INFLUENCE OF VARIOUS FACTORS ON PLANT HOMOLOGOUS RECOMBINATION

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Dedication

To my parents,

Ludmyla and Oleksandr Boyko

To the blessed memory of my grandparents,

Vasyl and Raisa Manenko

With love and gratitude

Abstract

The genome of living organisms is constantly subjected to the environmental influences that result in different negative, negligible or positive impacts. The ability to maintain the genome integrity and simultaneously provide its flexibility is the main determinant for the evolutionary success of any species. One of the important aspects of genome maintenance is the precise regulation of the DNA repair machinery. Results reported here indicate the existence of a tight, age-dependent regulation of homologous recombination, one of the two main DNA double-strand break repair pathways. We show that recombination is influenced by conditions such as the change of temperature (cold or warm), day length, water availability (drought or overwatering stress) and salinity. These stresses not only influence the genome stability of stress-subjected generations but also change the recombination in subsequent generations. This indicates the possible involvement of homologous recombination in plant evolution and development of plant stress tolerance.

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Abbreviations

ABA – abscisic acid

APX – ascorbate peroxidase

AsA – ascorbate

BM – MS basic media

CaMV – cauliflower mosaic virus

CAT – catalase

cDNA – complementary deoxyribonucleic acid

dpg – day post germination

DPM – decay per minute

DSB – double strand break

DSBR – double-strand break repair

GFP – green fluorescence protein

GR – glutathione reductase

GSH – glutathione

GT – gene targeting

GUS – *β–glucuronidase* gene

HR – homologous recombination

HRF – homologous recombination frequency

LUC – *luciferase* gene

mRNA – messenger ribonucleic acid

MS – Murashige and Skoog medium (Basic medium)

NHEJ – non-homologous end joining

NHR – non-homologous recombination

PCR – polymerase chain reaction

PM – point mutation

PMF – point mutation frequency

PMR – point mutation rate

PSII – photosystem II

pst1 – photoautotrophic salt tolerance mutant

R-gene – resistance gene

ROPS – random oligonucleotide primed synthesis

ROS – reactive oxygen species

RR – homologous recombination rate

RuBP – ribulose biphosphate carboxylase/oxygenase

SA – salicylic acid

SDSA – synthesis-dependent strand annealing

SOD – superoxide dismutase

SRS – systemic recombination signal

SSA – single-strand annealing

ssDNA – single stranded DNA

T-DNA – transferred DNA

Ti plasmid – tumor-inducing plasmid

TMV – tobacco mosaic virus

uidA – GUS gene

UV – ultraviolet radiation

1. INTRODUCTION

Plant interactions with the environment have been of primary importance for a long time (Levitt, 1980; Boyer, 1982). Initial experiments described physiological responses of plants to the environment, such as various types of tropisms, growth inhibition, change of pigmentation *etc* (Levitt, 1980; Boyer, 1982; Larcher, 1995). Later on, the plant responses were studied on a biochemical level via analysis of various biochemical reactions involving secondary plant metabolites (Govindachary, *et al.*, 2004; Stitt and Hurry, 2002; Savitch, *et al.*, 2001; Salvucci and Crafts-Brandner, 2004a; 2004b; Crafts-Brandner and Salvucci, 2002; Pittermann and Sage, 2000; Law, *et al.*, 2001; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002; Lawlor, 2002). Today, plant interactions with the environment are studied on a molecular level; different pathways and cross-talk between pathways have been identified revealing complex interactions between the plants and the surrounding environment (Hasegawa, *et al.*, 2000b; Zhang, *et al.*, 2000; Vranova, *et al.*, 2002; Winkel-Shirley, 2002). The influence of environmental stress on the genetic material of plants was not well studied until recently (Seki, *et al.*, 2002a; 2002b; Lucht, *et al.*, 2002; Ries, *et al.*, 2000a; 2000b; Kovalchuk, *et al.*, 2001; 2003b). External stress has a strong impact on plant genome stability and likely contributes significantly to evolution (Gorbunova and Levy, 1999; Lucht, *et al.*, 2002).

Genome stability may be described as the potential of a genome to maintain its integrity by resisting, preventing or reversing changes in DNA. DNA double strand breaks (DSBs) pose a major threat to genome integrity. The failure to repair a break can result in the loss of genetic information, chromosomal translocation and ultimately, cell

death (Critchlow and Jackson, 1998). The maintenance of genome stability is of primary importance for plants, since they cannot avoid the influence of environmental factors due to their stationary life. Moreover, the large content of repetitive DNA and multigenic, highly homologous gene families (Flavell, 1985) provide a perfect target for homologous recombination (HR), one of the two major DSB repair pathways. HR can result in permanent alterations of the genome and even lethal deletions if it functions improperly (Swoboda, *et al.*, 1994). Furthermore, somatic HR in plants is of special interest since plants do not contain a specialized cell line predetermined to produce the gametes. Genomic changes that occurs during vegetative growth can be transferred into gametes and thus into progeny (Walbot, 1985).

The first reports indicating the influence of environmental conditions on genome stability in plants were published in 1994 – 1995 (Swoboda, *et al.*, 1994; Puchta, *et al.*, 1995). Since that time, changes in genome stability have been reported for various abiotic stress conditions, such as light spectrum (Ries, *et al.*, 2000a), ultraviolet (UV) and ionizing radiation (Ries, *et al.*, 2000b; Kovalchuk, *et al.*, 1998), chemical mutagens (Brennan and Schiestl, 1998; Kovalchuk, *et al.*, 2001), toxic substances such as herbicides (Filkowski, *et al.*, 2003), temperature (Jiang, *et al.*, 2003; Lebel, *et al.*, 1993), salt (Puchta, *et al.*, 1995) and water stress (Kalendar, *et al.*, 2000). Genomic stability was shown to be affected by biotic stress – pathogen attack (Lucht, *et al.*, 2002; Kovalchuk, *et al.*, 2003b). The destabilization of plant genome upon stress could have potential impact on plant evolution (Gorbunova and Levy, 1999; Kovalchuk, *et al.*, 2003b).

The goal of our work was to assess the possible destabilization effect of various environmental factors on plant genome stability, particularly on HR. The hypothesis was

that different environmental influences will result in alteration in HR DSB repair pathway and, thus in genome stability. In order to reach the goal the following objectives were designed:

1. Select and analyze the several factors potentially influencing genome stability:

   A. **Internal**

      Development

   B. **External**

      Temperature

      Day length

      Salt stress

      Water-mediated stress

2. Estimate the influence of these factors on HR in plants.

3. Reveal the possible nature and mechanism of interaction between stress factors and the plant genome.

4. Develop a model for regulation of HR activity under the different environmental influences.

5. Analyze the impact of stress-induced changes of HR on plants and their ability to respond to environment.

The major purpose of this study was to expand current knowledge regarding mechanisms maintaining genome stability. To monitor homologous recombination frequency (HRF) as a reflection of genome stability under stress conditions, transgenic *Arabidopsis thaliana* plants harboring *β-glucuronidase* gene based recombination

constructs (Swoboda, *et al.*, 1994) were used. HRFs were assessed in plants subjected to different temperature and day length growth conditions as well as salt and water mediated stresses. Additionally, the dynamics of HR events during plant development were determined. The observed HRF was correlated to genome content of assayed plants, metabolic activity, level of reactive oxygen species (ROS), transgene activity, and level of DSBs. Finally, the inheritance of increased homologous recombination rate (RR) after stress application was also investigated and compared with DNA methylation levels. This provided evidence of the epigenetic nature of plant response to stress.

2. LITERATURE REVIEW

2.1. DNA DSB repair: mechanisms and main players

2.1.1. DSBs – a major threat to the integrity of the genome

DNA DSBs are well known factors influencing genome integrity. A variety of exogenous agents cause DSBs including ionizing and UV radiation, various chemicals and endogenous free radicals – the byproducts of oxidative metabolism (Mittler, 2002; Chatgilialoglu and O'Neill, 2001). Additionally, the over production of ROS can be induced by the influence of different environmental stresses of either a biotic or abiotic nature (Selote, *et al.*, 2004; Govindachary, *et al.*, 2004; Borsany, *et al.*, 2001). The repair of DSBs are essential for the maintenance of genomic stability. Failure to repair DSBs can result in the loss of genetic information, chromosomal translocation and ultimately, cell death (Weaver, 1995; Critchlow and Jackson, 1998; Kovalchuk, *et al*, 2003a). DSB repair is an essential task in plant development.

To control the negative influence of DSBs, eukaryotic cells possess a number of cell-cycle checkpoints for monitoring DNA damage, insuring that cells will inherit a full complement of intact chromosomes. These checkpoints are found at S phase prior to DNA replication, and at the G2 phase of the cell cycle prior to cell division (Cromie, *et al.*, 2001).

2.1.2. HR and non-homologous end joining – the main DSB repair pathways. HR events in somatic tissues

To repair DSBs, eukaryotic cells have evolved at least two evolutionarily conserved pathways: HR, which repairs the breaks by retrieving genetic information from a sister chromatid or homologous chromosome; and non-homologous end joining (NHEJ), which involves direct ligation of the DNA strand ends with reliance on little or no homology (Critchlow and Jackson, 1998). Several of the proteins involved in repair of DSBs by HR and NHEJ are also involved in sensing and signaling DNA damage during the cell cycle (van Gent, *et al.*, 2001; Thompson and Schild, 2001).

The ability to repair DSBs, occurring in plant somatic tissues, using the HR repair pathway has been shown for a variety of DNA-breaking factors (irradiation, transposons, rare-cutting endonucleases, and *in vivo* linearization) and various templates (homologous chromosomes, sister chromatid, direct or inverted repeats, and ectopic sequences) (Gorbunova and Levy, 1999). Indeed, the stimulating effect of DNA-breaking agents on HR in somatic tissues was shown at the molecular level by using experimental systems where recombination occurred between artificially constructed repeats (Tovar and Lichtenshtein, 1992; Lebel, *et al.*, 1993; Puchta, *et al.*, 1995). Moreover, the usage of the HR pathway can often be triggered by failure of another DNA maintaining pathway to repair damage. This was shown for *A. thaliana* mutants lacking photolyase, an enzyme involved in photoreactivation of UV-induced DNA damage. Plants exposed to UV-B light exhibited an elevated level of somatic HRF (Ries, *et al*, 2000a).

Once a DSB has occurred, different repair routes (either NHEJ or HR) can be taken (Gorbunova and Levy, 1999). Both, single-strand annealing (SSA) and synthesis-

dependent strand annealing (SDSA) pathways appear to be important mechanisms for DSB repair in plants. They can be used either for homologous or non-homologous recombination (NHR). For SSA the size of the annealing region where joining occurs determines whether the recombination event is homologous when it occurs at the region of long homology or non-homologous when a site of microhomology is used (SSA-like mechanism). For SDSA the invasion of the template determines the outcome of the event. If a non-homologous template is invaded this results in NHEJ and filler DNA. If a homologous template is invaded and gap repair occurs with no template switch this results in gene conversion (Gorbunova and Levy, 1999). Intermediate scenarios are possible where one end invades a homologous template and one end invades a non-homologous template, or where the process starts as HR and, following template switch, ends as NHR (Rubin and Levy, 1997).

The processing of DSBs through the HR using the SDSA model would be an ideal approach for DSB repair in plant somatic tissues resulting in only gene conversion events (no crossover would occur). Thus, the risk of undesirable translocations within the plant genome containing highly repetitive sequences (Flavell, 1985) will be reduced (Gorbunova and Levy, 1999).

Instead, processing of DSBs through HR using a DSB repair (DSBR) model results in the creation and resolution of Holliday junctions during meiosis and mitosis between homologous chromosomes, or between sister chromatids. This results in crossovers and generation of new genetic combinations (Gorbunova and Levy, 1999).

Therefore the aforementioned models have different applications. DNA repair with Holliday junction formation is predominant in meiosis where the fidelity of the

exchange is assured by the pairing of homologues. HR, based on the SDSA mechanism that excludes crossover, occurs mostly in somatic cells (Gorbunova and Levy, 1999).

2.1.3. HR and NHEJ – competitive interfering

The mechanisms and molecular components of DSB repair seem to be evolutionarily conserved (Hanin and Paszkowski, 2003), however, the use of these alternative repair pathways varies between organisms. It is believed that HR is prevalent in prokaryotes and yeasts whereas NHEJ is predominant in multicellular eukaryotes (Vergunst and Hooykaas, 1999). In higher plants as in mammals, DSBs are repaired preferentially by NHEJ although the repair mechanism in plants is less well understood than in yeast or vertebrates (Gorbunova and Levy, 1999).

The most recent model to explain the molecular mechanisms of competition between HR and NHEJ in eukaryotes (yeast) has been proposed by Ray and Langer (2002). According to the authors the DNA ends of the DSB play the role of substrate for the multiple-enzyme complex *Rad50-Rad58(Mre11)-Rad60(Xrs2)* which resects the ends by exonuclease activity to expose single strands. Interactions with *Rad52* lead to the assembly of another multiple enzyme complex, *(Rad51-Rad54-Rad55-Rad57)*, which finally leads to the DNA-sequence-homology-dependent pathways of DSBR or SDSA. SDSA requires continued participation of *Mre11-Xrs2* (Moore and Haber, 1996) but DSBR is more dependent on *Rad51* (Rattray and Symington, 1994). The utilization of either homologous or non-homologous templates result in different outcomes of DNA repair (Hohe and Reski, 2003).

Accordingly to the Ray and Langer (2002) model DSBR/SDSA and NHEJ compete for available DNA ends. This competition is mirrored at the molecular level in the equilibrium between *Rad52* on one hand and *Ku70-Ku80* on the other. If *Rad52* is not available a *Ku70-Ku80* complex binds to the DNA ends and triggers mitotic checkpoint arrest by signaling through DNA-dependent protein kinase. Binding of *Ku70-Ku80* complex allows the recruitment of DNA ligase IV (*Dn14*) and its accessory factor, *Lif1 (XRCC4)*. In this latter case the DNA ends are joined via NHEJ (Ray and Langer, 2002).

Interestingly, the level of *Ku70-Ku80* complex is much lower in meiotic mice cells as compared to somatic cells (Goedecke, *et al*, 1999). This implies that HR acts preferentially when the levels of NHEJ proteins are low. Reduced levels of *LIF1* expression in meiosis-competent yeast cells suggests that NHEJ is low when HR is high (Valencia, *et al.*, 2001). Certain alleles of *Rad50* in yeasts display mitotic hyper recombination and reduced NHEJ, suggesting the yeast NHEJ pathway is more sensitive to defects in the initial *Rad50-Mre11-Xrs2* complex than the DSBR pathway (Malone, *et al.*, 1990; Moore and Haber, 1996).

In the last few years great success has been achieved in the identification and characterization of plant genes involved in DSB repair. Plant homologues of *Ku70* and *Ku80* in *A. thaliana* – *AtKu70* and *AtKu80* (Tamura, *et al.,* 2002) and *Dn14* and *XRCC4* (West, *et al.*, 2002) have recently been described. Additionally plant orthologs of recombination and repair genes have been isolated (Klimyuk and Jones, 1997; Doutriaux, *et al.*, 1998; Gallego, *et al.*, 2000; Hartung and Puchta, 2000; Hays, 2002) and a homologue of *Rad51* in flowering plants has been described (Doutriaux, *et. al.*, 1998). The exclusive role of this protein in plant meiosis, particularly in homologous

chromosome pairing and meiotic recombination has been shown (Li, *et al.*, 2004). Additionally yeast *rad51* mutations were shown to result in the accumulation of meiosis-specific DSBs and reduced formation of physical recombinants (Shinohara, *et al.*, 1992). The *A. thaliana* homologue of *Rad51* (*AtRad51*) is not necessary under normal (non-meiotic) conditions for genome integrity (Li, *et al.*, 2004). This may explain the domination of NHEJ over HR in *A. thaliana* somatic tissues through vegetative development.

However, to date, no *Rad52* homologue is known in plants. Ray and Langer (2002) speculate that the absence of *Rad52* might be correlated with low levels of HR in flowering plants.

2.2. HR and plant development

2.2.1. Endoreduplication: advantages and threats

Endoreduplication (replication of DNA without nuclear and cell division which results in cells with nuclei that are larger than diploid nuclei (Mizukami, 2001)) is the process that leads to somatic polyploidisation in plants (Joubes and Chevalier, 2000). The importance of an increased mass of DNA in cellular function can be explained by the following:

1. Coordination of gene expression is required for the correct interaction of nuclear and organellar genomes. Endoreduplication of the entire genome rather than selective amplification of many nuclear genes involved in chloroplast (mitochondria) development and photosynthesis (respiration) can satisfy the requirements for increased levels of transcription from those nuclear genes in developing photosynthetic (non-photosynthetic) tissues (Galbraith, *et al.*, 1991);

2. There are interactions between organelle copy number, nuclear genome size, and cellular dimensions. Larger cells possess the capability to increase their volumes at rates greater than that of smaller cells. This can be advantageous in certain situations (Grime and Mowforth, 1982; Kondorosi, *et al.*, 2000; Joubes and Chevalier, 2000; Traas, *et al.*, 1998; Galbraith, *et al.*, 1991);

3. Endoreduplication in the somatic tissues, particularly in the leaf, by increasing the number of functional gene copies within each cell, acts to mitigate any adverse effect of environmental influence, like UV-irradiation, on the transcription of the genome (Brodsky and Uryvaeva, 1985).

As a main growth strategy endoreduplication provides plant cells not only with the advantages listed above but also poses a threat for genome integrity within one cell. This is especially important for plants since they contain large amounts of repeated DNA sequences consisting of non-coding elements and multigenic, highly homologous gene families dispersed in their genomes (Flavell, 1985). These sequences could provide targets for HR resulting in continuous alteration of the genome and the potential for lethal deletions (Swoboda, *et al.*, 1994). Thus the increase of ploidy level of somatic cells should be followed by the tight control over HR machinery to insure the genome integrity and protect from frequent rearrangements. Further, regulation of the HRF seems to be important for plants since the germline cells originate from somatic tissue. This enables the incorporation into the gametes and the progeny of genetic changes that occurred during the vegetative period (Walbot, 1985).

2.2.2. Endopolyploidy and somatic recombination

The presence of an age-specific pattern of endoploidy was described for *A. thaliana* (Galbraith, *et al.* 1991). Galbraith, *et al.* (1991) described, in general, older tissues show higher levels of multiploidy than younger tissues within the same plant, or homologous tissues of earlier developmental ages within different plants. Additionally, developmental control in switching from normal cell division to endoreduplication was determined for three different cell types of *A. thaliana*: hypocotyl cells (Gendreau, *et al.*, 1998), leaf epidermal pavement cells (Melaragno, *et al.*, 1993), and trichomes (Hulskamp, *et al.*, 1994). However, the number of endoreduplication cycles appears to be under the control of both developmental (Traas, *et al.*, 1998) and environmental signals

such as light (Gendreau, *et al.*, 1998). Despite this, developmental signals that control this switch remain to be found (Kondorosi, *et al.*, 2000).

The developmentally determined and possibly environmentally regulated increase of ploidy level in plant cells would result in the increased targets for HR within one genome. Extensive genome rearrangements can disrupt proper cell function and even result in cell death. Mechanisms must have evolved to restrict HR of particular genome segments or even to a particular cell type, ensuring a relatively stable yet flexible genome. The restriction of frequency with which the HR pathway is used can result in stimulation of error-prone NHEJ and lead to the persistence of numbers of small deletions and insertions. This however would prevent significant alteration of the genome due to chromosome rearrangements caused improper HR.

