 2003a). Thus, changes in DNA methylation patterns could be considered as part of the plant protection mechanism (Rizwana and Hahn 1999).

To analyze the methylation pattern of "P_of_I" and "P_of_C" plants, we used several assays based on differenttial digestion with methylation-sensitive enzymes. Over 30% of all plant cytosines are methylated (Adams and Burdon 1985). The most common sites for methylation are symmetrical CpG and CpNpG sites. Since both of these sites exist in the commonly methylated CCGG nucleotides, the recognition sequence of the methylation sensitive *Hpa*II and *Msp*I restriction enzymes would be an ideal target for study. Methylation of the external cytosine in CpNpG prevents digestion with *Msp*I and severely reduces (~3,000-fold) digestion with *Hpa*II (McClelland *et al.* 1994). Methylation of the external cytosine in CpG (internal in the restriction site) does not influence digestion with *Msp*I, but prevents digestion with *Hpa*II.

We analyzed the global genome methylation status of both progenies by digesting their genomic DNA using these enzymes. Methylation of internal, external or both cytosines alters the digestion pattern at these restriction sites and changes the amount of incorporated [^{3}H]dCTP. Lower radioactive counts reflect higher methylation levels, whereas higher radioactive counts reflect lower methylation levels. We have tested 5 independent "P_of_I" lines, and found four of them having significantly increased global genome methylation levels when compared to "P_of_C" lines (**Fig. 5**). A similar pattern of radionuclide incorporation was observed in both *Hpa*II and *Msp*I cut DNA (**Fig. 5**).

These data suggest that the difference in radionuclide incorporation in the global genome hypermethylation observed in "P_of_I" is primarily due to the methylation of external and internal cytosines (Boyko *et al.* 2007).

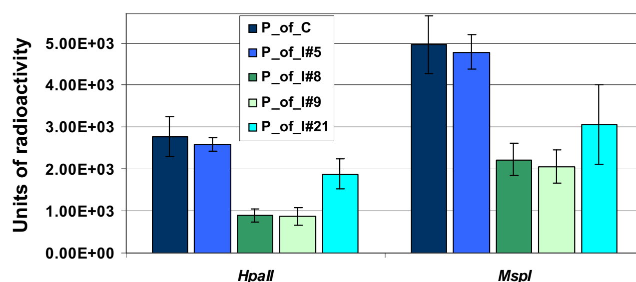

Fig. 5 Global genome methylation. Global genome methylation was analyzed by ROPS assay after digestion with either *Hpa*II or *Msp*I. "Y" axis shows radioactive incorporation (dpm/µg) in "P_of_C" (average from several lines) and four different "P_of_I" lines. Each bar represents the average (with SD) from 5 individual assays, each representing the readings from 5 plants.

R-gene loci are hypomethylated in "P_of_I" plants

In this review we have stated that high levels of rearrangements are generally associated with low levels of methyllation. In this case the fact that "P_of_I" plants exhibited a higher level of methylation when compared to "P_of_C" plants suggests that "P_of_I" plants should have lower levels of HR. This, however, does not coincide with the fact that we observed higher levels of rearrangements in meiotic tissue of treated plants. Similarly, several papers suggested a higher frequency of rearrangements in the progeny of stressed plants (Ries *et al.* 2000; Kovalchuk *et al.* 2003b; Boyko and Kovalchuk, unpublished data). One explanation was the hypothesis that there may be a differential pattern of methylation throughout the genome of "P_of_I" plants, whereas the majority of loci had higher methylation levels, while some loci, including the loci containing the transgene, could be hypomethylated.

To test the hypothesis of specific methylation patterns induced by viral stress, we analyzed the methylation pattern of the *N*-gene-like *R* genes. If the hypothesis is correct, we should observe hypomethylation of *R* genes in the "P_of_I" plants. Infection of SR1 plants that do not contain the *N* gene with TMV likely forces plants to elaborate other protection mechanisms. It is possible that one such mechanism would be the change in methylation status of the loci that carry homology to *N*-gene. Hypomethylation of these loci would allow them to have a higher degree of freedom for rearrangements (Engler *et al.* 1993; Bassing *et al.* 2002). Indeed, several assays, including methylation sensitive RFLP and COBRA showed that several *N*-gene-like *R*-gene loci were hypomethylated in "P_of_I" genomes. In several control loci, such as actin loci, repetitive elements in *N. tabacum* (RENT) and 5.8S rRNA loci were either equally methylated or partially hypermethylated in "P_of_I" genome (Boyko *et al.* 2007).

Stability of genomic loci

It is known that the increase in methylation in certain genome loci is correlated with a lower frequency of recombination, while the contrary is true for the loci that have a decrease in methylation (Engler *et al.* 1993; Bender 1998; Bassing *et al.* 2002). We hypothesized that the change in the methylation status of the *N*-gene-like *R* gene loci would change its stability, whereby hypomethylation would lead to more frequent rearrangement events.

The large variety of polymorphic *R* gene families apparently have evolved by extensive rearrangement mechanisms such as gene and chromosomal duplications, unequal crossing over, and deletions/insertions incited in plants challenged by pathogens. In fact, all of the above have been shown to exist in various clusters of *R* gene loci (Stahl *et al.* 1999; Tian *et al.* 2002; van der Hoorn *et al.* 2002; Mauricio *et al.* 2003). Therefore, in order to survive the constant battle with highly mutable pathogens, plants must continuously modify their *R* genes in order to recognize the pathogen *Avr* genes via gene-for-gene interactions (Madsen *et al.* 2003).