2.3. HR and abiotic stress

2.3.1. General tendencies of stress influence

2.3.1.1. Plant and stress

Drought, high salinity, high intensity light, UV and extreme temperatures are the most common environmental stress factors that influence plant growth and development placing major limits on plant productivity in cultivated areas worldwide. The responses of plants to various abiotic stresses have been important subjects of physiological studies (Levitt, 1980) and more recently of molecular and transgenic studies (Hasegawa, *et al.*, 2000b; Zhang, *et al.*, 2000). A number of genes down regulated by drought, salt or cold stress at the transcriptional level have been found (Hasewaga, *et al.*, 2000b; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999; Zhu, 2002).

Expression profiles has been used to identify more than 300 genes induced by drought, cold or salinity in *A. thaliana* and 40 stress inducible transcription factor genes were cloned (Seki, *et al.*, 2002a; 2002b). Microarray analysis of the *A. thaliana* transcriptome (approximately 8100 genes) under cold, salt and drought tolerance identified 2409 genes with a greater than 2-fold change when compared to the control suggesting that about 30% of the transcriptome is sensitive to regulation by common stress conditions (Kreps, *et al.*, 2002). A stress response was observed for 306 (68%) of the known circadian controlled genes supporting the hypothesis that an important function of the circadian clock is to "anticipate" predictable stresses such as cold nights.

The role of abiotic stress-activated signal molecule, abscisic acid (ABA), in stress tolerance is well documented (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). Analyses of the expression of stress-inducible genes in *A.*

thaliana have indicated that ABA-dependent and ABA-independent signal pathways function in the induction of stress-inducible genes. These studies indicate the existence of complex regulatory mechanisms between the perception of abiotic stress signal and gene expression (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002).

To date, our knowledge with respect to the influence of abiotic stress factors on genome stability is still fragmentary and requires further expansion. This is of special importance for plants that contain great amounts of repetitive DNA, including multigene families (Flavell, 1985) that provide targets for HR. The HRF was shown to be elevated by external factors (Lebel, *et al.*, 1993; Tovar and Lichtenstein, 1992; Ries, *et al.*, 2000a; 2000b; Lucht, *et al.*, 2002; Kovalchuk, *et al.*, 1998; 2001; 2003b). This elevation can lead not only to the alteration of the genome, but can provide genotypic variability helping plants to change according to different environmental factors.

2.3.1.2. ROS and their duel role in plant cell metabolism

One of the earliest biochemical responses to biotic and abiotic stresses for both plant and animal cells is the generation of ROS. Environmental stresses such as drought and desiccation, high temperature, chilling, salt, heavy metals, UV, intensive light, air pollutants such as ozone and SO_2, mechanical, nutrient deprivation and pathogen attack cause ROS generation in plants (Mittler and Zilinskas, 1994; Noctor and Foyer, 1998; Bowlet, *et al.*, 1994; Desikin, *et al.*, 2001; Allen, 1995). Pathogen attack and wounding have been shown to trigger the active production of ROS by NADPH oxidases (Hammond-Kosack and Jones, 1996; Orozco-Cardenas and Ryan, 1999; Cazale, *et al.*, 1999; Pei, *et al.*, 2000).

ROS, particularly superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), are also generated during normal cell metabolism in the cytoplasm, chloroplasts (Asada and Takahashi, 1987), mithochondria (Jimenez, *et al.*, 1998), peroxisomes (del Rio, *et al.*, 1992; Lopez-Huertas, *et al.*, 1999), and apoplasts (Hernandez, *et al.*, 2001). The uncontrolled accumulation of ROS generates oxidative stress and may cause membrane lipid peroxidation, inactivation of SH-containing enzymes, and RNA and DNA damage (Smirnoff, 1993; Menconi, *et al.*, 1995). Plants use a number of antioxidant enzymes and molecules such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbate (AsA), and glutathione (GSH) for scavenging ROS (Noctor and Foyer, 1998; Asada, 1994). In many cases, flavonoid compounds, like anthocyanins, may provide antioxidant activity indicating the similarity in stress signaling of different stress pathways (Winkel-Shirley, 2002).

The enhanced production of ROS during stress can pose a threat to cells on both structural (Smirnoff, 1993; Menconi, *et al.*, 1995) and genomic (Chatgilialoglu and O'Neill, 2001) levels. The increased intracellular concentration of ROS would lead to multiple DNA damage, challenging the repair machinery of the cell. However it is also thought that ROS acts as signals for the activation of the stress-response and defense pathways (Desikin, *et al.*, 2001; Knight and Knight, 2001).

ROS can be viewed as a cellular indicator of stress and as a secondary messenger involved in the stress-response signal transduction pathways. Additionally, the balance between different ROS-producing and ROS-scavenging mechanisms is tightly regulated (Mittler, 2002). The oxidative stress is managed either by controlling ROS generation or

by enhancing the antioxidant defense capacity may be an important cellular response, which results in acclimation (Selote, *et al.*, 2004).

2.3.2. Salt stress and genome stability

One of the most significant abiotic stresses in agriculture is soil salinity (Zhu, 2000). It is caused by the presence of NaCl in high concentration in the plants growth environment. Salt represents by itself a complex stress consisting of cellular osmotic and ionic stresses complemented with secondary oxidative stress (Zhu, 2000). Salinity interferes with plant growth as it leads to physiological drought through induction of osmotic stress and ion toxicity cased by excessive Na^+ (Zhu, 2002). It has been shown that salt and drought stresses induce common sets of plant genes (Zhu, 2001a; 2001b). However, the ionic aspect of salt stress is clearly distinct from other abiotic stresses such as drought and cold, and there are signaling pathways dedicated specifically to deal with ionic stress (Hasegawa, *et al.*, 2000b).

Salt stress conditions result in the disruption of cell metabolism on the molecular level, including the inhibition of enzyme activities in metabolic pathways, decreased uptake of nutrients in roots, decreased carbon-use efficiency, and the denaturation of protein and membrane structures (Tsugane, *et al.*, 1999). The ionic component of the salt stress results in the presence of toxic concentrations of Na^- ions in the cytoplasm and a deficiency of essential ions such as K^+ (Blumwaid, *et al.*, 2000). Deficiency of K^+ ions causes changes in the function of different ion transporters and often affects the level of their transcripts disrupting ionic homeostasis of the cell (Hasegawa, *et al.*, 2000b). Enhanced production of ROS as a secondary effect of stress can lead to DNA damage

and even result in apoptosis (Niu, *et al.*, 1995; Zhu, *et al.*, 1997; Hasegawa, *et al.*, 2000b).

The primary target for salinity is photosynthesis, one of the most important metabolic pathways in plants (Tsugane, *et al.*, 1999). ABA produced in response to salt stress (Leung, *et al.*, 1994) decreases turgor in guard cells and thus limits the CO_2 available for photosynthesis. During water stress, reduction of chloroplast stromal volume and generation of ROS, which were shown to be enhanced by salicylic acid (SA) (Borsani, *et al.*, 2001) are also thought to play important roles in inhibiting photosynthesis. Furthermore, plant salt stress defense pathways have been shown to interact with pathways for cell division and expansion (Zhu, 2000).

To tolerate salt stress plants have developed special signaling pathways. The important salt stress signal pathway was recently described (Zhu, 2000). Genetic and physiological studies defined five salt tolerance genes composing the aforementioned pathway: *SOS1* (Wu *et al.*, 1996), *SOS2* (Zhu *et al.*, 1998), *SOS3* (Liu and Zhu, 1997), *SOS4*, and *SOS5* (Zhu *et al.*, 2000). This Ca^{2+}-dependent pathway was shown to be involved in the regulation of ion homeostasis and salt tolerance (Gong *et al.*, 2001).

Molecular interaction and complementation analyses indicate that *SOS3* is the Ca^{2+}-binding protein that is required for activation of protein kinase *SOS2* regulating the expression of the *SOS1* transporter gene (Halfter, *et al.*, 2000). The order of the signal pathway is *SOS3*→*SOS2*→*SOS1* (Hasewaga, *et al.*, 2000a; Sanders, 2000; Zhu, 2000). Importantly, *SOS3*, *SOS2*, and *SOS1* were recently cloned and biochemically characterized in *A. thaliana* (Zhu, 2000).

2.3.3. Plant environment: temperature and light regimes

Both high and low temperature and light regimes are well known physiological factors influencing plants during their life, affecting plant performance and sometimes distribution (Boyer, 1982; Larcher, 1995; Govindachary, *et al.*, 2004). One of the primary metabolic targets for the aforementioned stresses is photosynthesis (Govindachary, *et al.*, 2004; Stitt and Hurry, 2002; Savitch, *et al.*, 2001; Salvucci and Crafts-Brandner, 2004a; 2004b; Crafts-Brandner and Salvucci, 2002; Pittermann and Sage, 2000; Law, *et al.*, 2001), particularly activity of the Ribulose biphosphate carboxylase/oxygenase (RuBP) enzyme (Kanechi, *et al.*, 1996, Feller, *et al.*, 1998; Law and Crafts-Brander, 1999; Crafts-Brandner and Law, 2000; Crafts-Brandner and Salvucci, 2000; Pittermann and Sage, 2000). Temperature induced metabolic changes may effect ROS production (Noctor and Foyer, 1998) and thus, pose a threat for both cell and genomic integrity. Heat shock has been found to not only influence photosynthesis but enhance HRFs in plant cells (Lebel, *et al.*, 1993).

2.3.3.1. Temperature

2.3.3.1.1. Low temperatures

Low temperature affects both plant performance and distribution (Boyer, 1982; Larcher, 1995). It slows down enzyme-catalyzed reactions and modifies conformation of lipids and other macromolecules with consequences for most biological processes including the inhibition of sucrose synthesis and photosynthesis, partially by inactivation of RuBP (Stitt and Hurry, 2002).

The biochemical and physiological adaptations of plants to low temperature include post-translational activation and increased expression of enzymes from the sucrose synthesis pathway, the changed expression of Calvin cycle enzymes and changes in the leaf protein content (Stitt and Hurry, 2002) that require induction of expression of diverse array of plant genes (Thomashow, 2001). Additionally, reduction or cessation of growth, transient increases in ABA concentration, changes in membrane lipid composition, the accumulation of compatible osmolytes and increased level of antioxidants (Xin and Browse, 2000) are important during cold acclimation.

The effect of cold acclimation on the light reaction of photosynthesis was recently examined *in vivo* (Savitch, *et al.*, 2001). *A. thaliana* plants showed a strong decrease in the rate of CO_2 assimilation as well as CO_2-dependent O_2 evolution when exposed to low-temperature stress. A similar effect on C4 photosynthesis also is well documented (Labate, *et al.*, 1991; Long, 1998). This negative effect might be explained by high thermal dependence of the RuBP V_{max}. The *in vivo* capacity of RuBP below 10-15°C is low relative to its value above 25°C (Pittermann and Sage, 2000). Moreover, low temperature affected photosynthesis not only on a biochemical but at the level of gene expression. Indeed, leaves of *A. thaliana* shifted to low temperature showed reduced expression of nuclear-encoded photosynthetic genes (Strand, *et al.*, 1997).

2.3.3.1.2. High temperatures

The influence of high temperature on photosynthesis has been extensively studied. Inhibition of photosynthesis by heat stress has been attributed to an impairment of electron transport activity caused in part by changes in membrane fluidity (Raison, *et al.*,

1982; Havaux, 1993; Murakami, *et al.*, 2000). Others (Bilger, *et al.*, 1987; Jones, *et al.*, 1998; Bukhov, *et al.*, 1999; Bukhov and Dzhibladze, 2002), supporting the idea that the initial site of inhibition is associated with a Calvin cycle reaction, specifically the inactivation of RuBP (Weis, 1981a; 1981b; Kobza and Edwards, 1987). This idea has been recently confirmed (Feller, *et al.*, 1998; Law and Crafts-Brander, 1999; Crafts-Brandner and Law, 2000; Crafts-Brandner and Salvucci, 2000) coupled with more thorough understanding of the biochemistry of the activation process (Andrews, 1996; Spreitzer and Salvucci, 2002; Portis, 2003).

Accumulated data from gas exchanger and biochemical analyses suggests that deactivation of RuBP caused at least at part by thermal inactivation of RuBP-activase (Salvucci, *et al.*, 2001), is the primary cause of inhibition of photosynthesis under moderate heat stress (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004b). Salvucci and Crafts-Brandner (2004b) showed that direct inhibition of photosynthesis occurred at temperatures higher than about 30°C mainly due to the low affinity of enzyme for CO_2 and its dual nature as an oxygenase. Moderate heat stress (30-42°C) generally reduced the rate of RuBP regeneration caused by disruption of electron transport activity, and specifically inactivation of the oxygen evolving enzymes of photosystem II (PSII).

The key role of RuBP-activase in inactivation of RuBP under heat shock was revealed in recent studies (Crafts-Brandner and Salvucci, 2002). It was shown that it was heat inactivation of this enzyme that caused changes in the rate of net photosynthesis. However, heat stress is known to affect alternative splicing of certain plant genes (Lazar and Goodman, 2000; Kinoshita, *et al.*, 2001), and could change the splicing pattern of

activase, culminating in the expression of a unique product(s) derived from this gene. Recently, induction of transcription of new form of activase induced by heat stress conditions was confirmed by studies of Law, *et al.* (2001) in cotton.

2.3.3.2. Light regimes

It is also well known that the activation state of RuBP is influenced by other conditions, such as light and CO_2 (Kanechi, *et al.*, 1996, Feller, *et al.*, 1998).

The light-induced decline in the photochemical activity of PSII or photosystem I is known as photoinhibition (Aro, *et al.*, 1993; Havaux and Davaud, 1994; Terashima, *et al.*, 1994). PSII is considered the primary target for excessive light. Based on *in vitro* studies, two mechanisms of photoinhibitory damage have been proposed (Ohad, *et al.*, 2000).

Photoinhibition occurs when the light-dependent reactions of photosynthesis produce ATP and NADPH in excess of what can be consumed by the reaction of the dark carbon mechanism. Therefore, when the environmental conditions do not promote carbon fixation, even weak or moderate light may become harmful (Havaux and Davaud, 1994; Terashima, *et al.*, 1994; Kudoh and Sonoike, 2002). Over reduction of the electron transport chain results in the generation of large amounts of ROS, such as superoxide anion radical or/and singlet oxygen (Asada, 1994) creating an extra challenge to DNA maintaining machinery (Mittler, 2002; Chatgilialoglu and O'Neill, 2001).

2.3.4. Water-mediated stress

2.3.4.1. Drought conditions

2.3.4.1.1. Molecular aspects of drought stress

Molecular and cellular responses to drought stress have been analyzed extensively at the biochemical level using different plant model systems (Ingram and Bartels, 1996; Thomashow, 1999; Bray, *et al.*, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2000) including *A. thaliana* (Liu, *et al.*, 1998; Iuchi, *et al.*, 2000). The early events of plant adaptation to environmental stresses are perception and subsequent stress-signaling transduction leading to activation of various physiological and metabolic responses, including stress-responsive gene expression (Tran, *et al.*, 2004). More than half of the drought inducible genes are also induced by high salinity and/or ABA treatments, indicating the existence of significant crosstalk between the drought, high-salinity and ABA responses (Seki, *et al.*, 2002a; 2002b). Additionally, about 10% of the drought-inducible genes were induced by cold stress (Tran, *et al.*, 2004). Interestingly, a decrease in polar lipid content was induced by drought as well as by chilling and senescence (Matos, *et al.*, 2000).

There are at least four independent regulatory systems for gene expression in response to drought stress in *A. thaliana*. Two are ABA-dependent and two are ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 2000). The genes activated by both of these pathways are thought to function in the accumulation of osmoprotectants, detoxification protection of the cells, protein turnover, stress signaling pathways, transcription regulation and *etc* (Bohnert, *et al.*, 1995; Bray, 1997; Ingam and Barteles, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997).

The influence of drought stress on genome integrity is partially mediated by ABA. It has been documented that ABA can cause an increased generation of O_2^- and H_2O_2, and induce the expression of antioxidant genes encoding Cu, Zn-SOD, Mn-SOD, Fe-SOD and CAT. Moreover, ABA also increases the activities of antioxidant enzymes such as SOD, CAT, APX, and GR in plant tissues (Jiang and Zhang, 2002). Thus, water stress-induced ABA accumulation may trigger the increased generation of ROS, which in turn, leads to the up regulation of the antioxidant defense system. Indeed, the accumulation during dehydradation of the lipoxygenase inhibitor and anthocyanins with antioxidant capability has been observed in *Craterostigma* leaves (Hoekstra, *et al.*, 2001).

2.3.4.1.2. Physiological aspects of drought stress

The general decrease in photosynthetic rate and resulting limitation of plant growth has been reported as the most common response to drought stress (Tabaeizadeh, 1998; Flexas, *et al.*, 2004). Water deficit induced stomatal closure, resulting in reduced CO_2 assimilation, triggering to NADPH accumulation (Lawlor and Khanna-Chopra, 1984) and subsequent leakage of e^- towards O_2 leading to enhanced ROS generation (Asada, 1999). ROS also originate in the cell by non-enzymatic mechanisms such as Fe-catalyzed oxidation of NAD(P)H (Selote, *et al.*, 2004). The analyses suggest that stomatal closure is the earliest response to drought and the dominant limitation to photosynthesis at mild to moderate drought. However, progressive down regulation or inhibition of metabolic processes leads to decreased RuBP content, which becomes the dominant limitation at severe drought and thereby inhibits photosynthetic CO_2 assimilation (Flexas and Medrano, 2002; Lawlor, 2002; Cornic and Fresneau, 2002; Tang, *et al.*, 2002).

2.3.4.2. Overwatering conditions

At the same time, the data about plant behavior under flood/over-watering stress is still fragmentary. Some important effects of flooding include reduction in the water and nutrient uptake and decrease in metabolism. Prolonged soil flooding will also ultimately lead to anoxia conditions with profound effects on the plant respiratory mechanism (Dat, *et al.*, 2004). Hypoxic and anoxic conditions were shown to trigger morphological adaptation such as formation of adventitious roots (Lorbieske and Sauter, 1999; Mergemann and Sauter, 2000), aerenchyma, and a number of a complex metabolic adaptations (Drew, 1997; Liao and Lin, 2001) such as switch to the different type of fermentation pathway (Ismond, *et al.*, 2003). These can provide a better energy status due to higher rates of glycolysis and ethanol fermentation (Peng, *et al.*, 2001), limitation of lactate accumulation, and better regulation of cytoplasmic pH (Ismond, *et al.*, 2003).

Genetic variation in flooding tolerance has been found in rice, maize, barley and *A. thaliana* (Ismond, *et. al.*, 2003). Zhang, *et al.* (2000) overexpressed a gene involved in cytokinin biosynthesis and demonstrated improved flooding tolerance in *A. thaliana*. It is now established that flooding responses are characterized by enhanced ethylene production, accompanied by a signaling cascade which includes a network of hormones and other common secondary signaling molecules (Dat, *et al.*, 2004).

Recently, the redox-derived signaling during flooding stress was reported (Dat, *et al.*, 2004). Additionally, induction dependence of alcohol dehydrogenase on NADPH-oxidase activity, which is a known ROS generator, indicates the importance of ROS during hypoxia conditions (Dat, *et al.*, 2004). Furthermore, the transient increase in transcript levels of a respiratory burst oxidase in *A. thaliana* root cultures during low-

oxygen growth condition has been found (Klok, *et al.*, 2002). However it is still unclear which signals and which sensory mechanisms are responsible for triggering the plant response to flood stress. The data accumulated to the date are not complete to draw any conclusion. Moreover, data lacks describing the possible genotoxicity of over-watering stress.

2.4. HR and pathogen induced stress

One of the first successful attempts to examine the impact of pathogens on plant genome stability was performed by Lucht, *et al.* (2002). The authors used transgenic *A. thaliana* plant line 651 that carries the GUS reporter gene as the experimental model and oomycete pathogen *Peronospora parasitica* to induce a defense response.

Once the pathogen is recognized, different signaling pathways are activated in plants to transduce this stimulus (McDowell and Dangl, 2000). One pathway involves SA as an important intermediate. Accumulation of SA is required for the activation of several pathogenesis-related genes as well as for a secondary defense responses that results in systemic immunity against pathogen attack (Yang, *et.al.,* 1997; McDowell and Dangl, 2000). The same response could be stimulated by external application of SA analogs (Lawton, *et al.*, 1996).

Lucht, *et al.* (2002) found that chemical inducers of the SA – dependent pathogen response pathway stimulate HR in reporter transgenes of differing structure and at different positions in the plant genome. Crosstalk between the defense signal-transduction pathway and the HR machinery of plants has been confirmed with the help of new *cim3 A. thaliana* mutant. This mutant has constitutively increased the levels of SA and increased resistance against infection with *P. parasitica* (Lucht, *et al.*, 2002). Mutant hemizygous *A. thaliana* plants having dominant *cim3* mutation and one copy of the recombination reporter construct showed 4 – 5 fold increase in HRF. Several plants lines carrying reporter constructs at different positions in the genome were analyzed. All lines showed an increase of somatic HRF under the pathogen infection or application of SA analogs in absence of pathogen (Lucht, *et al.*, 2002). Interestingly, SA was also shown to

potentiate the effects of salt and osmotic stresses by enhancing ROS generation during photosynthesis and germination of *A. thaliana* (Borsani, *et al.*, 2001).

We can conclude that induction of somatic recombination by pathogen-induced stress is a general effect that may influence homologous sequences throughout the plant genome. However it is still unclear whether effects on recombination require the presence of the pathogen or represent a systemic plant response.

Kovalchuk, *et al.* (2003b) studied plant genome stability under pathogen-induced stress. They found that Tobacco Mosaic virus (TMV) infection of tobacco plants can elicit a systemic recombination signal (SRS) that can move through the plant triggering genomic changes. Grafting experiments revealed that the signal can be transmitted from plant to plant in the complete absence of the virus that originally incites its production. Additionally, SRS was shown to result in increased frequency of meiotic and/or inherited late somatic recombination (Kovalchuk, *et al.*, 2003b).

Other authors have supported these findings. Exposure of maize plants to barley stripe mosaic virus activated transposable elements (Dellaporta, *et al.*, 1984; Johns, *et al.*, 1985) and resulted in the mutations in the non-infected progeny of infected plants (Brakke, 1984).

The stimulation of somatic recombination events by pathogen stress and their transmission to progeny may be important in the process of evolving of plant resistance-gene clusters and new pathogen specificities.

2.5. DSB repair: implication in plant genome evolution

Evolution is based on genetic variability and subsequent phenotypic selection. Mechanisms that modulate the rate of mutation according to changes in the environment, and thus control the balance between genetic stability and flexibility, may provide a distinct evolutionary advantage (McClintock, 1984; Radman, *et al.*, 1999; Caporale, 2000; Rosenberg, 2001) by facilitating evolutionary adaptation of plant populations to stressful environment (Lucht, *et al.*, 2002). The importance of genetic variability for plants can be illustrated by the fact that animals can adapt to a changing environment with behavioral changes, unlike plants. Plants can use other adaptation methods such as rapid genomic change. The germline of plants is not set-aside early in development. Therefore, somatic genomic changes can be transferred to the next generation. In addition, the high degree of plasticity in the plant body structure and physiology likely allows plants to tolerate larger quantities of genomic rearrangements as compared to animals. One of the most interesting and evolutionarily significant ways to increase the diversity of genomic content within a single plant population is the enhanced level of somatic RR after exposure to stress (Walbot, 1996; Kovalchuk, *et al.*, 2003b).

Different abiotic and biotic stresses often result in more frequent occurrence of DSBs within plant genome. Therefore plants can utilize their unique feature, their more error-prone character of DSBs repair than in other eukaryotes (Gorbunova and Levy, 1997; Rubin and Levy, 1997; Salomon and Puchta, 1998), to provide a fast evolutionary adaptation of the genome to new environmental conditions.

The stimulation effect of DSBs and other forms of DNA damage on HR in plants is well documented (Puchta and Hohn, 1996; Puchta, *et al*, 1995). The high copy number

of retrotransposon sequences within plant genome (Kumar and Bennetzen, 1999), along with highly homologous gene families dispersed in plants genomes (Flavell, 1995), make HR important for plant genome evolution. Recently, the model explaining the role of retrotransposons in plant evolution was revealed in tobacco (Beguiristain, *et al.*, 2001). According to the authors retrotransposons have adapted to their host genome through the evolution of a highly regulated promoter similar to those that stress-induced plant genes have. Stress influence will result in an increase of the transcriptional level of the retrotransposons inducing their mutagenicity. All three different subfamilies of *Tnt1* retrotransposons analyzed were induced by stress but their promoters showed a different response to different stress-associated signaling molecules (Beguiristain, *et al.*, 2001). Moreover it was shown that exposure of maize plants to pathogen resulted in the activation of transposons (Dellaporta, *et al.*, 1984; Johns, *et al.*, 1985) and causing mutations in the non-infected progeny of infected plants (Brakke, 1984).

It is well documented that exposure of plants to different stress conditions results in global transcriptional changes (Hasewaga, *et al.*, 2000a; 2000b; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999; Zhu, 2002; Seki, *et al.*, 2003; Kreps, *et al.*, 2002). Indeed, global transcriptional changes after pathogen stress affect about 1 in 25 *A. thaliana* genes (Maleck, *et al.*, 2000). This would likely affect somatic recombination at multiple loci of the genome due to the positive influence of increased transcription of locus on somatic HR in the vicinity (Saxe, *et al.*, 2000).

Possible substrates for somatic recombination events in plants are the large number of disease-resistance genes (R-genes) involved in the perception of pathogen attack that are spread in clusters throughout the genome (Young, 2000; Ellis, *et al.*,

2000). The evolution of the R-genes and the generation of novel disease-resistance specificities have often been associated with recombination events (Ritcher and Ronald, 2000; Tinland, *et al.*, 1994).

The evolutionary significance of the increase in somatic RR after exposure to stress may lie in elevated genetic variability of the progeny as stable genetic changes occurring in somatic tissue can be transmitted to the next generation (Walbot, 1996; Kovalchuk, *et al.*, 2003b). The exposure to UV-B radiation (Ries, *et al.*, 2000a; 2000b) or pathogen attack (Kovalchuk, *et al.*, 2003b) results in the appearance of an increased number of *A. thaliana* progeny plants carrying a completely recombined reporter construct. This indicates the importance of this DNA maintaining pathway for plant evolution.

2.6. HR and T-DNA integration. Application of HR for gene targeting technique

2.6.1. *Agrobacterium*-mediated plant transformation

Agrobacterium-mediated plant transformation and its application for plant genetic engineering (Gelvin, 2003a) is based on the ability of *Agrobacterium* to cause trans-kingdom DNA transfers (Stachel and Zambryski, 1989). The transferred DNA (T-DNA), which is the part of bacterial tumor-inducing (Ti) plasmid, is integrated into the host genome and expressed using host transcription machinery (Gelvin, 2000; Gelvin, 2003b; Tzfira and Citovsky, 2000; Tzfira and Citovsky, 2002; Zupan, *et al.*, 2000).

Processing, transport and nuclear import of the T-DNA is performed by *Agrobacterium Vir* genes. It is thereby believed that T-DNA carries no specific targeting signal and does not encode any transport or integration function (Tzfira, *et al.*, 2004). It is generally believed that plant factors and not only the bacterial *Vir* proteins have a crucial role in the process of T-DNA integration into the host genome (Gelvin, 2000; Friesner and Britt, 2003; van Attikum, *et al.*, 2003; Mysore, *et al.*, 2000; Gallego, *et al.*, 2003). Recent reports have revealed some functional features of host proteins in the transformation process, particularly in its last two steps: T-DNA nuclear import and integration (Gelvin, 2003b; Tzfira and Citovsky, 2002). Unfortunately our understanding of T-DNA integration mechanism into the recipient genome is limited (Tzfira, *et al.*, 2004).

2.6.2. DSBs repair plant pathways and T-DNA integration

It is believed that DSBs are the preferable sites for T-DNA integration (Tzfira, *et al.*, 2003). Data supports enhanced host cell transgene integration following DSBs inducing (Leskov, *et al.*, 2001) X-ray irradiation (Kohler, *et al.*, 1989). Integration of the transgene into the DSBs may represent a default mode for T-DNA entry into the recipient's genome (Chilton and Que, 2003). Based on this model naturally occurring DSBs and/or DSB-associated DNA repair activity may act as molecular stimuli for incoming into the nucleus T-DNA molecules (Tzfira, *et al.*, 2003).

Utilizing a yeast-based system T-DNA integration has been directed into the yeast genome by either HR (Bundock, *et al.*, 1995; Risseeuw, *et al.*, 1996) or NHR pathways (van Attikum and Hooykaas, 2003). This made it possible the examination of the role of various NHEJ proteins (van Attikum, *et al.*, 2001; van Attikum, *et al.*, 2003). It was shown that *Ku 70*, *Rad50*, *Mre11*, *Xrs2*, *Lig4* and *Sir4* are all required for T-DNA integration by NHR (van Attikum, *et al.*, 2001), however T-DNA integration provided through HR mechanism required only *Rad51* and *Rad52*, but not *Rad50*, *Mre11*, *Xrs2*, *Lig4* or *Ku70*. In the absence of *Ku70* T-DNA integration occurs via the HR pathway (van Attikum, *et al.*, 2001), whereas in the absence of *Rad52*, transgene integration was possible only by NHR (Ristic, *et al.*, 2003). Simultaneous mutations in both *Ku70* and *Rad52* genes resulted in blocking both HR and NHR pathways led to complete inhibition of T-DNA integration (van Attikum, *et al.*, 2003). Results derived from yeast-based system suggest there is a key role for *Ku70* and *Rad52* enzymes in transgene integration (van Attikum, *et al.*, 2003; van Attikum, *et al.*, 2001).

In plants the NHEJ proteins have been suggested to be key players in T-DNA integration. They direct process via NHR even when T-DNA shares high homology with the host genome (Tzfira, *et al.*, 2003). This can be explained in part by the fact that a *Rad52* homologue has yet to be identified in plants (Ray and Langer, 2002). Two additional *A. thaliana* proteins, DNA ligase IV *AtLIG4* (West, *et al.*, 2002) and *AtKu80* (Tamura, *et al.* 2002; West, *et al.*, 2002) both essential for NHEJ, were studied for their role in T-DNA integration without conclusive results. *AtLIG4* was dispensable for T-DNA integration in *A. thaliana* (van Attikum, *et al.*, 2003) using a root tumor formation assay (Zhu, *et al.*, 2003) but was reported to be either required (van Attikum, *et al.*, 2003) or dispensable (van Attikum, *et al.*, 2003) for transgene integration using an *in planta* transformation procedure that targets the host germ-line cells (Ye, *et al.*, 1999). The involvement of *AtKu80* in T-DNA integration is even less conclusive as it was reported to be both required (Friesner and Britt, 2003) and dispensable (Gallego, *et al.*, 2003) for transgene integration using the same *in planta* transformation assay.

2.6.3. HR and gene targeting: future perspectives

Great efforts have been undertaken for establishing a gene targeting (GT) system in plants (Puchta, 2002). Almost all attempts have been unsuccessful, because NHEJ is the prevailing process for transgene integration in flowering plants. The lower frequency of HR repair of DSBs in plants than in other higher eukaryotes is a principal obstacle to the practical application of gene-replacement techniques.

The integration of T-DNA by NHR has a number of disadvantages. It results in the random integration of a transgene often in unpredictable copy numbers (Vergunst and

Hooykaas, 1999; Kumar and Fladung, 2001). HR results in the precise integration of transgenic DNA at the homologous site of the host genome, thus allowing GT. The later one, on other hand, is widely used as a basic tool in functional genomics for unraveling unknown gene function via reverse genetics (Hohe and Reski, 2003).

The logical goal of GT is to initiate a shift in the competition between DSBs repair pathways in plants by inhibiting NHEJ to encouraging HR. Our future ability to alter the higher plant genome in a more directed way rather than by random plant transformation will depend on our understanding of the mechanisms of HR and its genetic control. In this respect the definition of factors that may control the level of chromosomal HR in plant somatic cells could be important.

The identification of cellular factors that may control the level of chromosomal HR in plant somatic cells is required. Evidence for evolutionary conservation should help to decipher mechanisms of plant HR and possibly detect limiting factors. Furthermore the identification of abiotic stresses that are not influencing DNA metabolism but are able to induce HR in plants could be an interesting approach to improve GT and *Agrobacterium*-mediated plant transformation techniques. Previously reported data indicate the possible induction of recombination by salt (NaCl) stress (Puchta, *et al.*, 1995). However to date, no attempt has been made to extend knowledge in this direction or to determine the mechanism of such influence.

There are several reports that indicate the possible enhancement of HR competence with respect to T-DNA integration. Previously discussed reports indicated dispensability of *AtKu80* (Gallego, *et al.*, 2003) and *AtLIG4* (van Attikum, *et al.*, 2003) for Agro-transformation suggest that an alternative pathway must exist in plants.

Ninomiya, *et al.* (2004) obtained promising data that showed the *Ku70* and *Ku80* mutant strains of yeasts being efficient recipients for gene targeting. Authors hypothesized that blocking NHEJ function can increase the rate of homologous integration of exogenous DNA.

Despite the fact that there was little success in gene targeting, including the integration of the transgene at the defined location, it seems possible in the future. This will depend on our ability to optimize various parameters influencing the efficiency of chromosomal HR. Knowledge gained in this area will help us understand regulatory relationships among the various DSB repair pathways in plant. This will result in the improved precision of plant genome engineering.

3. MATERIALS AND METHODS

3.1. Plant cultivation

In the current work, *A. thaliana* wild type (Columbia ecotype) plants and transgenic *A. thaliana* lines 11; 651 and 699 were used. All plants were obtained from Friedrich Miescher Institute (Basel, Switzerland).

3.1.1. Growth conditions

3.1.1.1. Growing plants on soil

A. thaliana seeds were sown on *All purpose potting soil* (Plant *Etc*; Lethbridge, AB, Canada) were left for vernalization at +4°C in the dark for two days and after were moved to germinate and grow in a growth chamber (Enconair, Winnipeg, MB, Canada) with constant humidity 65%. Plants were grown with the light (32.8 $\mu Em^{-2}s^{-1}$) provided by Cool White Fluorescent bulbs (Sylvania, Mississauga, ON, Canada) and Longlife Incandescent bulbs (Sylvania, Mississauga, ON, Canada) for 16 hours each day at 22°C and without light 8 hours at 18°C.

3.1.1.2. Growing plants under sterile conditions

A. thaliana seeds sown on sterile medium contained in an 100 mm Petri dish were incubated at +4°C in the dark to break dormancy then moved to the growth chamber with a constant humidity of 65% for germination and growth. Plants were grown utilizing high light condition (32.8 $\mu Em^{-2}s^{-1}$) provided by Octron T8 Fluorescent bulbs (Sylvania,

Mississauga, ON, Canada) for 16 hours each day at 22°C and without light 8 hours at 18°C. Humidity was maintained at 65% for the duration of all experiment.

3.1.2. Sterilization of seeds

Seeds were rinsed in 70% ethanol for two minutes, sterilized with a solution of 0.5% sodium hypochloride, 0.05% Tween-80 for five minutes and rinsed twice with a large excess of sterile distilled water for five minutes each. Following this seeds were planted on basic medium (BM).

3.1.3. Growth medium composition

BM of the following composition was used to grow plants under the standard, control condition (Table 3.1). All components of BM were prepared separately and combined together prior to use. Media was solidified with agar (8 g/L). The pH was adjusted to 5.7 before autoclaving; sucrose and vitamins were filter sterilized using 0.45 nm Nalgene filters (Nalgene, New York, USA) and added to the cooled medium after autoclaving.

Table 3.1 The standard chemical composition of basic medium

Group of chemicals or single chemical	Chemicals included to the particular group	Final amount in growth medium
MS macro	NH_4NO_3	20.6 mM
	KNO_3	18.8 mM
	$CaCl_2$	3 mM
	$MgSO_4$	1.5 mM
	KH_2PO_4	1.25 mM
Microelements	$CoSO_4$	0.00025%;
	Na_2MoO_4;	0.0025%
	H_3BO_3	0.062%
	$ZnSO_4$	0.086%
	$CuSO_4$	0.00025%
	$MnSO_4$	0.169%
	KJ	0.0083%
Vitamins	Nicotinic Acid	0.1%
	Pyridoxin HCl	0.1%
	Thiamin HCl	1%
	Inositol	10%
Ethylenediamine Tetraacetic Acid (EDTA)		1.5%
$FeCl_3$		0.54%
2-(N-Morpholino)-ethanesulfonic Acid monohydrate (MES)		0.05%
Sucrose		2%

3.2. Assays for the detection of marker gene activity

3.2.1. Measurement of HRF

HRF was measured using the *uidA* (GUS) reporter gene, which served as a recombination substrate. It based on two overlapping (500-1200 bp) truncated non-functional parts of the *uidA* gene cloned either in direct or indirect orientation (Figure 3.1a). *A. thaliana* homozygous lines 11 and 651 carried the reporter gene cloned in direct (line 11) or indirect (line 651) orientation (Swoboda, *et al.*, 1994; Ilnytskyy, *et al.*, 2004). Recombination transgenic markers were driven by 35S cauliflower mosaic virus (CaMV) promoter. This promoter has previously been shown to be active in virtually all plant tissue (Benfey and Chua, 1990). Upon somatic recombination event between regions of homology the reporter gene is restored and becomes functional, thus displaying activity of the *β–glucuronidase* (GUS) (Figure 3.1c).

3.2.2. Measurement of point mutation frequency

The point mutation frequency (PMF) was measured using the GUS reporter gene. *A. thaliana* homozygous line 699 carries one copy per haploid genome of the stop-codon inactivated *uidA* gene (Kovalchuk, *et al.*, 2000). The reporter gene was inactivated by generating in the open reading frame of a *uidA* gene nonsense mutation (GGA→TGA) in position 166 (number of the mutated nucleotide starting from ATG) (Figure 3.1b). Plants carrying the stop-codon inactivated GUS gene are not capable of producing a functional protein. Reversion to the original nucleotide or mutation to another amino acid that allows protein function would restore gene activity (Figure 3.1c).