To test our hypothesis, we performed RFLP analysis using the 4th exon of the *N*-gene as a probe. This assay revealed nearly 30 loci with substantial degree of homology to the *N*-gene (**Fig. 6**). Analysis of the profile of more than 150 plants in each group revealed an over 5-fold increase in instability of these loci in “P_of_I” plants (Boyko *et al.* 2007). It is important to note that the stability of the control loci, such as actin and 5.8S was not changed.

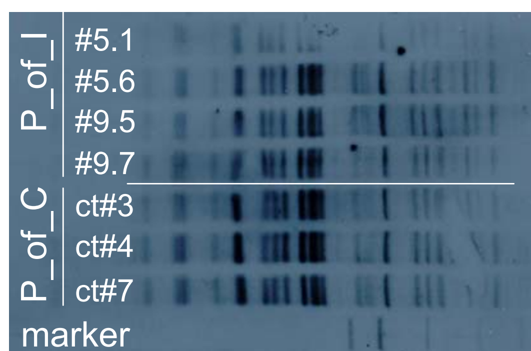

Fig. 6 RFLP analysis of the *N*-gene-like *R* gene loci. Stability of the N-gene-like R gene loci was analyzed by hybridization of the 4th exon of the N-gene to the genomic DNA of the progeny of infected SR1 tobacco plants. Figure shows the hybridization pattern, confirming that SR1 plants, despite the fact that they do not have an active *N*-gene, have approximately 30 loci with different (over 60%) degree of homology to the LRR region of the *N*-gene. Representative blot contains the genomic DNA extracted from individual “P_of_I” lines (plants from line #5 and line #9 are shown) and “P_of_C” lines.

CHANGES IN METHYLATION ARE DIRECTLY LINKED TO GENOME INSTABILITY

Since methylation is a well-explored process of genome maintenance, whereby methyl groups tend to make chromatin less accessible to various remodeling processes, hypomethylation could be suggested as a mechanism that facilitates the rearrangement of *R* gene loci. This phenomenon is apparently not unique to plants. The high rate of recombination in the V(D)J region of immunoglobulin and T-cell receptor genes of the mammalian immune system is also related to lower levels of DNA methylation (Bassing *et al.* 2002). 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, whereas methylated loci were much more stable. Previous studies have also shown that highly duplicated genes present a high level of DNA methylation (Bender 1998). Moreover, the removal of extra gene copies resulted in a reduction of the methylation density of the remaining copies (Bender 1998).

Plant genomes (especially those that are relatively large) are known to be highly repetitive and thus, highly methylated. In this case, the methylation could be a mechanism of stabilization of the genome, preventing rearrangements (Puchta and Hohn 1996). The hypermethylation of the rest of the genome, as could be assumed from our global genome methylation data, prevents the deleterious effect of this genome reshuffling at unfavorable loci. Additionally, this could explain why some loci, such as the actin loci, seem to be less duplicated or found in clusters, since these configurations would result in higher conservation (Kroymann *et al.* 2003; Mauricio *et al.* 2003).

We do not know what triggers the methylation change in “P_of_I” plants. It has been shown previously that exposure of somatic cells to stress results in a decrease in DNA methylation (Duthie *et al.* 2000). The hypomethylation in this instance could either be associated with replication or with direct repair synthesis (Duthie *et al.* 2000). A variety of DNA repair mechanisms, such as a double strand break, long-patch base excision, and global nucleotide excision repair would result in the replacement of methylcytosines with cytosines (Duthie *et al.* 2000). This mechanism, however, cannot be directly applied to our case, as we observed changes in methylation in the progeny of treated plants.

The methylation pattern of every individual organism is established after fertilization and is specific to each individual organism. The changes in methylation, observed in our experiments, suggest that exposure to a stress apparently "rewrites" the methylation pattern according to the needs of the organism, as dictated by a particular stress. It would be correct to assume that external stresses of various intensities are capable of changing the methylation status in somatic tissue and, more importantly, in tissue destined to future generations (Wada *et al.* 2004; Weaver *et al.* 2004).

Our report is not the first evidence of the existence of a specific mechanism directed toward genome rearrangements in stressed plants. Previously, a number of publications reported heritable changes in inbred flax in response to specific, defined environmental changes, such as nutrients balance and temperature regimes (Schneeberger and Cullis 1991; Cullis *et al.* 1999). These conditions apparently change the activity of a transposon-like sequence, LIS1, that assembles and inserts itself into the genome of stressed plants (Chen *et al.* 2005). The new “genotroph” appears to be stabilized, as no further changes in LIS1 activity occur in plants upon the exposure to additional stresses (Cullis *et al.* 1999). It is difficult to explain what activates the transposon, as methylation studies were not performed.

It remains to be determined whether changes observed in “P_of_I” plants were virus-specific or pathogen-wide. Future studies are needed to show whether the infection with bacterial pathogens lead to rearrangements in *N*-gene-like loci, or perhaps any other type of *R*-gene loci, and whether the methylation status and genome stability return to normal in the progeny of “P_of_I” plants that would be grown at normal conditions. It also remains to be established whether plants with higher instabilities and methyllation changes acquire any changes in the tolerance to similar (TMV) or different pathogens, or even other stresses.

CONCLUSION

We discussed various cases of stress-induced changes in the stability of the plant genome. It is noteworthy that stress not only promotes changes in non-treated somatic tissue, but transgenerational changes as well. Our research suggests the existence of a specific, epigenetically controlled mechanism that promotes rearrangements in *R* gene loci in the progeny of plants infected with a compatible pathogen. As such, this phenomenon suggests a second, more flexible level of inheritance, regulated by stress and responding with immediate changes in the progeny or treated plants. Future studies are clearly needed to understand the specificity of such reg-

ulation, including signal production, maintenance and "inheritance".

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