3.2.3. Visualization of recombination and point mutation events

Recombination or point mutation (PM) events that yielded an intact, functional copy of the GUS gene were visualized following a histochemical staining procedure. Plants were harvested and suspended in a GUS staining buffer containing: 500 μg/mL of X-glu (Rose Scientific LTD.; Edmonton, AB, Canada) 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 10 min 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. Chlorophyll was removed 48 hours later 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 with blue spots indicating the location of GUS activity (Figure 3.1c). The number of blue spots (HR or PM events) for each plant was counted.

3.2.4. Calculation of HR and PM frequencies and rates

The HRF/PMF was calculated by counting number of HR/PM events (sectors) in each plant separately, summing and then relating the data to the number of plants in the tested populations. It represents the number of HR/PM events per single plant.

The 'Number of genome present' represents the number of haploid genomes present per single plant. It was calculated by relating the yield of total DNA (in micrograms per plant) to the mean DNA content (0.16 pg) of an *A. thaliana* haploid cell (Swoboda, *et al.*, 1993) and the number of plants used for DNA preparation.

The HR/PM rate (PMR) was calculated by relating HRF/PMF to the number of haploid genomes present per single plant. It represents the number of HR/PM events per single haploid genome.

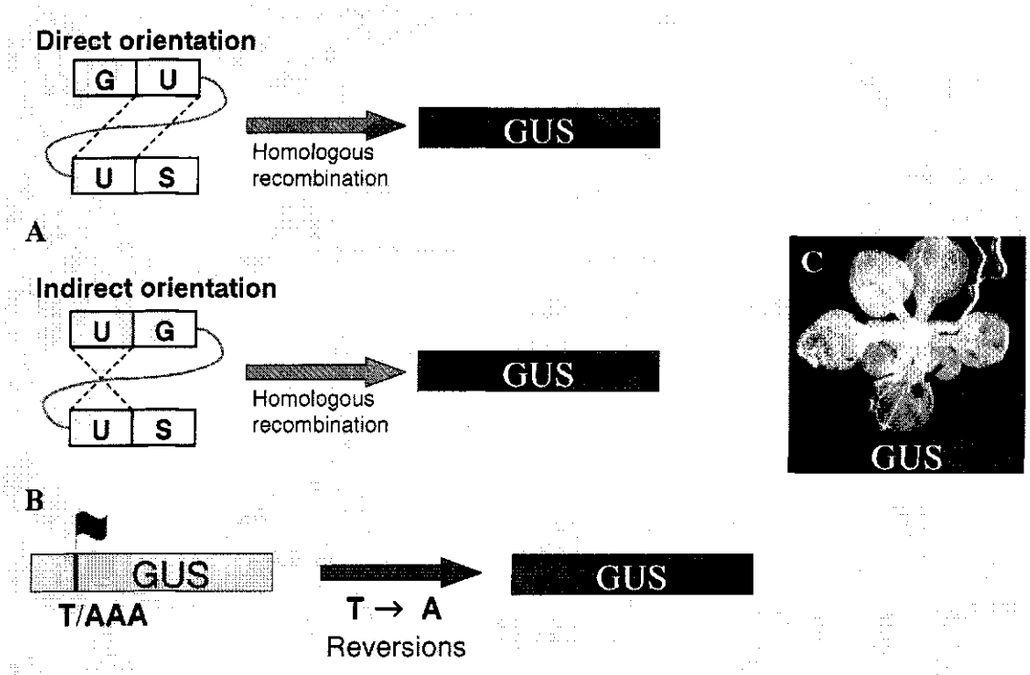

Figure 3.1 Detection of recombination and point mutation events in transgenic *A. thaliana* plants

A – the structure of the GUS-based reporter construct cloned in the direct or indirect orientation that served as the substrate for somatic HR; ***B*** *– structure of the stop-codon inactivated GUS-based reporter construct used for detection of PM events in the site of nonsense mutation (red flag indicates nonsense mutation in position 166);* ***C*** *– detection of recombination events using histochemical staining (arrow indicates recombination event (blue spot) in leaf tissues)*

3.3. Main experimental procedures

3.3.1. DNA extraction

Tissue from four 3 week-old *A. thaliana* plants were snap frozen, ground, homogenized in 400 µL of extraction buffer (200 mM Tris-HCl pH 5.0; 250 mM NaCl; 25 mM EDTA; 0.5% SDS), transferred to a 1.5 mL Eppendorf tube and vortexed. After addition of 6 µL of 2-mercaptoethanol, the samples were vortexed and kept at 65°C for 30-45 min with occasional vortexing. After 5 min centrifugation at 1100 g the supernatant was transferred to new tubes. An equal volume of phenol was added and tubes were mixed vigorously for 20 to 30 seconds. The aqueous (upper phase) was transfered to new tubes after centrifugation at 16100 g for 10 minutes. An equal volume of chloroform was added, the contents were mixed well and tubes were centrifuged at 16100 g for 10 min. The upper aqueous phase was transfered to new Eppendorf tubes and RNAse was added to a final concentration of 20 µg/mL. Samples were incubated 60 min at 37°C then a 1/10 volume of 3 M sodium acetate, pH 5.0, and 1 volume of cold isopropanol were added. The mixture was incubated 30 min at –20°C and centrifuged at 16100 g for 15 minutes. Pellets were washed with 1 mL of cold, 70% ethanol and centrifuged at 16100 g for 10 minutes, dried and resuspended in sterile distilled water.

3.3.2. DNA and RNA quantification

Aliquots (10 µl) of each sample were diluted to 200 µl with 10 mM Tris-HCl pH 5.0 and quantified using UV/Visible Spectrophotometer Ultrospec 1100 Pro (Biochrom Ltd, Cambridge, UK) at wavelength 260 nm.

3.3.3. Evaluation of transgene activity

Total RNA was prepared using Trizol reagent (Invitrogen). After quantification, 5 μg of RNA was taken for cDNA preparation (Revertaid H-Minus First Strand cDNA synthesis kit, Fermentas). The transgene activity was detected by amplification of a 150 bp N-terminal region of the *uidA* transgene. The following primers were used for amplification: forward – 5' CAGACTCAGACTAAGCAGGTG 3', reverse – 5' GATCAATTCCACAGTTTTCGCG 3'. *Real time* PCR was performed in a total volume of 25 μL using 1 μL of the 1st strand cDNA synthesis mixture as a template, 300 nM forward primer, 300 nM reverse primer and 1.25 μL of 5x SYBRGreen and 1.5 units of *Taq* polymerase (Applied Biosystems). Duplicate reactions were carried out with 1:3 and 1:15 dilutions of the 1st strand cDNA synthesis mixture. A SmartCycler (Cepheid, Sunnyvale, CA) was used to perform the PCR cycles and fluorescence was quantified against standards. The cDNA was amplified under the following conditions: (i) 95°C for 150 s for one cycle; (ii) 94°C for 30s, 66°C for 30s for 35 cycles. The standards for gene expression were amplified from the cDNA of following dilutions: 1μL, 1:4, 1:20, 1:100. Equal loading of each amplified sample was determined by the control AtActin-1 PCR product (forward primer: 5' TGGACAAGTCATAACCATCGGAGC 3'; reverse primer: 5' TGTGAACAATCGATGGACCTGAC 3').

3.3.4. RuBP activity assay

To assess the metabolic activity of leaf tissues the activity of the most abundant photosynthetic enzyme RuBP, was evaluated using the following procedure. Fifty to 100 mg of plant tissue (5-6 plants) were weighed and frozen in liquid nitrogen. Mortars and pestles cooled to a –20°C were used to grind leaf tissue with 30 mg of polyvinyl polypyrrolidone, 200 mg of sand, and 4 mL of extraction buffer consisting of 100 mM Tris-HCl (pH 8.0), 1 mM EDTA-NaOH (pH 7.0), 5 mM $MgCl_2$, 5 mM DDT, 0.2% (w/v) BSA. For total activity, the $MgCl_2$ and $NaHCO_3$ concentrations were brought to 30 mM and 10 mM, respectively. The homogenate was filtered through 1 layer of Miracloth (Calbiochem, Rose Scientific Ltd.) into flasks embedded in ice and subsequently spun at 10,000 g for 2 min at 1°C. The extract was stored on ice until used. The assay solution was warmed to 30°C and consisted of 50 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid (HEPES) – KOH, 10 mM $NaHCO_3$, 0.2 mM NADH, 2.5 mM ATP, 10 mM KCl, 1 mM EDTA-NaOH (pH 7.0), 20 mM $MgCl_2$, 5 mM DDT, 5 mM phosphocreatine, 6 units/mL of assay solution glycerate phosphokinase, 6 units/mL of assay solution GAPDH and 20 units/mL of assay solution creatine phosphokinase. Activity was determined by mixing 20 μL of extract with 920 μL assay solution. 60 μL of a 10 mM RuBP (final concentration 0.6 mM) was added to start the reaction. The solution was vortexed and the absorbance read at 340 nm after 1min had lapsed.

3.3.5. Measurement of peroxide content

To analyze free radical activity in plant tissues the aqueous and organic form of H_2O_2 was measured using the following procedure. Plant tissue was ground thoroughly in liquid nitrogen and 10 mL of 2:1 chloroform:methanol solution was added. The extract was shaken in the fume hood for 1 hour and then transferred to 50 mL conical tubes and centrifuged at 9300 g for 5 min. The supernatant was filtered through a cotton-stuffed Pasteur pipette into 50 mL conical tubes. Two mL of a 0.9% NaCl solution was added to the filtrate and mixed well. The content was centrifuged again at 9300 g for 5 minutes. Evaporation of the top (aqueous) and bottom (organic) phases was accomplished in two rotovap vials. Dried extracts were re-dissolved in 6 mL of 90% methanol for the organic half of the extract, and in 1 mL of water for the aqueous half of the extract. Peroxide content was determined using the PeroxiDetect KIT (PD-1, Sigma) according to the manufacturers protocol.

3.3.6. Global methylation assay

A 1 μg aliquot of *A. thaliana* DNA was digested with 10 units of methylation-sensitive *Hpa* II restriction endonuclease (Fermentas; Burlington, ON, Canada) and 1x enzyme buffer in total volume of 20 μl overnight at 37°C. This enzyme leaves a 5' G overhang at the cleavage site (C↓CGG), which provides an opportunity for radiolabeled nucleotide incorporation; i.e., specifically [^{3}H]-dCPT (PerkinElmer; Boston, MA, USA). Following digestion, 10 μl of each sample underwent a cytosine extension assay (Pogribny, *et al.*, 1999) by incubation in a total volume of 25 μl containing 1.5 mM $MgCl_2$, 0.5 units of *Tag* polymerase (Fisher Scientific, Mississauga, ON, Canada), and 0.1 μl of [^{3}H]-dCPT at 56°C for 1 hour. Following extension, the 25 μl reaction volume of each sample was applied to 25 mm DE-81 ion-exchanging filter paper (Whatman), washed with 500 mM sodium phosphate buffer (pH 7.0) for 10 min, repeated three times. Subsequently, filters were thoroughly dried and transferred to a vial containing 5 mL of scintillation cocktail. Radiation levels (^{3}H decays per minute (DPM) were detected in a scintillation counter (Beckman LS 5000CE; Fullerton, CA, Canada). As a control, each sample also underwent the initial incubation in the absence of *Hpa* II enzyme and was subjected to identical extension condition.

3.3.7. ROPS assay

Quantification of 3'OH DNA breaks was performed using the random oligonucleotide primed synthesis (ROPS) assay (Basnakian and James, 1996). It provides a high level of sensitivity not attainable with other available methods.

The assay is based on the ability of the *Klenow fragment* polymerase (New England Biolabs) to initiate random oligonucleotide-primed synthesis from the re-annealed 3'OH ends of single-stranded (ss)DNA. After a denaturation-reassociation step, the ssDNA serves as its own primer by randomly re-associating itself or to other ssDNA molecules. Under strictly defined reaction conditions the incorporation of [^{3}H]-dCPT into newly synthesized DNA will be proportional to the initial number of 3'OH ends (breaks). A 1 μg aliquot of plant DNA just prior to ROPS reaction was denatured at 100°C for 5 min and then cooled on ice. The reaction mixture for one sample contained: 1 μg heat denatured DNA, 2.5 μl 0.5 mM 3dNTPs (dGTP, dATP and dTTP mix), 2.5 μl 10x *Klenow fragment* buffer (New England Biolabs), 0.45 μl 33 μM dCTP, 5 units *Klenow* enzyme (New England Biolabs), 0.5 μl [^{3}H]-dCPT. Reaction volume was adjusted to 25 μl with distilled sterile water. After incubation at 25°C for 30 min, the reaction was stopped by the addition of an equal volume of 25 mM EDTA pH 8.0. Following this, the 50 μl reaction volume of each sample was aliquoted to three 25 mm DE-81 ion-exchanging filter paper (Whatman). Washing and detection procedure was performed as described above for the *Global methylation assay*.

3.4. Developmental dynamics of HR

Genome stability during plant development was analyzed using *A. thaliana* line 11 plants. HR estimated in nine groups of plants, which were germinated on soil and sampled for histochemical staining at 2, 3, 5, 7, 10, 13, 16, 19 and 22 days post germination (dpg). Total DNA of the respective transgenic lines was isolated from whole plants at different developmental stage (60 plants of ages of 2, 3, and 5 dpg, and 4 to 20 plants of 7, 10, 13, 16, 19 and 22 dpg were used). Additionally, RRs were correlated with transgene and metabolic activity at different developmental stages.

Additionally, estimation of the number of DSBs present in plants was performed at aforementioned time-points using ROPS assay. Total DNA for ROPS assay was isolated from leaf tissues at different developmental stage (60 plants of ages of 2, 3, and 5 dpg, and 4 to 20 plants of 7, 10, 13, 16, 19 and 22 dpg were used). Obtained results were recalculated to get number of DSBs present per single haploid genome for each respective experimental group.

3.5. HR in plants grown under different temperature and light conditions

To analyze genome stability of *A. thaliana* plants exposed to different temperature and light regimes we used homozygous transgenic lines 651 and 11. Plants were germinated on soil at 22°C and at 2 dpg were moved to grow under 4°C, 22°C and 32°C. Separate groups of plants were grown under consistent 22°C temperature but different day/night regimes: 8/16, 12/12, 16/8 and 24/0. All plants were grown under high light conditions (32.8 $\mu Em^{-2}s^{-1}$) provided by Octron T8 Fluorescent bulbs (Sylvania). Sampling for histochemical staining, DNA and RNA preparation was done at the age of 19-21 days. Additionally, assessment of transgene activity and analysis of metabolic and radical activity were performed.

3.6. The influence of salt stress on HR and genomic stability in plants

3.6.1. NaCl concentration gradient

Plants of *A. thaliana* line 11 were planted on the modified BM (Table 3.2). BM was supplemented with 50 mM, 75 mM and 100 mM of NaCl (final concentration). Standard, unmodified BM was used as the control. Plants were harvested at 22^{nd} dpg for histochemical staining and DNA preparation.

3.6.2. Contribution of Na^+ or Cl^- ions

Seeds of *A. thaliana* line 11 plants were sown on modified BM medium (Table 3.3). Standard, unmodified BM was used as the control. To deliver Na^+ and Cl^- ions separately Na_2SO_4 and $MgCl_2$, correspondingly in final concentration of 25 mM were used. Experimental medium was supplemented with either Na^+ or Cl^- to a final concentration of 50 mM. To assess the possible influence of Mg^{2+} and SO_4^{2-} ions delivered to the media along with the Na^+ and Cl^- ions we established a $MgSO_4$ treated control group. Within this group, plants were grown on medium supplemented with 25 mM of $MgSO_4$. Plants were harvested on 22^{nd} dpg and used for histochemical staining procedure and DNA preparation.

Table 3.2 Chemical composition of experimental basic medium used for analysis of salt stress influence on homologous recombination

			NaCl gradient all final concentrations listed in mM			
MS macro standard components	**Final conc. in media, mM**	**MS macro modified components**	**BM control**	**NaCl 50**	**NaCl 75**	**NaCl 100**
NH_4NO_3	20.6	NH_4NO_3	20.6	20.6	20.6	20.6
KNO_3	18.8	KNO_3	18.8	18.8	18.8	18.8
$CaCl_2$	3	$CaCl_2$	3	3	3	3
$MgSO_4$	1.5	$MgSO_4$	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	KH_2PO_4	1.25	1.25	1.25	1.25
		NaCl	**0**	**50**	**75**	**100**

Modified or newly added MS macro components shown in bold; NaCl was used to simulate salt stress conditions

Table 3.3 Chemical composition of experimental basic medium used to separately deliver Na^+ and Cl^- ions to the medium

			Na^+ Cl^- separate gradient all final concentrations listed in mM			
MS macro standard components	**Final conc. in media, mM**	**MS macro modified components**	**BM control**	**Cl^- 50**	**Na^+ 50**	**$MgSO_4$ 25**
NH_4NO_3	20.6	NH_4NO_3	20.6	20.6	20.6	20.6
KNO_3	18.8	KNO_3	18.8	18.8	18.8	18.8
$CaCl_2$	3	$CaCl_2$	3	3	3	3
$MgSO_4$	1.5	$MgSO_4$	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	KH_2PO_4	1.25	1.25	1.25	1.25
		Na_2SO_4	0	0	**25**	0
		$MgCl_2$	0	**25**	0	0
		$MgSO_4$	0	0	0	**25**

Modified or newly added MS macro components shown in bold; NaCl was used to simulate salt stress conditions. To deliver Na^+ or Cl^- ions separately, Na_2SO_4 and $MgCl_2$ were used; to assess the possible influence of Mg^{2+} and SO_4^{2-} ions, $MgSO_4$ treated control was established.

3.6.3. KCl gradient

To determine the mechanism of influence of Cl^- ions on activation of HR components or damaging effect to DNA we substituted Na^+ with K^+ ions (Table 3.4). To deliver Cl^- ions in media we applied KCl in final concentrations of 18.8 mM (1x), 47 mM (2.5x) and 94 mM (5x). Note: 1x stands for the concentration of K^+ ions in standard BM. Two different transgenic lines were used. One carried the recombination substrate (*A. thaliana* line 11) and another – PM substrate (*A. thaliana* line 699). Line 699 allowed the measurement of different types of DNA damage and was used to check whether Cl^- damaged DNA directly. Plants of two lines were harvested on the 22nd dpg and somatic RRs or PMRs were calculated.

3.6.4. Genome stability of progeny of NaCl treated plants

To analyze the inherant of changes in HRF plants of *A. thaliana* line 11 were exposed to 25 and 75 mM NaCl and HR was checked in their progeny grown on basic medium. Progeny of plants grown on BM with no NaCl added were used as a control. Parental plants were grown on modified medium (Table 3.5) for 22 days and then were transplanted to the soil for propagation. Obtained seeds were planted on BM (note, no NaCl was added) and harvested for histochemical staining and DNA preparation on 22nd dpg. Additionally, global methylation status of the progeny of salt treated and untreated plants was assessed.

Table 3.4 Chemical composition of experimental basic medium used for determination of the mechanism of influence of Cl^- ions on the induction of homologous recombination

			Cl^- gradient all final concentrations listed in mM			
MS macro standard components	**Final conc. in media, mM**	**MS macro modified components**	**BM control**	**KCl 1x**	**KCl 2.5**	**KCl 5x**
NH_4NO_3	20.6	**NH_4NO_3**	**20.6**	**41.2**	**41.2**	**41.2**
KNO_3	18.8	**KNO_3**	**18.8**	**0**	**0**	**0**
$CaCl_2$	3	$CaCl_2$	3	3	3	3
$MgSO_4$	1.5	$MgSO_4$	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	**KH_2PO_4**	**1.25**	**0**	**0**	**0**
		$NH_4H_2PO_4$	**0**	**1.25**	**1.25**	**1.25**
		KCl	**0**	**18.8**	**47**	**94**

Modified or newly added MS macro components are shown in bold; KNO_3 was substituted with KCl to deliver Cl^- ions; additionally, KH_2PO_4 was substituted with $NH_4H_2PO_4$ in the same final concentration to provide only one source of K^+ ions in medium. To compensate withdrawal of NO_3^- with KNO_3 the concentration of NH_4NO_3 applied to the experimental medium was increased from 20.6 to 41.2 mM (1.5x).

Table 3.5 Chemical composition of experimental basic medium used for growing plants under salt stress condition for further analysis of genomic stability of their progeny

			NaCl gradient all final concentrations listed in mM		
MS macro standard components	**Final conc. in media, mM**	**MS macro modified components**	**BM control**	**NaCl 25**	**NaCl 75**
NH_4NO_3	**20.6**	NH_4NO_3	20.6	20.6	20.6
KNO_3	**18.8**	KNO_3	18.8	18.8	18.8
$CaCl_2$	**3**	$CaCl_2$	3	3	3
$MgSO_4$	**1.5**	$MgSO_4$	1.5	1.5	1.5
KH_2PO_4	**1.25**	KH_2PO_4	1.25	1.25	1.25
		NaCl	**0**	**25**	**75**

Modified or newly added MS macro components shown in bold; NaCl was used to simulate salt stress conditions

3.7. Influence of water-mediated stress on HR

The drought/overwatering stresses influences on the genomic stability of *A. thaliana* line 11 plants were analyzed. Seeds were sown on soil mixture that contained 2/3 *"All purpose potting soil"* (Plants *Etc.*) and 1/3 *"Metro-mix 2900 growth media"* (W.R. Grase and Co of Canada Ltd). The final density of the growth mixture was 0.29 g/cm^3, while individual densities were 0.18 and 0.49 g/cm^3 for "growth media" and "potting soil" respectively. Additionally, 750 ml of water was added per each kg of soil mixture. Seeds were incubated at +4°C for 48 hours to break dormancy and then moved for germination and growth to a Conviron growth chamber (Winnipeg, MB, Canada). The experiment was started on the 5^{th} dpg. Five experimental groups were designed:

1. Control (normal watering, moisture was maintained on a certain level; ~30%)
2. Drought (no water was allowed after a certain dpg; soil dries by itself)
3. Semi-drought (moisture was maintained on a certain level lower than control; ~25%)
4. Overwatering (moisture was increased during first 6 days until the complete saturation of soil is reached. After this point the complete saturation of soil (~80%) was maintained for all duration of the experiment)
5. Drastic overwatering (moisture was increased drastically during first couple hours, until the complete saturation of soil (~80%) is reached. After this point the complete saturation of soil was maintained for the duration of the experiment)

Readings of soil water pressure for all duration of experiment were taken using Soil Moisture Logger THLog Type ML2x (Dynamax, Houston, Texas, USA). The HRF was analyzed at 0, 4, 8, 12 and 16^{th} day after experiment started. Additionally, at the same time points, tissues were harvested for subsequent DNA extraction.

3.8. Statistical analysis

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 Excel (Microsoft).

4. RESULTS

4.1. Developmental dynamics of HR

4.1.1. RRs at different stages of development

To reveal information about the dynamic of HR events during plant development of *A. thaliana* line 11, HRF was analyzed at nine different time-points: 2, 3, 5, 7, 10, 13, 16, 19 and 22 dpg. Our results coupled with those previously reported showed that DNA content in leaves increases linearly until 20 – 25 dpg (Draper and Hays, 2000). Therefore, it was not surprising that HRF was increased by a factor of 28.0 from 0.09 per plant at the age of 2 dpg to 2.52 by the age of 22 dpg (Table 4.1). However, the calculation of number of genomes present showed that there was as much as 185.7-fold ($7.2x10^7$ versus $3.9x10^5$) increase from 2 to 22 dpg. The RR was calculated by relating the HRF to the number of genomes present. The obtained results were surprising. Although the number of HR events from day 2 to 22 increased (28-fold), the RR dropped significantly and by 22 dpg consisted of 15% of RR at 2 dpg. Only one transient increase in RR was observed from day 2 to 3 by a factor of about 1.5. Started from day 3 on, RR began to decline and two major drops in RR were detected, from 3.4E-07 to 2.0E-07 between day 3 and 5; and from 2.0E-07 to 6.6E-08 between day 5 and 7 (Table 4.1). After day 7 RR remained unchanged. Previously performed evaluations of ploidy levels in *A. thaliana* revealed that most cell divisions are finished by the age of 7-8 days; ploidy level changed slightly until day 6-7 and increased significantly from day 7 on. Interestingly, the number of genomes present per plant increased 24.7-fold from day 2 (3.9E+05) until day 7 (9.5E+06) then increased only by 7.5-fold between days 7 (9.5E+06) and 22 (7.2E+07) (Table 4.1).

Table 4.1 Recombination frequency and recombination rate at different developmental stages

Plant age	HRF	Fold	Genomes per plant	RR	Fold	*uidA*/actin
2 days	0.09±0.03	1.0	3.9E+05	2.3E-07	1.00	0.7
3 days	0.15±0.04	1.7	4.4E+05	3.4E-07	1.46	1.6
5 days	0.36±0.06	4.0	1.9E+06	2.0E-07	0.83	4.4
7 days	0.63±0.08	7.0	9.5E+06	6.6E-08	0.28	15.7
10 days	1.11±0.20	12.3	2.4E+07	4.7E-08	0.20	21.2
13 days	1.05±0.25	11.7	1.9E+07	5.6E-08	0.24	28.3
16 days	1.95±0.33	21.7	3.2E+07	6.2E-08	0.26	25.8
19 days	2.70±0.55	30.0	4.3E+07	6.3E-08	0.27	32.1
22 days	2.52±0.42	28.0	7.2E+07	3.5E-08	0.15	29.4

HRF – recombination frequency (with SD) per plant as calculated in 200 plants per group; "Fold" stands for the ratio of HRF or RR at a certain plant age to HRF or RR at 2 dpg. "uidA/actin" shows the activity of the transgene standardized to the activity of actin.

4.1.2. Transgene and metabolic activity at different developmental stages

To analyze whether the difference of RR is not due to extremely low expression of the transgene at later developmental stages we analyzed transcription by *Real time* PCR. The *uidA* transgene activity rose exponentially until day 22. On 22nd day it was 42.0-fold higher than on day 2nd (Table 4.1). This showed the drop in RR was not due to the lower transgene activity. In fact, it is possible that RR at early developmental stages is even underestimated.

By correlating of RR with metabolic activity of plants at various developmental stages, RuBP activity (as calculated per single plant) was found to be similar at all stages except in plants harvested at 2 dpg and 3 dpg (as 50% lower) (Table 4.2). RuBP activity calculated per mg of tissue dropped 20-fold from 2 to 19 dpg (Table 4.2).

Our data showed 6-fold drop of RR from 2 to 22 dpg. We propose the possible explanation for this phenomenon, which is based on the possible harmful effect of highly active HR pathway in polyploid plant cells possessing a great amount of homologous and repetitive DNA that can result in error-prone repair mechanism.

Table 4.2. Ribulose biphosphate carboxylase/oxygenase activity in plants at different developmental stages

Plant age	Activity	Weight	N of plants	Act/mg	Act/plant
2 days	0.54±0.12	27.2±5.2	100	0.020	5.4E-03
3 days	0.75±0.14	32.6±6.1	100	0.023	7.5E-03
5 days	1.18±0.22	45.9±8.5	100	0.026	1.2E-02
7 days	0.95±0.21	49.4±7.6	75	0.019	1.3E-02
10 days	0.55±0.17	57.1±11.1	50	0.010	1.1E-02
13 days	0.54±0.23	78.0±12.2	50	0.007	1.0E-02
16 days	0.23±0.02	59.6±9.4	25	0.004	0.9E-02
19 days	0.28±0.03	93.2±15.2	25	0.003	1.1E-02
22 days	0.12±0.01	87.4±18.7	15	0.001	0.8E-02

Plant age reflects dpg. "Activity" – units of activity of RuBP enzyme as measured in 15-100 plants (the average of 3 measurements with SE). "Weight" shows the total weight (with SE) of all plant tissue. "N of plants" shows number of plants taken for analysis, depending on the plant age. "Act/mg" shows activity of RuBP per mg of analyzed tissue. "Act/plant" stands for the activity of RuBP per single plant.

4.1.3. Frequency of DSBs at different developmental stages

HR is an essential DSB repair mechanism. The drop in HR rate at later developmental stages could also mean that there are significantly less breaks per single genome of older plants.

DSBs frequency was analyzed at different dpg in leaf tissues. DSB frequency was assessed at eight different time points: 3, 5, 7, 10, 13, 16, 19 and 22 dpg. Importantly, only leaf tissues were used for DNA extraction and following ROPS assay. The level of DSBs increased significantly from day 3 to day 5 by 1.8-fold (Table 4.3). At the other time points the level of DSBs dropped slightly but still remained higher than at day 3. Overall, the level of DSBs remained steady from day 10 on.

Table 4.3 Occurrence of double strand breaks in leaves at different developmental stages

Plant age	^{3}H DPM	Fold A	Fold B
3 days	290.89±69.5	1.00	1.00
5 days	516.00±19.4	1.77	1.77
7 days	385.40±43.1	1.32	0.75
10 days	478.60±65.6	1.65	1.24
13 days	305.60±29.2	1.05	0.64
16 days	354.30±35.3	1.22	1.16
19 days	404.60±62.7	1.39	1.14
22 days	311.80±47.2	1.07	0.77

1 μg of DNA was analyzed; ^{3}H DPM – decay per minute (with SE); "Fold A" stands for the ratio of ^{3}H DPM at a certain plant age to ^{3}H DPM at 3 dpg; "Fold B" stands for the ratio of ^{3}H DPM at a certain plant age to ^{3}H DPM at previous plant age.

4.2. Influence of various temperatures and light regimes on plant genomic stability

4.2.1. Temperature

To analyze the influence of temperature on the genome stability of plants, we germinated and grew on soil *A. thaliana* plants from two transgenic lines (11 and 651). Importantly, low temperature drastically affected plant size: plants grown at this temperature were approximately half of the size of plants grown at 22°C. This trend was observed for both lines. Plants grown at 32°C were also smaller in size, as compared to plants grown at 22°C, although the difference was not so drastic. The HRF in plants grown at sub-optimal (4°C and 32°C) conditions was 2-fold lower than in plants grown at optimal, 22°C conditions (Table 4.4).

Table 4.4 Rates of homologous recombination in plants grown at different temperatures

Line	HRF	Genomes per plant	RR	A	*uidA* activity	B
#11, 4°C	1.64	5.9E+06	2.8E-07	3.3	0.18	18.33
#11, 22°C	2.71	3.2E+07	8.5E-08	1.0	1.0	1.0
#11, 32°C	1.27	2.6E+06	4.9E-07	5.8	3.78	1.53
#651, 4°C	0.17	4.1E+06	4.2E-08	1.6	0.29	5.52
#651, 22°C	0.74	2.9E+07	2.6E-08	1.0	1.0	1.0
#651, 32°C	0.41	1.3E+06	3.2E-07	12.4	2.75	4.51

"A" shows the ratio of RR in plants grown at either 4℃ or 32℃ to 22℃. "uidA activity" shows the ratio of GUS activity at either 4℃ or 32℃ to 22℃. "B" represents the ratio of RR in plants grown at either 4℃ or 32℃ to plants grown at 22℃ standardized to the activity of the transgene.

4.2.2. Analysis of genome number and transgene activity in plants grown at various temperatures

The small size of plants grown under sub-optimal conditions correlated with smaller numbers of genomes present. We found that both lines grown at 4°C and 32°C had an order of magnitude fewer number of genomes at the time of histochemical staining as compared to plants grown at 22°C. To correlate the HRF with number of genomes present we related it to genome content of single plant. We found that RR was significantly higher among plants of both transgenic lines grown under sub-optimal conditions (Table 4.4). It was 3.3- and 5.8-fold higher for line 11 plants grown at 4°C and 32°C, respectively; and 1.6- and 12.4-fold higher for line 651 grown at the same sub-optimal conditions (4°C and 32°C, respectively). Finally, RR standardized to transgene activity was significantly higher (1.5 – 18.3-fold) in plants grown at sub-optimal temperatures (Table 4.4).

4.2.3. Day length

To investigate the possible influence of day length on plant genome stability we grew on soil transgenic *A. thaliana* line 11 and 651 plants. HRF was the lowest in plants grown under the lowest day length; a statistically significant difference between plants grown at 8h/16h (1.0±0.11) and plants grown at 12h/12h (2.0±0.13) was observed (*P<0.001*) (Figure 4.1). The HRF was even higher in plants grown at 16h/8h, although the difference was not statistically significant (Figure 4.1). Plants grown under full day length condition exhibited lower HRF as compared to plants grown at 16h day length

(*P<0.01*). Thus, the HRF was the highest in plants grown at optimal 16/8 (day/night) conditions.

4.2.4. Analysis of the genome number and transgene activity in plants grown at various day lengths

The previously described effect of sub-optimal growth conditions (temperature) on plant size was observed also for plants grown at sub-optimal light growth conditions. Plants of both lines grown at 8h day length were the smallest and had 5 leaves on average. At the same time, plants grown at 16h day were the largest and had 8-9 leaves on average. Finally, plants grown at 12h and 24h day had intermediate size and 7-8 leaves on average. Interestingly, we found strong positive correlation between the day length and the number of genomes per plant (*r=0.98; P<0.01*) (Table 4.5). To correlate the observed HRF with number of genomes present RR was calculated. It was found that plants grown at longest day length exhibited the lowest RR (Table 4.5). Overall, a strong negative correlation between the day length and RR standardized to transgene activity was found (*r=-0.94; P<0.01*).

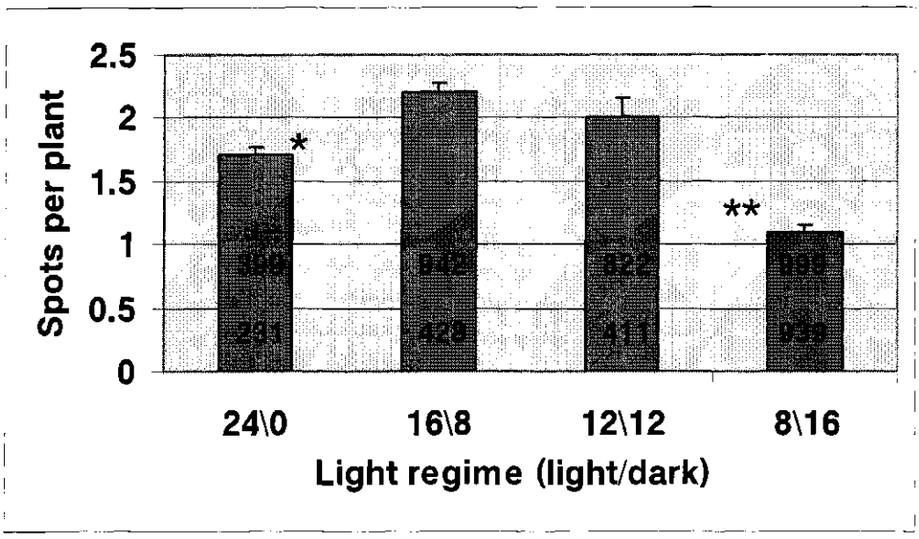

Figure 4.1 Frequency of homologous recombination in *A. thaliana* line 11 plants grown at different light/dark regimes

*Bars represent mean values ± SE. Numbers in the columns are: the upper one – total number of HR events; the lower one – number of plants screened. Statistically significant differences are shown as asterisks (** - P<0.01; *** - P<0.001; F-test, two samples variance).*

Table 4.5 Rates of homologous recombination in plants grown at different day length

Light\dark	HRF	Genomes per plant	RR	A	*uidA* activity	B
24\0	1.7	4.9E+07	3.5E-08	0.51	2.04	0.25
16\8	2.2	3.2E+07	6.9E-08	1.0	1.0	1.0
12\12	2.0	1.7E+07	1.2E-07	1.74	0.55	3.16
8\16	1.1	1.4E+07	7.9E-08	1.14	0.27	4.22

"A" shows the ratio of RR in plants grown at 24h, 12h, 8h day length to plants grown at the 16h day length condition. "uidA activity" shows the ratio of uidA activity at either 24h, 12h, 8h day length to 16h day length. "B" represents the ratio of RR in plants grown at 24h, 12h, 8h day length to plants grown at 16h day length standardized to the activity of the transgene.

4.2.5. Correlation between the HR and plant metabolic activity

One of the reasons for the difference in RR could be the difference in metabolic activity in plants exposed to various temperature and light regimes. To analyze whether the RR in plants grown at various temperature and day length regimes correlate with metabolic rate, we investigated the activity of the most abundant photosynthetic enzyme, RuBP. We found that it was much higher in plants grown at 24h and 12h day length as compared to plants grown either at 16h or 8h days (Table 4.6). At the same time, the RuBP activity was higher in plants grown at 22°C as compared to those grown at sub-optimal temperatures; the enzyme activity at 4°C was 4% and at 32°C – 0.2% of the activity at 22°C. Interestingly, comparison of RuBP activity with RR revealed the strong negative correlation ($r=-0.97$; $P<0.05$) for plants grown at different temperatures and moderate negative correlation ($r=-0.55$; $P<0.05$) for plants grown at different day length.

Table 4.6 Activity of Ribulose biphosphate carboxylase/oxygenase in plants grown under different conditions

Light\dark	Act/plant	A	RR_{st}
24\0	2.1	11.0	0.25
16\8	0.2	1.0	1.0
12\12	1.2	6.0	3.16
8\16	0.1	0.5	4.22
4°C	0.01	0.04	5.52
22°C	0.25	1.0	1.0
32°C	0.0005	0.002	4.51

"Act/plant" stands for average (per plant) RuBP activity as calculated from 6-30 plant per each treatment. "A" represents the ratio of RuBP activity at sub-optimal conditions (light - 24\0, 12\12 and 8\16; temperature - 4℃ and 32℃) to optimal conditions (light - 16\8; 22℃). "RR_{st}" stands for RRs standardized to the transgene activity.

4.2.6. Radical activity in plants grown at different temperatures and day length

Higher metabolic activity could result in the increase of production of radicals. To analyze whether RuBP activity and the rates of HR correlated with the radical production in plants, we measured aqueous and organic forms of H_2O_2. The assay revealed that plants grown at sub-optimal temperatures exhibited higher levels of both peroxide forms (Figure 4.2). This positively correlated with RRs (*r=0.74, 0.76; P<0.01*) - plants that had higher radical production exhibited higher RR.

The lowest radical production was found in plants grown at 8h days, whereas the highest radical production was in plants grown at 16h days; plants grown at 24h and 12 hours days exhibited the radical levels that were similar to the 16h/8h group (Figure 4.2). No correlation between radical concentration and RRs was found.

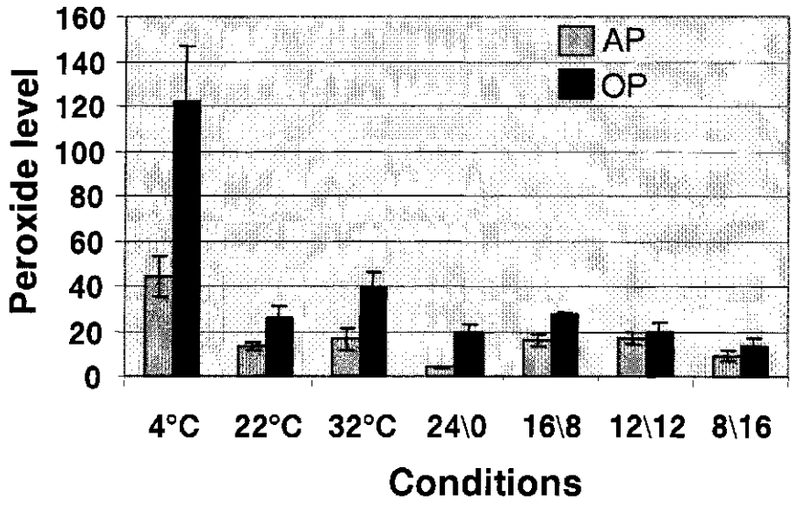

Figure 4.2 The levels of aqueous and organic peroxide produced in plants

*Bars represent mean values ± SE;n=4. Two different kinds of peroxide were measured – the aqueous solution (**AP**) reflecting actual level of H_2O_2 in the cell and "organic peroxide" (**OP**) reflecting organic compounds oxidized by peroxide.*

4.3. Salt stress and genomic stability

4.3.1. Influence of NaCl on HR in plants

Previously reported studies (Swoboda, *et al.*, 1994) and our own trials revealed that concentration of NaCl in growth medium below 100 mM did not result in negative changes in plant phenotype; plants grown in presence of 100 mM and lower of NaCl looked healthy and did not show growth inhibition (data not shown). To provide assessment of the influence of salt stress of different strength on genome stability we used *A. thaliana* line 11 plants. Plants were grown on BM medium supplemented with NaCl in final concentration of 0 mM, 50 mM, 75 mM and 100 mM, respectively. On 22 dpg all plants grown in the presence of NaCl exhibited enhanced level of both HR and RR as compared to NaCl untreated control (Table 4.7). The genome content of stress-subjected plants has not been drastically affected by the applied stress conditions and was comparable with one calculated for control group. Our study revealed that an application of 50 mM NaCl caused 2.0-fold increase of HRF and 1.64-fold increase of RR, respectively ($P<0.001$). Supplementation of growth medium with 75 mM of NaCl resulted in approximately 2.8-fold increase of HRF. Finally, presence of 100 mM of NaCl significantly up regulated the HRF and RR by factor of 3.88 and of 3.93, respectively, as compared to untreated control ($P<0.001$).

Table 4.7 Influence of NaCl mediated salt conditions of different strength on homologous recombination

	Genomes per plant, E+06	Fold	HRF	Fold	RR, E-06	Fold
Control basic media	21.455	1.00	1.61±0.2	1.00	0.075	1.00
NaCl 50mM	26.208	1.22	3.22±0.27	2.00	0.123	1.64
NaCl 75 mM	19.720	0.92	4.44±0.34	2.76	0.225	3.00
NaCl 100 mM	21.154	0.99	6.25±0.5	3.88	0.295	3.93

HRF-recombination frequency with SE; "Fold" stands for the ratio of either 'genomes per plant' or HRF or RR in a certain experimental group to either 'genomes per plant' or HRF or RR in control group n=91 (control), n=119 (NaCl 50 mM), n=148 (NaCl 75 mM), n=135 (NaCl 100 mM) P<0.05

4.3.2. Contribution of Na^+ and Cl^- ions to genomic stability

To examine whether the effect of salt stress on HR is related to the specific ion or required the presence of both, sodium and chlorine ions, we delivered them to growth BM medium separately in final concentration of 50 mM. The working concentration of 50 mM was chosen as the ideal concentration that did not have a negative effect on plants. For delivering of aforementioned ions we used Na_2SO_4 and $MgCl_2$ in final concentration of 25 mM. This created the equivalence of the presence of 50 mM of NaCl in the media. Additionally to check whether Mg^{2+} or SO_4^{2-} have any influence on the genome stability plants were grown in presence of $MgSO_4$. Our study revealed that the simultaneous presence of either Mg^{2+} and SO_4^{2-} ions or Na^+ and SO_4^{2-} ions did not result in the increase of HRF and RR. However the presence of 50 mM Cl^- ions in the growth media drastically increased the HRF by factor of 5 ($P<0.001$) as compared to BM control (Figure 4.3). To provide solid support for our data two, independent trials were performed and both revealed the same pattern of response to moderate salt stress (Figure 4.3). A strong positive correlation between these two experiments was found ($r= 0.99$ and $r=0.98$, $P<0.05$ for HRF and RR, respectively) (Figure 4.4 and 4.5).

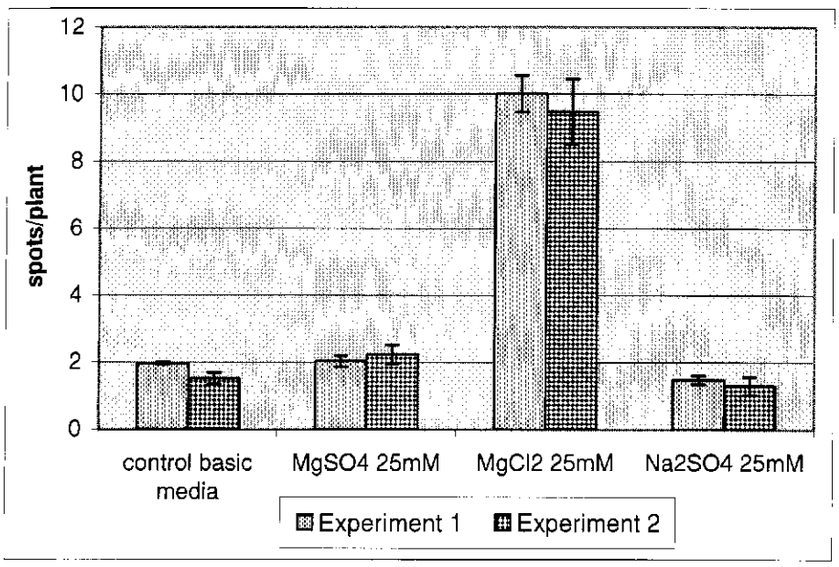

Figure 4.3 Frequency of homologous recombination in *A. thaliana* line 11 plants grown on basic medium supplemented with either Na^+ or Cl^- ions during two independent trials

Bars represent mean values ± SE; Experiment 1 (P<0.001): n=1326 (control), n= 379 ($MgSO_4$), n=288 ($MgCl_2$), n=214 (Na_2SO_4); Experiment 2: (P<0.001) n=214 (control), n= 190 ($MgSO_4$), n=155 ($MgCl_2$), n=75 (Na_2SO_4)

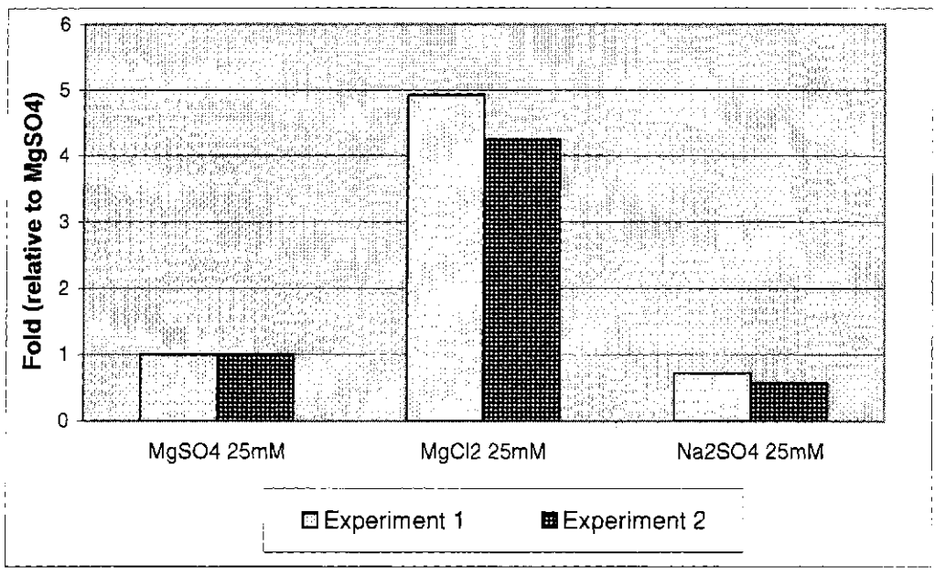

Figure 4.4 Homologous recombination frequency (related to $MgSO_4$) in *A. thaliana* line 11 plants grown on basic medium supplemented with either Na^+ or Cl^- ions during two independent trials

Experiment 1: n= 379 ($MgSO_4$), n=288 ($MgCl_2$), n=214 (Na_2SO_4); Experiment 2: n= 190 ($MgSO_4$), n=155 ($MgCl_2$), n=75 (Na_2SO_4) (r=0.99, P<0.05)

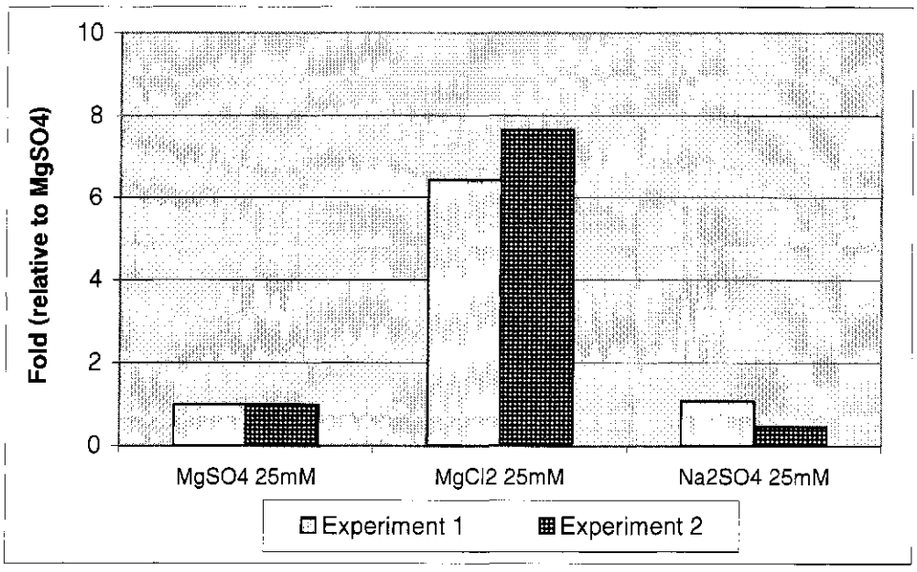

Figure 4.5 Recombination rate (related to $MgSO_4$) in *A. thaliana* line 11 plants grown on basic medium supplemented with either Na^+ or Cl^- ions during two independent trials

Experiment 1: n= 379 ($MgSO_4$), n=288 ($MgCl_2$), n=214 (Na_2SO_4); Experiment 2: n= 190 ($MgSO_4$), n=155 ($MgCl_2$), n=75 (Na_2SO_4) (r=0.98, P<0.05)

4.3.3. Mechanism of influence of Cl^- ions

The exact mechanism of the influence of Cl^- ions on HRF and RR in plants remains unclear. We attempted to determine the mechanism of the observed HRF and RR increase. It was possible that Cl^- ions were either directly damaging DNA or stimulating the sensitivity of HR repair enzymes. Plants of *A. thaliana* line 11 and 699 were grown for 22 days on BM medium supplemented with different concentrations of KCl. Previous experiments showed that K^+ does not increase the HRF and RR. Thus, any HRF and RR increase would be contributed to Cl^- ions. Line with recombination substrate (*A. thaliana* line 11) showed the increase of RR with application of KCl to the growth media as compared to plants grown on basic media without KCl ($P<0.001$ for *A. thaliana* line 11) (Figure 4.6). Application of 18.8 and 47 mM of KCl to growth medium resulted in 9.2-fold and 15-fold induction of RR as compared to KCl untreated control (Figure 4.6). At the same time, we did not observe induction of PMF (Figure 4.7). Application of 18.8 mM and 47 mM of Cl^- ions even reduced the PMF by 9-fold ($P<0.05$) and 1.8-fold (statistically insignificant), respectively.

Since Cl^- ions do not increase the PMF, it is possible that Cl^- ions increase the RR by activating the enzymes involved in HR.

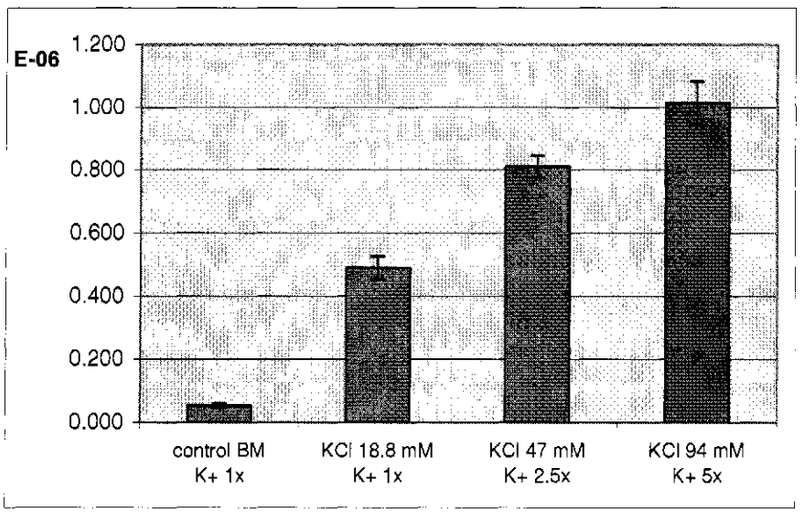

Figure 4.6 Recombination rate in *A. thaliana* line 11 plants grown on basic medium supplemented with KCl of different concentrations

Bars represent mean values ± SE; n=172 (control), n=132 (KCl 18.8 mM), n=107 (KCl 47 mM), n=144 (KCl 94 mM) (P<0.001)

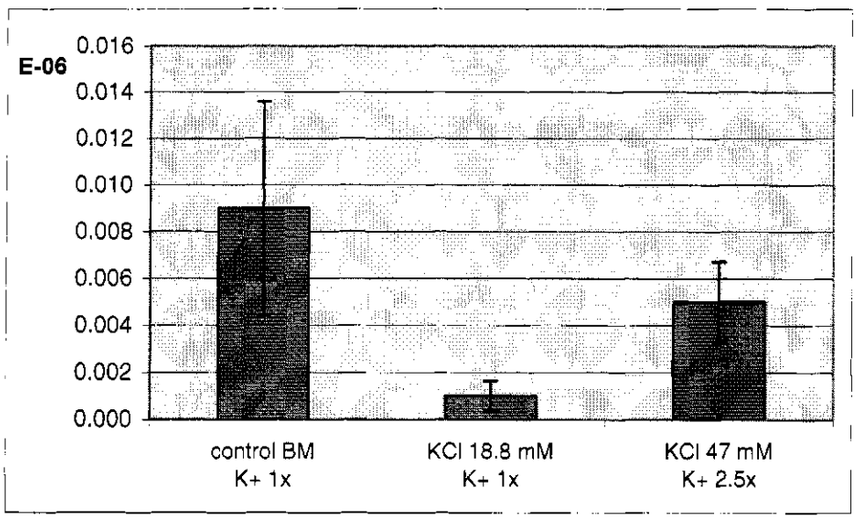

Figure 4.7 Point mutation rate in *A. thaliana* line 699 plants grown on basic medium supplemented with KCl of different concentrations

Bars represent mean values ± SE; n=42 (control), n=102 (KCl 18.8 mM), n=96 (KCl 47 mM) (for control and KCl 18.8 mM P<0.05)

4.3.4. Induction of HR in the progeny of salt treated plants

To check whether the increase of HRF can be inherited, we analyzed HRF in the progeny of *A. thaliana* line 11 plants grown on NaCl of 0 mM, 25 mM and 75 mM. The parental generation was exposed to salt for 22 days. Obtained seeds were planted on BM and harvested for analysis of HRF and RR on 22 dpg. To our surprise, the progeny of salt treated plants showed the statistically significant higher RR ($P<0.001$) (Table 4.8). The HR was higher in the progeny of plants exposed to higher NaCl concentrations; while the progeny of 25 mM treated plants exhibited 1.89-fold increase in RR, the progeny of 75 mM treated plants showed induction of RR by the factor of 2.34 ($P<0.001$). The similar pattern was observed for HRF. The progeny of all groups had comparable number of genomes per single plant (Table 4.8).

4.3.5. Global methylation changes in progeny of NaCl treated plants

To detect possible inherited epigenetic changes we assessed the global methylation status of the progeny of salt treated and untreated plants. Surprisingly, progeny of 25 and 75 mM treated plants had hypomethylated genomes as compared to the progeny of untreated plants (Table 4.9). The progeny of plants exposed to 25 mM and 75 mM of NaCl exhibited 1.35- and 1.17-fold lower methylation as compared to control (Table 4.9).

Table 4.8 Rates of homologous recombination in progeny of NaCl treated plants

Progeny of	Genomes per plant, E+06	Fold	HRF	Fold	RR, E-06	Fold
Control basic media	8.817	1.00	1.13±0.08	1.00	0.128	1.00
NaCl 25mM	8.916	1.01	2.16±0.12	1.91	0.242	1.89
NaCl 75 mM	9.708	1.10	2.90±0.13	2.57	0.299	2.34

HRF-recombination frequency with SE; "Fold" stands for the ratio between data for NaCl and control (for genomes per plant, HRF and RR), n=504 (control), n=346 (NaCl 25 mM), n=514 (NaCl 75 mM), P<0.05

Table 4.9 Global methylation analysis of progeny of NaCl treated plants

Progeny of	^{3}H DPM (corrected)	Fold
Control basic media	5834.6±751.9	1.00
NaCl 25mM	7892.0±252.4	1.35
NaCl 75 mM	6838.95±450.2	1.17

1 µg of DNA was analyzed; DNA was incubated overnight at 37 °C either in the presence of Hpa II (10un/µgDNA) or without enzyme; "^{3}H DPM (corrected)" stands for difference in counts (with SE) obtained for Hpa II digested and non-digested DNA and reflects the methylation level; "Fold" stands for the ratio between ^{3}H DPM of progeny of NaCl treated plants and untreated control.

4.4. Water-mediated stress and genomic stability

4.4.1. Influence of drought on HR

Stress was applied to 5 days old *A. thaliana* line 11 plants. The differences between treatments were successfully established (Figure 4.8). The moisture percentage remained nearly steady for 'control' group and consisted of 30%. The moisture percentage of 'drought' and 'semi-drought' group dropped gradually and resulted in 12% and 21% at the day 16, respectively. HRF and RR were analyzed at various time points: day'0' (5 dpg); day '4' (9 dpg); day '8' (13 dpg); day '12' (17 dpg); day '16' (21 dpg).

On day "0" (the 5 dpg), when the experiment was started, all groups, including controls, exhibited a similar HRFs (Figure 4.9). However on day 4, HRF for the 'drought' group dropped and consisted of 50% of the HRF observed for 'control' group. Importantly, the genome content of 'control' and 'drought' groups remained the same. The HRF and RR continued to decline and on day 16 consisted only of 8.5% and 14% of those for 'control' group, respectively (Figure 4.9 and 4.11). Importantly, the 'drought' treated plants were much smaller in size, showing growth inhibition and accumulation of visible amounts of anthocyanins. The latter ones are well-known indicators of drought conditions (Winkel-Shirley, 2002) (Figure 4.10). Importantly, 'semi-drought' treated plants did not show growth inhibition and were comparable in size with control plants. By the day 16 these plants showed only 1.4-fold lower RR as compared to respective control group. Interestingly, calculation of RR for 'control' group revealed previously reported in our study phenomenon; while number of genomes present significantly increased with age, RR drastically dropped and on day 16 after experiment started (21 dpg) consisted of 5% from those on the day 0 (5 dpg) ($P<0.05$).

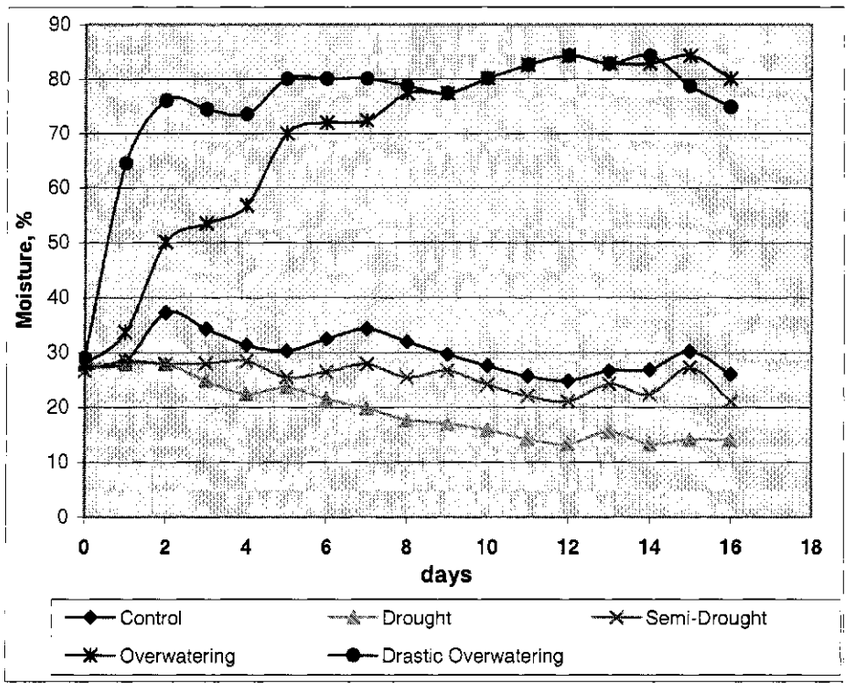

Figure 4.8 Soil moisture conditions for drought/overwatering experiment

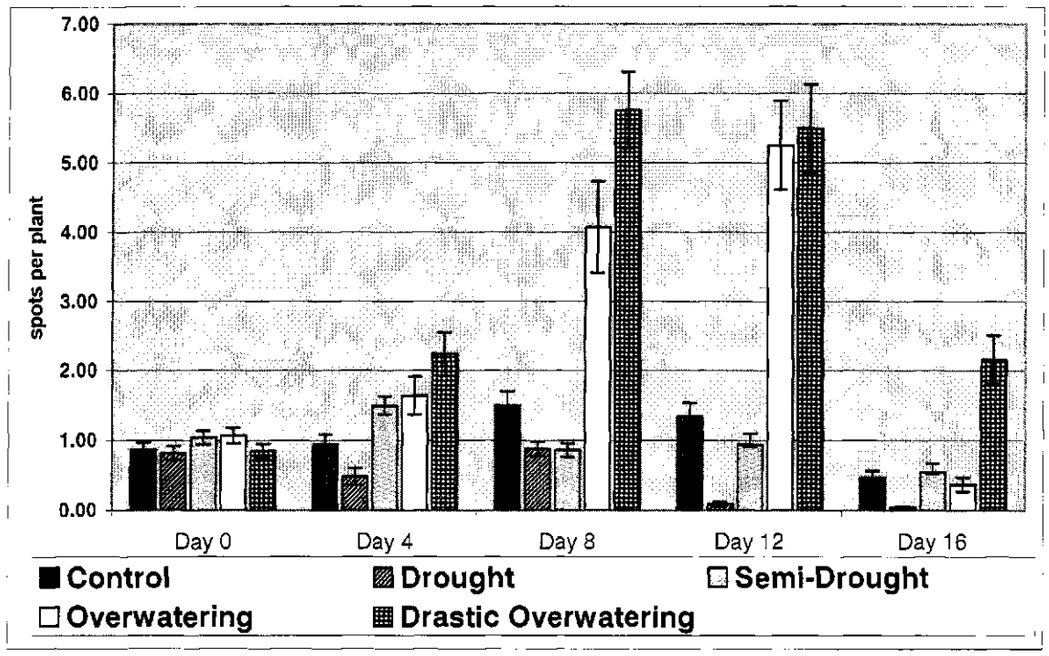

Figure 4.9 Frequency of homologous recombination in *A. thaliana* line 11 plants grown on soil under different drought/overwatering stress conditions

Bars represent mean values ± SE; for day 0: n=102 (control), n=107 (drought), n=101 (semi-drought), n=92 (overwatering) n=104 (drastic overwatering); for day 4: n= 86(control), n=88 (drought), n=81 (semi-drought), n=97 (overwatering) n=96 (drastic overwatering); for day 8: 4: n=95 (control), n=120 (drought), n=127 (semi-drought), n=91 (overwatering) n=106 (drastic overwatering); for day 12: 4: n=102 (control), n=101 (drought), n=94 (semi-drought), n=107 (overwatering) n=113 (drastic overwatering); for day 16: 4: n=119 (control), n=104 (drought), n=115 (semi-drought), n=98 (overwatering) n=98 (drastic overwatering) (for each 'day' group P<0.001)

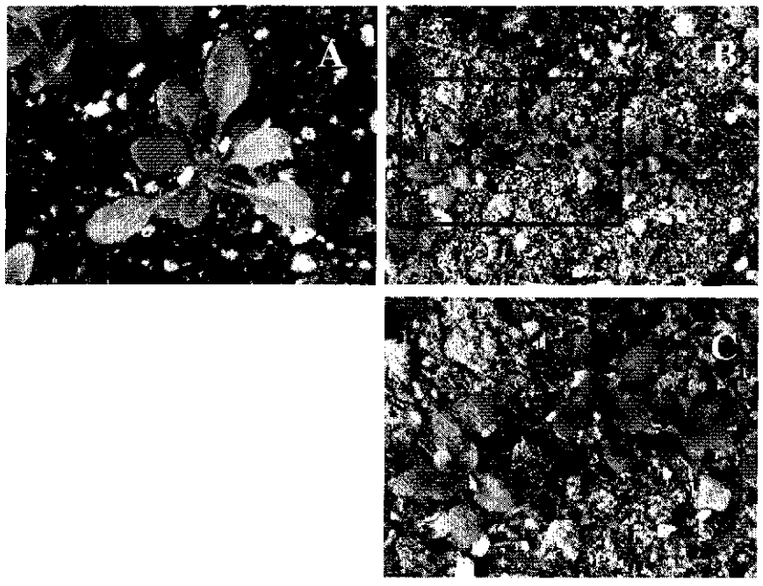

Figure 4.10 The effect of drought stress on plants

A – control plants; B – drought treated plants; C – zoom in (zoom area indicated with red frame) into B. Note: magnification of A and B is identical

4.4.2. Influence of overwatering stress on HR

To analyze the influence of overwatering stress conditions we established two experimental groups subjected to stress conditions of different intensity: 'drastic overwatering' and 'overwatering' (Figure 4.8). This allowed us to compare abrupt and gradual stress conditions. Experiment started on 5 dpg, simultaneously with drought stress; the same 'control' group (~ 30% moisture) was used for both experiments.

Two independent trials revealed the similar results. HRF and RR were significantly induced by overwatering stress ($P<0.001$ between control and drastic overwatering stress for each day analyzed). At day 4 of the experiment, the HRF and RR were induced by a factor of 1.7 and 1.8 in the 'overwatering' group and by a factor of 2.4 and of 2.2 in the 'drastic overwatering' group, respectively. Interestingly, the RR of plants exposed to drastic overwatering was higher than in plants exposed to overwatering by the factor of 1.5 at all days of the experiment, except for the day 16, when the observed difference was even more drastic, 8-fold (Figure 4.11). It is important to note that neither overwatering nor drastic overwatering conditions had any influence on plant appearance. Similarly to the previously reported experiments, there was an age-dependence for RR; RR was lower in older plants exposed to either of two stresses.

The phenomenon observed for water stress is an interesting one. Whereas the drought conditions reduced RR, the overwatering resulted in drastic increase of RR. We will discuss possible mechanisms of this influence, but clearly this work requires further experimentations in the future.

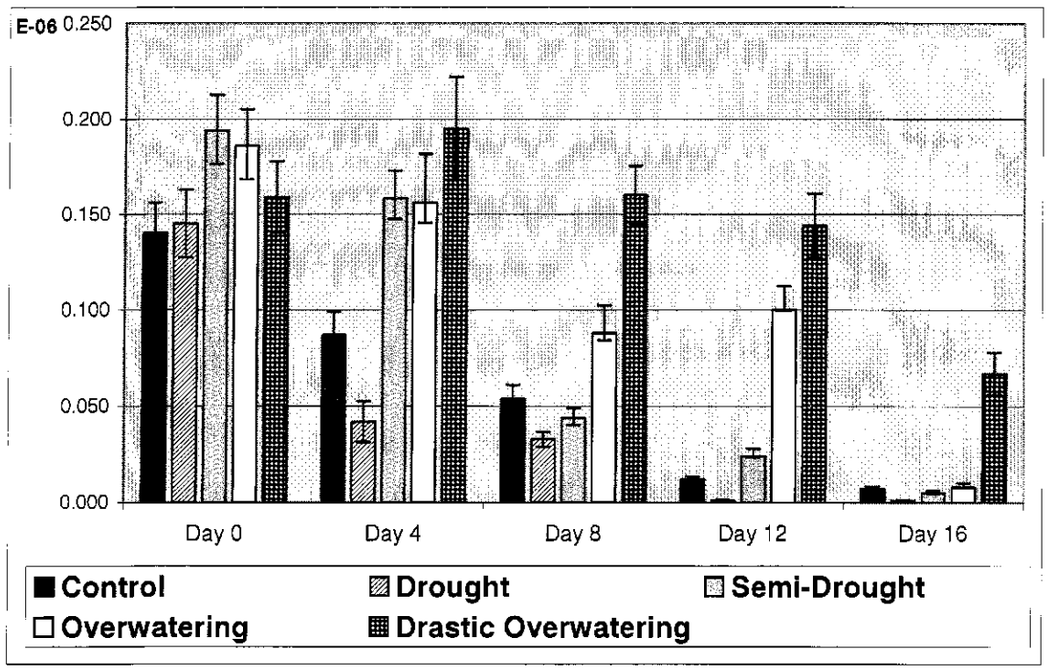

Figure 4.11 Recombination rate in *A. thaliana* line 11 plants grown on soil under different drought/overwatering stress conditions

Bars represent mean values ± SE; for day 0: n=102 (control), n=107 (drought), n=101 (semi-drought), n=92 (overwatering) n=104 (drastic overwatering); for day 4: n= 86(control), n=88 (drought), n=81 (semi-drought), n=97 (overwatering) n=96 (drastic overwatering); for day 8: 4: n=95 (control), n=120 (drought), n=127 (semi-drought), n=91 (overwatering) n=106 (drastic overwatering); for day 12: 4: n=102 (control), n=101 (drought), n=94 (semi-drought), n=107 (overwatering) n=113 (drastic overwatering); for day 16: 4: n=119 (control), n=104 (drought), n=115 (semi-drought), n=98 (overwatering) n=98 (drastic overwatering) (for each 'day' group P<0.001)

5. DISCUSSION

5.1. Developmental dynamics of HR

Despite the number of reviews available describing DNA repair in *A. thaliana* the important aspects of developmental dynamic of DSBs occurrence and their repair remain unclear. A recent review in this field (Hays, 2002) proposed that most of the HR events (95%), if occurring randomly, would occur during the last two cell divisions. Until now, the question of whether HR occurs randomly over the growth period of *A. thaliana* and whether it is directly proportional to cell division or genome replication, has not been answered. Our data show that the occurrence of HR is not random and does not directly depend on the number of genomes present in plant. It supports the previously reported data that suggested that the accuracy of DSBs repair in plants couldn't simply be correlated with the genome size or with the amount of repetitive DNA (Gorbunova and Levy, 1999).

We found HR events to be age-dependent. They were more frequent at early developmental stages and became more rare as the number of genomes present in plant increased. After certain developmental stages the occurrence of cell division decreases drastically while genome duplication may continue (Traas, *et al.*, 1998; Galbraith, *et al.*, 1991; Mizukami, 2001; Kondorosi, *et al.*, 2000; Sugimoto-Shirasu and Roberts, 2003). Endoreduplication will result in the significant increase of number of genomes per single cell providing the opportunity for enhanced cell growth and metabolism (Grime and Mowforth, 1982; Kondorosi, *et al.*, 2000; Joubes and Chevalier, 2000; Traas, *et al.*, 1998; Galbraith, *et al.*, 1991). The endoreduplication increases the number of genomes per plant and thus increases the presence of highly homologous sequences, possible HR substrates.

It is expected that the number of mutation events would double for each round of DNA duplication. This would inevitably increase the number of gene rearrangements, duplications, insertions and other types of alteration of original sequences as a result of DNA repair malfunction. Despite the low probability that cells having high ploidy level will give rise to a new generation, the increase in genome alterations is unfavorable and perhaps even harmful. Therefore we could expect plant cells to possess a specialized mechanism for controlling HR in leaf epidermal cells, which are known to experience a high frequency of endoreduplication (Traas, *et al.*, 1998; Melaragno, *et al.*, 1993). The previously asked question – whether the genome fidelity functions as efficiently during endoreduplication as during DNA replication in dividing cells is now answered. Nevertheless it remains unclear whether the NHEJ is able to compensate a decrease in HR rate during plant development.

Another interesting phenomenon observed during our studies was the age-dependent increase of *uidA* activity. There is not a clear explanation for the increased activity of 35S promoter driving *uidA* gene. A similar situation was recently described for 35S promoter driving green fluorescent protein (GFP) reporter gene (Sunilkumar, *et al.*, 2002). It has been shown to be both developmentally and tissue-specifically regulated. The expression of GFP was increased from barely detectable levels in the cotton embryo to strong expression level in mature tissues. This increase was occurring progressively throughout plant growth (Sunilkumar, *et al.*, 2002). However further studies with other transgenic lines are required to understand this phenomenon. Besides, the development of transgenic lines with different recombination substrate driven by other than 35S promoter seems to be promising for analysis of it's activity at different developmental stages.

Significantly higher activity of 35S promoter at later developmental stages probably masks even higher differences in RR, as the frequency of the occurrence of HR events at early stages was probably underestimated.

To correlate HRF with the abundance of DSBs in leaves we analyzed their persistence through the ontogenesis of plant. Using the ROPS assay we detected an increased number of DSBs at the time of the intensive cell division (from 3 to 5 dpg; 1.77-fold increase). The level of strand breaks slightly decreased from day 5 on and from day 10 remained constant (Table 4.3). Noting that DSBs occurred with the same frequency throughout plant development was very important. It suggested existence of a tight age-dependent regulation of HR and raises the question whether the other mechanisms such as NHEJ are able to compensate a for the decrease in HR rate in leaf cells during plant development.

5.2. Influence of temperature and light regimes on plant genomic stability

In this study we tried to extend our previous work and work of others on the understanding how various environmental and physiological conditions influence plant genome stability (Ries, *et al.*, 2000a; 2000b; Puchta, et. al., 1995; Kovalchuk, *et al.*, 1998; 2003b).

We analyzed genome stability of *A. thaliana* plants grown at different temperatures and light regimes. We found that plants grown at sub-optimal temperatures (either 4°C or 32°C) had a significantly higher rate of HR as compared to plants grown at 22°C (Table 4.4). Plants grown at 4°C or 32°C were smaller in size and contained proportionally less DNA (Table 4.4). One possible explanation is the reduced level of endoreduplication at non-physiological temperatures. It is known that endoreduplication can be used to provide plant cells with a higher capacity to increase volumes and number of essential metabolic organelles such as mithochondria and chloroplasts (Galbraith, *et al.*, 1991; Mizukami, 2001; Traas, *et al.*, 1998; Kondorosi, *et al.*, 2000; Sugimoto-Shirasu and Roberts, 2003). We can speculate that non-physiological temperatures affected metabolism and reduced the endoreduplication level leading to plant growth inhibition. However we have not found information determining if the process of endoreduplication in plants could be regulated by temperature. Even similar HRFs would correspond to a different RRs in plants with different number of genomes. That is the more genomes present, the lower the RR would be in plants that have an equal number of HR events.

The fewer genomes present in plants grown at 4°C or 32°C is understandable. Plants grown at these temperatures were significantly smaller in size when compared to

plants grown at 22°C. It goes in parallel with the observation that plants grown at 8 and 12h days had fewer genomes and were smaller than plants grown at 16h days.

The spectrum of light has been reported to have an influence on ploidy level of plant and thus endoreduplication frequency (Gendreau, *et al.*, 1998). It was shown that far red light caused the arrest of the third endoreduplication cycle and resulted in the absence of 16C nuclei in wild type hypocotyls of *A. thaliana*. Unfortunately there is a lack of information regarding the influence of different light regimes on the regulation of endoreduplication in plants.

Transgene activity has been previously shown to be effected by different temperature and light growth conditions. This influence is based on the sensitivity of the 35S promoter to various environmental conditions. Schnurr and Guerra (2000) have reported that the changes in day length as well as temperature result in a change of 35S promoter activity. Similarly to our data, the promoter activity decreased in plants grown at 4°C (Schnurr and Guerra, 2000). Moreover, the activity of the 35S promoter increased in plants shifted from 16h to 8h day length (Schnurr and Guerra, 2000). In contrast, in our experiment it decreased (Schnurr and Guerra, 2000). The difference could be explained by the fact that Schnurr and Guerra (2000) first germinated and grew tobacco plants at normal 16h day length until the third leaf fully developed and then shifted plants to 8h day length for 3 days. In our experiments the day length for plants (*A. thaliana*) was determined immediately following germination and maintained for a period of 3 weeks.

Other reports have confirmed the instability of 35S promoter activity under the different stress conditions. Goddijn, *et al.* (1993) showed significant down regulation of the 35S promoter in nematode-induced feeding structures of transgenic plants.

Additionally, it has been shown that the activity of the GUS gene driven by the 35S promoter is significantly repressed by *Activator (Ac)* transposase (MacRae, 2002). Also, the 35S promoter has been demonstrated to be developmentally regulated – a gradual increase in activity of a 35S driven green fluorescence protein was observed in developing cotton embryos (Sunilkumar, *et al.*, 2002). Our results have demonstrated that the 35S promoter changes it's activity with the changes of the temperature and day length. Environmental conditions that differed from optimal (22°C and 16h day) were shown to affect activity level of this promoter. This phenomenon requires further investigation as it would be interesting to analyze whether transgene activity in plants grown at sub-optimal conditions change when they are shifted to optimal light or temperature conditions. Similar activity restoration has been confirmed in tobacco plants that carry the GUS transgene driven by the 35S promoter (Schnurr and Guerra, 2000).

We found metabolic activity (RuBP enzyme) negatively correlated with RR. RuBP enzyme activity reflects the photosynthetic rate of a plant. We found significantly higher (6 – 11-fold) RuBP activity in plants grown at 12 and 24h days as compared to plants grown at 8 or 16h days. This resulted in the negative correlation with RR since plants grown at longer day length had lower RR. The strong negative correlation was observed for plants grown at different temperatures. Metabolic activity in plants grown at either 4 or 32°C was much lower (25 – 50-fold) than in plants grown at 22°C.

The negative influence of different stresses on photosynthetic activity of plants is well documented (Pittremann and Sage, 2001; Savitch, *et al.*, 2001; Crafts-Brander and Salvucci, 2002; Bukhov, *et al.*, 1999; Labate, *et al.*, 1991). Similarly various other

stresses including the change of CO_2, ozone, water stress, and changes of light intensity were shown to inhibit the photosynthetic rate (Pittremann and Sage, 2001).

The negative correlation observed between RuBP activity and RR could have different explanations. It is possible that a high metabolic rate could be a threat to DNA integrity due to the increased production of free radicals through different cells pathways (Asada and Takahashi, 1987; Jimenez, *et al.*, 1998; Lopez-Huertas, *et al.*, 1999; Hernandez, *et al.*, 2001). This may lead to a higher level of alert for various defense mechanisms involving radical scavenging and DNA repair enzymes. We found that plants with higher RuBP activity exhibited lower levels of peroxide. This may be due to induction of production of radical scavenging enzymes by high photosynthetic activity. This could lead to enhanced antioxidant defense capacity and the lower level of DNA damage. Decreased DNA damage would result in the positive correlation between the amount of radicals produced and the RRs observed.

Our results indicate that even "moderate" stresses like growth temperature and day length are able to substantially influence plant genome stability and perhaps plant genome evolution.

5.3. Salt stress and genomic stability

5.3.1. Influence of salt stress on HR in plants

The influence of salt stress on plants is well documented on different levels (Zhu, 2000; Hasegawa, *et al.*, 2000a; 200b; Tsugane, *et al.*, 1999; Leung, *et al.*, 1994; Borsani, *et al.*, 2001). While the physiological influence of this stress is well understood the possible genome toxicity effects remains unclear. We analyzed a broad concentration range and detected the ion specificity in the influence of NaCl on both somatic and meiotic recombination frequency.

Different concentrations of sodium chloride in the growth medium were reported to have a negative influence on plants. Interestingly, various reports have suggested different impacts for the same concentrations of NaCl on *A. thaliana*. Sodium chloride (100 mM) was reported to have either no negative influence on plant phenotype (Puchta *et al.*, 1995), significant effect on plant phenotype (Tsugane, *et al.*, 1999) or to be in range of concentration that plants are able to tolerate (Zhu, 2000). We performed pilot trials that revealed *A. thaliana* line 11 plants are capable of growth for 1 month in the presence of up to 100 mM of NaCl without visible growth inhibition. Analysis of RR in plants grown on media supplemented with 100 mM of NaCl showed 3.93-fold induction (Table 4.7). This correlates with previously reported 2.5-fold increase of RR in *A. thaliana* plants in the presence of the same amount of NaCl (Puchta, *et al.*, 1995).

The possible influence of NaCl on HRF and RR could be due to the accumulation of ROS triggered by osmotic stress. The significantly elevated activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) enzymes involved in H_2O_2 scavenging was observed in wild type plants and *A. thaliana pst1* salt-tolerant mutants subjected to

the salt stress (Tsugane, *et al.*, 1999). The ability of *pst1* mutants to grow at the presence of 200 mM of NaCl, at which wild type died, was dependent on the more efficient detoxification of ROS due to the increase in activities of SOD and APX (Tsugane, *et al.*, 1999). The enhancement of ROS production might be the result of the influence of salt stress on CO_2 uptake by leaves that leads to the change of photosynthesis rate (Tsugane, *et al.*, 1999; Vranova, *et al.*, 2002). Indeed, it has been shown that salt stress sensitivity is increased in *A. thaliana* exposed to moderate light intensities (Tsugane, *et al.*, 1999).

It is possible that the main mechanism of the genome destabilization effect of NaCl is the change of the equilibrium between ROS-producing and ROS-scavenging mechanisms.

5.3.2. Contribution of Na^+ and Cl^- ions to the plant genomic instability

The application of salt stress by itself usually results in ionic and osmotic stresses by which secondary effect is oxidative stress (Zhu, 2000; Hasegawa, *et al.*, 2000b). As an acclimation to osmotic stress, plants were shown to accumulate large amounts of compatible solutes such as mannitol (Hoekstra, *et al.*, 2001), which on the other hand, were reported to have the antioxidant activity (Smirnoff and Cumbes, 1989; Orthen, *et al.*, 1994). This raises doubts that ROS plays the main and exclusive role in enhancing the HRF in plants exposed to salt stress.

To examine whether the effect of salt stress on HRF in somatic tissues is related to the specific ion or required the simultaneous presence of both, sodium and chlorine, we delivered them separately to growth media in final concentrations of 50 mM. Our data

suggest an exclusive role of Cl^- ions in induction of HRF under salt stress conditions (Figure 4.3). At the same time role of Na^+ seems to be negligible. It is especially surprising because the impact of salt stress is usually related to the metabolic toxicity of excessive amounts of Na^+ ions (Zhu, 2001; Zhu, 2000). At the same time, the role of Cl^- ions has not been established yet. We found only one report discussing the role of Cl^- ions in NaCl mediated salt stress (Niu, *et al.*, 1995) on the level of trans membrane pumps. Below, we propose the model that could explain the influence of Cl^- ions on HRF.

5.3.3. Mechanism of influence of Cl^- ions on HR

The enhancement of somatic HRF in response to the presence of excessive amounts of Cl^- ions can be explained by two major points of view. First, Cl^- can directly damage DNA thereby challenging the DNA repair machinery. Second, it can stimulate/activate HR pathways by unknown mechanism without physically damaging DNA.

Analysis of influence of Cl^- ions on HRF and on PMF showed Cl^- primarily increases HRF and decreases the PMF (Figure 4.6 and 4.7).

Our results indicate non-toxic concentration of the Cl^- ions have a positive effect on HR. Furthermore this influence is not directly associated with DNA damage, as PMF does not increase. PMs are a frequent outcome of mistakes in NHEJ. The decreased PMR observed in this experiment could be a result of Cl^- shifting DSB repair from NHEJ toward HR.

5.3.4. Genome stability of progeny of NaCl treated plants. Inheritance of enhanced HR response

Several recent reports show genome destabilization in somatic tissues could be transmitted to next generation (Kovalchuk, *et al.*, 2003b). The progeny of plants exposed either to UV or pathogen stress revealed higher HRFs at non-damaging to plant conditions (Ries, *et al.*, 2000a; 2000b; Kovalchuk, *et al.*, 2003b). This effect is likely explained by epigenetic regulation of gene expression. We hypothesized that the influence of Cl^- ions on HRF could also be based on changes in expression level of different genes and possibly on their methylation. These epigenetic changes can be inherited and lead to the enhanced HRF in progeny of plants never exposed to salt stress.

Analysis of genome stability of progeny of salt stress exposed plants revealed an interesting phenomenon. The RR in the progeny of plants exposed to 25 mM and 75 mM of NaCl was 1.9 and 2.3-fold higher than in the progeny of control plants ($P<0.001$) (Table 4.8).

To provide additional support for the theory of inherited epigenetic changes we analyzed the global methylation status of progeny genomes. Our data showed that progeny of salt treated plants had hypomethylated genomes. We suggest that this hypomethylation reflects the epigenetic changes in parental plants caused by their exposure to salt stress. IThe 2.1-fold higher hypomethylation was observed in the progeny of 25 mM treated plants as compared to progeny of 75 mM treated plants. We can speculate that this likely reflects a higher efficiency of mild stress in signaling stress conditions as compared to more severe ones. However to make a final conclusion, further analysis of several plant generations are required.

5.4. Water-mediated stress and genome stability

5.4.1. HR under drought stress conditions

It is well known that plants subjected to drought stress undergo numerous physiological and metabolic changes (Tabaeizadeh, 1998). Drought stress triggers various alterations on a molecular level that can result either in cell death or plant acclimation (Ingram and Bartels, 1996; Thomashow, 1999; Bray, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2000; Tran, *et al.*, 2004). Recent studies revealed the existence of some common sets of plant genes induced not only by drought but also by salt and cold stresses as well. The presence of shared by aforementioned stresses activator-signals was shown (Zhu, 2001). While similarities and differences in response to salt, cold and drought stress were thoroughly discussed (Seki, *et al.*, 2003; Zhu, 2001), little is still known about the effect of those on the genome integrity. In our study we tried to define the influence of mild drought conditions on the HRF in plant somatic tissues using *A. thaliana* line 11 plants grown under the different watering regimes.

It is well documented that one of the first and main responses to drought stress is the generation of different ROS (Smirnoff, 1993; Beihier and Fock, 1996; Zhang and Kirkham, 1996), mainly superoxide radical and H_2O_2 (Chowdhury and Chowdhury, 1985; Quartacci, *et al.*, 1994). Both are known to be important DNA damaging agents and to play a significant role in stress signaling (Vranova, *et al.*, 2002; Selote, *et al.*, 2004) and acclimation (Vranova, *et al.*, 2002). Indeed, we observed the accumulation of anthocyanins in drought-subjected plants indicating the presence of oxidative stress (Winkel-Shirley, 2002) (Figure 4.10).

The second common response to the drought stress is a general decrease in photosynthetic rate (Tabaeizadeh, 1998) partially due to the initiation of stomatal closure and thus lower CO_2 diffusion into leaves (Flexas and Medrano, 2002; Flexas, *et al.*, 2004). Additionally, H_2O_2 produced under mild water-mediated stress inactivates the enzymes of Calvin cycle (Charles and Halliwell, 1980; Bowler, *et al.*, 1994). This results in growth inhibition. Furthermore, limitation of CO_2 fixation has been shown to reduce the $NADP^+$ regeneration by the Calvin cycle and over reduce the electron transport chain, forming superoxide radicals (Krause, 1994). The reduced growth and other energy-requiring processes in plants will lead to the over reduction of mithochondria electron transport chain and production of additional quantities of superoxide radicals (Purvis, 1997). The overproduction of ROS will be therefore challenge the DNA repair machinery.

Based on the results of our studies, we report while plants grown under mild drought stress conditions exhibited the main symptoms of stress influence such as growth inhibition and anthocyanins accumulation, the HRF was not increased, and in fact even decreased by the factor of 17.8. The calculated RR showed the same surprising pattern. RR gradually declined and by day 16 consisted of 0.7% of values measured on day 0. During the same period RR of control group decreased to 5% (Figure 4.11). The decreased RR in drought treated plants is hard to explain.

It has been previously reported that plants exposed to mild drought stress were capable of acclimating to stress conditions (Jiang and Zhang, 2002; Selote, *et al.*, 2004) partially due to ABA accumulation followed by induction of an antioxidant defense system (Jiang and Zhang, 2002). Furthermore, accumulation of compatible solutes

(Hoekstra, *et al.*, 2001) with antioxidant activity (Smirnoff and Cumbes, 1989; Orthen, *et al.*, 1994) resulted in low levels of superoxide radicals (Selote, *et al.*, 2004). We suggest that the exposure of plants to moderate drought stress (first 4-7 days of experiment) resulted in the plants ability to limit ROS accumulation during subsequent severe water stress (7-16 days after experiment started). One important aspect of this acclimation might be the tight regulation of HR. Considering the presence of great amounts of repetitive DNA including multigene families (Flavell, 1985), it would otherwise result in numerous genome aberrations under the influence of high ROS amounts. From this speculative point of view we can explain more than 7-fold (5% versus 0.7%) difference in reduction of RR by day 16 of the experiment between the 'control' and 'drought' groups (Figure 4.11).

Interestingly, the 'semi-drought' treated plants did not show anthocyanin accumulation and growth inhibition (they were comparable in size with control plants). They exhibited 1.8-fold induction of RR on the day 4 as compared to the respective control. This indicated an absence of inhibition of RR by stress conditions. Moreover, on day 16 RR of semi-drought treated plants was only 1.4-fold lower than the respective control group.

We understand that the model proposed here is speculative. However additional support for our hypothesis may be provided by reports describing the role of sublethal amounts of ROS in plant acclimation to abiotic stress conditions. Pre-treatment of maize seedlings with H_2O_2 or menadione, superoxide-generating compound, induced chilling tolerance (Prasad, *et al.*, 1994). Plants regenerated from potato nodal explants treated with H_2O_2 were significantly more thermotolerant than control plants (Lopez-Delgado, *et*

al., 1998). Similar evidences were reported for *A. thaliana.* Leaves pre-treated with H_2O_2 became tolerant to excess of light (Karpinski, *et al.*, 1999). Additionally partial exposure of *A. thaliana* plants to excess of light resulted in systemic acclimation of unexposed leaves to photo-oxidative stress (Karpinski, *et al.*, 1999). Exposure of wheat seedlings to the mild drought stress was shown to trigger plants acclimation to severe drought stress (Selote, *et al.*, 2004).

Growth inhibition on drought stress can be viewed as a strategy plants have adopted to tolerate stress. The ability to reduce cell division under unfavorable conditions may not only allow conservation of energy for defense purpose, but may also limit the risk of heritable damage (May, *et al.*, 1998). Furthermore the signaling role of ROS in these adaptive changes has been proposed (Reichheld, *et al.*, 1999). Low concentrations of menadione impaired the G1-to-S transition, retarded DNA replication and delayed entry into mitosis (Reichheld, *et al.*, 1999).

Nevertheless, it remains unanswered other types of strand break repair mechanism such as NHEJ are able to compensate for a decrease in the HR rate under mild drought conditions. Further studies should be performed to correlate abundance of DSBs, ROS accumulation, and activity of the main enzymes involved into DNA repair and ROS-scavenging enzymes under mild and sever drought conditions.

5.4.2. Overwatering stress and HR

While there are a number of papers describing the influence of drought stress on plants ranging from molecular to organism levels, there is still insufficient information about the influence of overwatering stress on plants on molecular level. Recently published studies shed some light on the fermentative sensing and signaling pathways under this type of stress (Ismond, *et al.*, 2003; Peng, *et al.*, 2001; Liao and Lin, 2001; Dat, *et al.*, 2004). At the same time the possible influence of this stress on genome stability in plants remains unclear. We analyzed the influence of gradual and drastic overwatering stresses on somatic HRF in *A. thaliana* line 11 transgenic plants.

Our studies revealed that overwatering stress significantly ($P<0.05$) upregulated both HRF and RR. Over production of ROS triggered by alteration of plant metabolism may explain this phenomenon (Ismond, *et al.*, 2003; Peng, *et al.*, 2001; Liao and Lin, 2001; Dat, *et al.*, 2004). It has been shown that an adaptation to overwatering in plants is the shift in their fermentation pathway (Ismond, *et al.*, 2003; Peng, *et al.*, 2001; Liao and Lin, 2001). The stimulated expression of alcohol dehydrogenase gene (ADH) was found in *A. thaliana* subjected to overwatering conditions (Peng, *et al.*, 2001). At the same time, the dependence of ADH on NADPH-oxidase activity has been reported (Klok, *et al.*, 2002). NADH-oxidase has been regarded as a source of ROS for the oxidative burst, which is typical of plant – pathogen incompatible interactions (Lamb and Dixon, 1997). Furthermore, stomatal closure triggered by water stress (Liao and Lin, 2001) will result in reduction of leaf CO_2 incorporation and thereby induce additional overproduction of ROS (Krause, 1994; Asada, 1994; 1999; Purvis, 1997). Therefore, the ROS accumulation seems to be a favorable reason for explaining the induction of HR.

Another interesting observation was made when we compared effects of drastic and gradual application of stress conditions. While both, 'drastic overwatering' and 'overwatering' resulted in an increase of RR, the effect of drastic stress was stronger (Figure 4.11). This could be explained by the ability of plants to adapt to gradual stress. The increase of RR by overwatering and not by drought could also mean that there are some unknown parameters that change and influence genome stability. These molecules may change the equilibrium between HR and NHEJ. Further studies are clearly required to explain this puzzling phenomenon.

6. SUMMARY

The data presented here suggest the existence of a tight age-dependent mechanism for regulating the activity of the HR pathway of DSBs repair in plants. The age-dependent pattern of HR may reflect the involvement of different growth strategies, either cell division or endoreduplication, in order to reach the maximum biological performance plant organism. This may prevent the destabilization of duplicated genomes. The HR increase in plant somatic tissues can result in dual events. The presence of high numbers of potential targets for HR such as great amounts of repetitive non-coding DNA sequences dispersed within plants genomes makes the frequent use of this pathway dangerous for the genome integrity. The increased number of genomes per cell or the increase in stress-induced strand breaks would result in continuous alteration of the genome. However the stress induced genome rearrangement could also contribute to the relatively fast evolutionary adaptation of the genome to new environmental conditions. The existence of stress-regulated RR increase reported here is of special importance for understanding genome evolution. Our studies indicated that there is number of environmental conditions including temperature, light regimes, salt and water-mediated stresses. These stresses can drastically affect the activity of HR pathway in plants. The application of sub-optimal (stress) conditions in most cases upregulated RR rate. This was consistent with low metabolic activity (temperature and light/dark regime stresses) and higher peroxide production (temperature stress). However, on our opinion the increase of RR cannot be simply explained by the increase of ROS production and direct DNA damage. ROS were shown to be involved in a variety of acclimation responses to abiotic stresses. It is possible that tight regulation of HR can be a part of this acclimation

response as shown in our study of water-mediated stress influence. We found that salt stress resulted in induction of HR of treated plants as well as increased HR of untreated progeny of salt-exposed plants. This phenomenon was previously described for UV and pathogen stress. We believe our data showed that stress factors not only change the genome stability but also contribute to plant evolution. Among the major mechanisms is apparently HR. This mechanism of strand breaks processing in not only the repair mechanism but also a powerful way of shaping genomes.

7. FUTURE DIRECTIONS

The role of HR and NHEJ in the maintenance of genome stability and plant evolution remains to be specified. While the age-dependent regulation of HR seems to be clear, the possible age-dependent management of NHEJ has yet to be determined. It is still unclear what mediates the shift in the equilibrium between HR and NHEJ or how either of two contributes to development of stress tolerance. In the future more work should be done using transgenic lines that have major HR and NHEJ repair genes targeted. Possible candidates are *Ku70* and *Ku80* for NHEJ and *Rad51* for HR. It would be interesting to analyze the response and adaptation abilities of these plants to stress. Our data suggested the possibility of significant up regulation of HR in plants without affecting the plant's physiological performance. The identification of internal (cellular) or external (stress) factors that may control the level of chromosomal HR seems promising. These factors may have great potential in GT since they do not damage DNA directly but change the activity of HR proteins. Finally, although the effect of different abiotic conditions is known and seems to be heritable, the analysis of increased HRF in non-treated progeny of stress-subjected plants during several generations after the stress application could be interesting. It is important to determine the mechanism of inheritance invoking these changes. This would help us understand the role of different DNA repair pathways in providing relatively fast evolutionary adaptation of the genome to the new environmental conditions.

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